Review Paper

Public health and clinical importance of amoebiasis in Malaysia: A review

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Abstract. Entamoeba histolytica, the causative agent of human amoebiasis remains a significant cause of morbidity and mortality in developing countries and is responsible for up to 100,000 deaths worldwide each year. Entamoeba dispar, morphologically indistinguishable from E. histolytica is more common in humans in many parts of the world. Similarly Entamoeba moshkovskii, which was long considered to be a free-living amoeba is also morphologically identical to E. histolytica and E. dispar, and is highly prevalent in some E. histolytica endemic countries. Humans are the host of infection and there would not appear to be other meaningful animal reservoirs of E. histolytica. Entamoeba. histolytica can be present in sewage and contaminated water. The infection is mainly transmitted via ingestion of water or food contaminated by faeces containing E. histolytica cysts. Clinical features of amoebiasis range from asymptomatic colonization to amoebic dysentery and invasive extraintestinal amoebiasis, which is manifested most commonly in the form of abscesses in liver and lungs. The epidemiology of amoebiasis has dramatically changed since the separation of E. histolytica and E. dispar species and the worldwide prevalence of these species has not been estimated until recently. Moreover, E. moshkovskii, another morphologically indistinguishable human parasitic Entamoeba was not mentioned or considered as a contributor to the prevalence figures in endemic areas. Amoebiasis is still a major health problem especially in aboriginal settlements and amongst people living in remote area in Malaysia. However, until now there is only one data currently available to indicate the true prevalence and incidence of E. histolytica and E. dispar. Further studies are needed to determine the burden of E. histolytica, E. dispar and E. moshkovskii infections in Malaysia. In the present review, we briefly summarize all methods use in diagnosing Entamoeba species, ranging from microscopic identification to molecular detection such as culture and isoenzyme analysis, antibody detection tests, antigen detection tests, immunochromatographic assays, conventional PCR, real-time PCR and loop-mediated isothermal amplification (LAMP).

INTRODUCTION

The genus Entamoeba contains many species, six of which Entamoeba histolytica, Entamoeba dispar, Entamoeba moshkovskii, Entamoeba polecki, Entamoeba coli, and Entamoeba hartmanni reside in the human intestinal lumen. Entamoeba histolytica is the only species definitely associated with pathological sequelae in humans; the others are considered non-pathogenic and rarely cause intestinal disease in humans (Clark & Diamond, 1991; Garcia & Bruckner, 1997). During the 1990s, enough evidence had accumulated to support the formal separation of two morphologically identical species of amoeba: the non-pathogenic E. dispar from the potentially pathogenic E. histolytica (Abd-Alla et al., 1993; WHO, 1997; PAHO, 1997).
Entamoeba histolytica infections are commonly observed in tropical and subtropical regions of the world including Malaysia. In developed countries E. histolytica infections are commonly seen in travelers, recent immigrants, homosexual men and inmates of institutions. Humans are the primary host and the main source of infection is the cyst passing chronic patient or asymptomatic carrier. The infection is mainly transmitted via ingestion of water or food contaminated by faeces containing E. histolytica cysts (Leber & Novak, 1999; Stanley, 2003). Within the last few decades, many studies have reported the occurrence of this infection in homosexual men usually as the result of oral-anal and oral-genital sexual contact (Yi Chen et al., 2007; Stark et al., 2008). The infection of E. histolytica is known as amoebiasis and is globally considered a leading parasitic cause of human mortality (WHO, 1997; Haque et al., 2003; Haque & Petri, 2006). It infects hundreds of millions of people per year. Analysis based on published data by Walsh (1986) indicated that 10% of the world’s population was infected by E. histolytica and only 1% of the infected individuals developed invasive form of the disease, whilst 9% were asymptomatic.

Humans are the host of infection and there would not appear to be other meaningful animal reservoirs of E. histolytica. Entamoeba histolytica can be present in sewage and contaminated water. Cysts may remain viable in suitable aquatic environments for several months at low temperature. The potential for waterborne transmission is greater in the tropics, where the carrier rate sometimes exceed 50%, compared with more temperate regions, where the prevalence in the general population may be less than 10%. Person-to-person contact and contamination of food by infected food handlers appear to be the most significant means of transmission, although contaminated water also plays a substantial role. Ingestion of faecally contaminated water and consumption of food crops irrigated with contaminated water can both lead to transmission of amoebiasis. The transmission of E. histolytica by contaminated drinking water has been confirmed (Marshal et al., 1997). The cysts are relatively resistant to disinfection and may not be inactivated by chlorination practices generally applied in the production of drinking water. Within a Water Safety Plan (WHO, 2004), control measures that can be applied to manage potential risk from E. histolytica include prevention of source water contamination by human waste, followed by adequate treatment and protection of water during distribution. Owing to the resistance of the cysts to disinfectants, E. coli (or, alternatively thermotolerant coliforms) cannot be relied upon as an index of the presence/absence of E. histolytica in drinking water supplies.

Morbidity and mortality data which existed prior to this time pertaining to cases of invasive disease were not greatly affected because all invasive disease was known to be caused by E. histolytica. However, because most prevalence data previously collected were associated with asymptomatic individuals, and a majority of asymptomatic individuals with cysts detected in their stool were actually infected with non-pathogenic E. dispar, the definite prevalence of E. histolytica has became a matter of speculation. On the other hand, the role of E. moshkovskii, a free living amoeba that is indistinguishable in its cyst and trophozoite forms from E. histolytica and E. dispar, plays in human infections is yet to be adequately defined. Entamoeba moshkovskii was first isolated from sewage effluents and recognized as an ubiquitous free-living organism in 1941 (Tshalaia, 1941). A recent study reported a high prevalence and association of E. moshkovskii with E. histolytica and E. dispar infections in young children in Bangladesh (Ali et al., 2003). To date, human isolate of this species has been reported from many studies all over the world (Clark et al., 1991; Haque et al., 1998; Parija & Khairnar, 2005; Fotedar et al., 2007; Tanyuksel et al., 2007; Beck et al., 2008; Ben Ayed et al., 2008; Nazemalhosseini Mojarad et al., 2010; Hamzah et al., 2010).

Some of the original epidemiologic descriptions distinguishing E. histolytica from E. dispar infections originated from study conducted among asymptomatic semi
rural population in South Africa. Culture and zymodeme analysis were used for the identification of Entamoeba sp. and the findings reported an overall prevalence of E. histolytica-E. dispar complex of approximately 10%. On further analysis it was revealed that 90% of the asymptomatic individuals were infected with the non-pathogenic E. dispers and 10% asymptptomatically harboured E. histolytica (Gathiram & Jackson, 1985). It was subsequently noted that approximately 10% of asymptomatic carriers of E. histolytica would develop invasive disease while a majority of others spontaneously cleared their infection by 1 year (Blessmann et al., 2002a; Haque et al., 2002).

In developing countries, waterborne gastrointestinal parasite pathogens such as Cryptosporidium parvum, Giardia lamblia, and E. histolytica are frequently associated with morbidity, particularly in children. In developed nations, outbreaks of E. histolytica infections have been caused by sewage contaminated water supplies (Barwick et al., 1999). Many studies of Entamoeba species have used specimens from stool and and liver abscess samples. Investigations of the occurrence of Entamoeba species in surface and waste water in Thailand was carried out by Sirilak Sukprasert et al. (2008). In this study, DNA of 137 surface and waste water samples collected from Pathum Thani Province, Thailand were examined for Entamoeba spp. using PCR and genus-specific primers that amplify DNA of E. polecki, Entamoeba chattoni, E. dispar, E. histolytica, E. hartmanni, E. coli, and E. moshkovskii. The results showed that 27% of the samples were positive for Entamoeba species. When the positive samples were further examined by a single-round PCR assay specific for E. histolytica, E. dispar and E. moshkovskii, all were negative. In contrast of their study, two out of six water samples (32%) collected from Ankara river in Turkey were positive for E. histolytica by PCR (Bakir et al., 2003). These results extend our knowledge on E. histolytica as waterborne parasite and such occurrence information on waterborne pathogens assists the management and treatment of municipal water.

Following this, many studies all over the world reported the true prevalence of E. histolytica, E. dispar and E. moshkovskii as summarized in Table 1. PCR, ELISA - antigen detection and isoenzymes analysis were the common techniques used for the specification of all three species of Entamoeba. Studies had shown that all the three techniques for species identification of E. histolytica in fresh stool showed excellent correlation with TechLab E. histolytica antigen detection test; TechLab E. histolytica antigen detection test was also found to be both rapid and technically simple (Haque et al., 1998). In exception studies by Al-Hindi et al. (2005) in Gaza and Noor Azian et al. (2006) in Malaysia, all of the studies reported high prevalence of E. dispar as compared to E. histolytica. For example, the earliest community study in South Africa by Gathiram & Jackson (1985) reported a high prevalence of E. dispar as compared to E. histolytica. Subsequent community and hospital studies carried out a decade later confirmed the findings (Samie et al., 2006; Fotedar et al., 2007; Saeed et al., 2007; Beck et al., 2008; Nazemalhosseini Mojarad et al., 2010).

A study to determine the prevalence and species distribution of E. histolytica and E. dispar was carried out in South Africa by examining stool samples collected from public hospitals and primary schools using ELISA and nested polymerase chain reaction (PCR). The results showed that E. histolytica was detected in 18.8% and 2.1% samples of patients from public hospitals and primary school children respectively, whereas 25.3% and 8.5% had E. dispar respectively (Samie et al., 2006). A similar clinical setting study was also carried out in Sweden using established PCR and the analysis showed that 79.7% were positive for E. dispar and only 4.8% were positive E. histolytica (Lebbad et al., 2005). A latest study in Iran reported that out of 3,825 stool samples examined using single-round PCR assay 3.5%, 91.4% and 3.5% were reported positive for E. histolytica, E. dispar and E. moshkovskii respectively (Nazemalhosseini Mojarad et al., 2010).
Table 1. Prevalence of intestinal *E. histolytica*, *E. dispar* and *E. moshkovskii* infections

<table>
<thead>
<tr>
<th>Method of diagnosis</th>
<th>Population studied</th>
<th>Prevalence (%)</th>
<th>Author</th>
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</thead>
<tbody>
<tr>
<td>Isoenzyme analysis</td>
<td>Ecuador; community study; school children</td>
<td><em>Ed</em> is 3.7 times higher than <em>Eh</em></td>
<td>Gatti et al., 2002</td>
</tr>
<tr>
<td></td>
<td>UK; male homosexual</td>
<td>0.0</td>
<td>20.0</td>
</tr>
<tr>
<td></td>
<td>South Africa; community study; all ages</td>
<td>1.0</td>
<td>9.0</td>
</tr>
<tr>
<td>ELISA: Antibody detection</td>
<td>Malaysia; community study; all ages</td>
<td>33.4</td>
<td>24.6</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>8.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>8.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mexico; community study; all ages</td>
<td>8.4</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Malaysia; community study; adults</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Orang Asli</td>
<td>9.7</td>
<td></td>
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<tr>
<td></td>
<td>Malay</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Indian</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chinese</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Malaysia; community study – Orang Asli; children</td>
<td>70.0</td>
<td>87.0</td>
</tr>
<tr>
<td></td>
<td>adult</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELISA: Antigen detection</td>
<td>Nigeria; hospital study; all ages</td>
<td>ND</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>South Africa; HIV patients</td>
<td>4.0</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Saudi Arabia; hospital study; all ages</td>
<td>2.7</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Saudi Arabia; hospital study; all ages</td>
<td>4.3</td>
<td>95.7</td>
</tr>
<tr>
<td></td>
<td>South Africa; hospital study (all ages) school children</td>
<td>18.8</td>
<td>25.3</td>
</tr>
<tr>
<td></td>
<td>Bangladesh; hospital study; children community study; urban slum; children community study; rural village; children</td>
<td>4.2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.3</td>
<td>ND</td>
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<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>ND</td>
</tr>
<tr>
<td>PCR</td>
<td>Iran; hospital study; all ages</td>
<td>3.45</td>
<td>91.4</td>
</tr>
<tr>
<td></td>
<td>South Africa; HIV patients</td>
<td>ND</td>
<td>5.0</td>
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<tr>
<td></td>
<td>Australia; hospital study; all ages</td>
<td>3.4</td>
<td>33.7</td>
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<tr>
<td></td>
<td></td>
<td>36.0</td>
<td><em>Ed</em> + <em>Em</em></td>
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<td></td>
<td></td>
<td>1.1 <em>Eh</em> + <em>Em</em></td>
<td></td>
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<tr>
<td></td>
<td>India; hospital study; all ages</td>
<td>3.5</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
<td>Thailand; hospital study; all ages</td>
<td><em>Eh:Ed</em> = 1:6</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Malaysia; community study – Orang Asli; all ages</td>
<td>13.2</td>
<td>5.6</td>
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<tr>
<td></td>
<td>Brazil; community study; all ages</td>
<td>ND</td>
<td>74.2</td>
</tr>
<tr>
<td></td>
<td>Gaza Strip, Palestine; Hospital study; children</td>
<td>69.6</td>
<td>22.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.6 <em>Eh</em> + <em>Ed</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sweeden; hospital study; all ages</td>
<td>4.8</td>
<td>79.7</td>
</tr>
<tr>
<td></td>
<td>India; hospital study; all ages</td>
<td>1.7</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>Vietnam; community study; adults</td>
<td>11.2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Ghana; community study; all ages</td>
<td>82.8</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Philippines; community study; all ages</td>
<td>1.0</td>
<td>7.1</td>
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</tbody>
</table>
Outbreaks of amoebiasis have been reported in several countries. Lalla et al. (1992) reported an outbreak of *E. histolytica* and *G. lamblia* infections in travellers returning from the tropics. Of 160 travellers from various regions in Italy who had taken part in a five-day organized trip to Phuket, Thailand, and been accommodated in the same luxury hotel, 17 showed either amoebic abscess or colitis. A pretested questionnaire that focused on the consumption of foods and beverages well known to be a source of intestinal infection in endemic areas was available from these 17 patients as well as from 41 out of 74 asymptomatic travellers. Stool samples for parasitological examination were also available. In patients affected with amoebic abscess, antibodies to *E. histolytica* were also determined. Overall, parasitological examinations were negative in eight (13.8%) patients, and 50 out of 58 (86.2%) were found to be positive. The prevalence of *E. histolytica* was 72.4% and 28 subjects (48.3%) were stool positive for both protozoa. No other intestinal parasites were found. The consumption of drinks with ice, ice cream and raw fruit in ice was significantly associated with *E. histolytica* infections. Vreden et al. (2000) reported an outbreak of amoebiasis in a family in The Netherlands. The index case was a 5 year old girl who presented with a 6 week history of abdominal pain and bloody diarrheal stools without fever. Stool cultures for bacterial pathogens remained negative. Because the girl had never been abroad, the possibility of amoebiasis was not considered by the physician until her mother gave a history of amoebic dysentery and persistent cyst passage after return from India 13 years back. In addition to the index case, the household contacts of the mother were her husband, a 4 year old son, a 2 year old son, and a nanny. None of the household contacts had traveled outside Western Europe or had developed signs or symptoms that were consistent with dysentery or extraintestinal invasive disease. However, the oldest son had an episode of abdominal cramps and nonbloody diarrhoea a few weeks after his sister had developed amoebic dysentery. Trophozoites and/or cysts of *E. histolytica/E. dispar* were found in stool specimens from the index case, her mother, and other household contacts. This outbreak of amoebiasis demonstrates that even with Western standards of hygiene, persistent cyst passage may result in transmission of *E. histolytica* to household contacts many years later. In Japan, Niichiro et al. (1999) reported an amoebiasis outbreak among the residents of two institutions for the mentally retarded in Osaka City. In February 1996, a liver abscess due to *E. histolytica* was found in a resident of institution A. In June, several residents in institution B complained of diarrhoea. Their stool contained *E. histolytica*. The stool specimens from the residents, their parents and the staff of the two institutions were examined for *Entamoeba*. Thirteen among 79 residents in institutions A (16.5%) and 29 among 69 residents in institution B (42.0%) carried *E. histolytica* cysts. The relationship of the outbreaks in the two institutions remains obscure but they could be related since four of the residents in institution A had once been residents of institution B and since one short stay individual in institution B acquired the parasite.

**Pathogenesis**

*Entamoeba histolytica* was first described by Lamb in 1859 and the pathogenesis of this infection was first described in St Petersburg by Fedor Losch in 1875 in the stool of a Russian suffering from dysentery. Infection of *E. histolytica* develops following ingestion of the quadrinucleate cysts of *E. histolytica*; encystation of the cysts in the intestinal lumen produces trophozoites and thereby colonizes the large intestine. The trophozoite may remain confined to the intestinal lumen feeding on bacteria and cellular debris. Galactose and N-acetyl-D-galactosamine (Gal/GalNAc)-specific lectin are used by the trophozoites to adhere to colonic mucins and colonize the large intestine (Petri et al., 2002); aggregation of the trophozoites in the mucin layer will trigger encystations; cysts are then excreted in stool.

Colitis results when the trophozoites penetrate the intestinal epithelium. The ability of trophozoite to invade intestinal mucosa depends on their genetic,
immunoenzymatic profile, their ability to produce proteolytic enzymes and enabling resistance to complement mediated lyses. Trophozoites invasion are initiated by killing of epithelial cells and inflammatory cells (neutrophils and lymphocytes); interaction of the amoeba with intestinal epithelium causes an inflammatory response marked by the activation of nuclear factor and secretion of lymphokines (Eckmann et al., 1995; Seydel et al., 1998). The development of epithelial response may depend on trophozoite virulence factors and this will lead to intestinal abnormalities through neutrophil-mediated damage. In early invasive lesion with superficial ulceration, three main consecutive events occur and there are focal superficial epithelium erosion followed by small glandular foci of microinvasion and mild to moderate neutrophil infiltration of lamina propria.

The mechanism involves in superficial epithelium erosion is complex; once protective mucus barrier has been broken down, trophozoites of *E. histolytica* will adhere and make a contact-dependent damage and lyses of epithelium cells. Lectins, amebapores and proteases are the three molecules involve in this event (Martinez-Palomo et al., 1985). The second event is characterized by continuing lyses of cells with the aid of proteolytic enzymes (i.e. cysteine proteinase, phospholipase and haemolysin) that degrade elastin, collagen and fibrinogen. Tissues penetration of trophozoites is also assisted through its locomotion activities and proteolytic degradation of extracellular matrix components of the colonic mucosa (Espinosa-Cantellano & Martinez-Paloma, 2000). In the final event, infiltration of neutrophil and other inflammatory cells around trophozoites leads to rapid lyses of inflammatory cells and tissue necrosis (Espinosa-Cantellano & Martinez-Paloma, 2000). Peptide-mediated lysosome enzymes released by the lysed inflammatory cells contribute to the destruction of host tissue and extend of the lesion. Neutrophil representing over 90% of the circulating granulocytes, respond to a variety of cytokines and soluble factors during the inflammatory process. The first sign of colonic aggression visualized by sigmoidoscopy is nonspecific thickening of the mucosa or pin head-size micronodules.

The late invasive lesion is characterized by extension of mucosal ulcer deep into a larger area of the submucosa. Once the interglandular epithelium has been invaded by trophozoite, the underlying tissue offers little resistance, allowing extension of the ulcer laterally, creating the classical flask-shaped amoebic ulcer (Stanley, 2003). Histopathology shows necrotic areas and vascular congestion with minimal inflammation in contrast with the extension of the lesion. Trophozoites may be found in the surface layer of the ulcers; bacterial infection causing deficiency of blood supply leads to necrosis, haemorrhage and gangrene and subsequent perforation of the intestinal wall (Haque et al., 2003). Bowel complications occur in 1-4% of patients and invasive extraintestinal complication is very uncommon and may be present in 0.1 to 1% of symptomatic patients.

**Clinical features**

Clinical features of amoebiasis range from asymptomatic colonization to amoebic dysentery and invasive extraintestinal amoebiasis, which is manifested most commonly in the form of abscesses in liver and lungs. It has been recognized that disease expression of amoebiasis varies geographically. For example, invasive disease in Egypt is predominantly amoebic colitis (Abd-Alla et al., 2002) whereas in South Africa there is an excessive rate of amoebic liver abscess (ALA). In fact, in Hue City, Vietnam, an overall estimated frequency of ALA was recently reported to be as high as 21 cases per 100,000 inhabitants (Blessmann et al., 2002b). Although 90% of *E. histolytica* infections remain asymptomatic, approximately 50 million people have invasive disease, resulting in 100,000 deaths per year (WHO, 1997), placing amoebiasis as the second leading cause of death from parasitic diseases worldwide (Stanley, 2003).

Individuals harbouring *E. histolytica* (asymptomatic carriers) can develop antibody titers in the absence of invasive...
disease (Jackson et al., 1985; Gathiram & Jackson, 1987; Ravdin et al., 1990). Asymptomatic colonization with *E. histolytica*, if left untreated can lead to amoebic dysentery and a wide range of other invasive diseases, but more often the infection resolves spontaneously without the development of diseases (Gathiram & Jackson, 1987; Haque et al., 2001; Blessmann et al., 2002b). Cohort studies reported that when asymptomatic individuals were followed up for 1 year, 4 to 10% of them developed colitis or extraintestinal diseases (Gathiram & Jackson, 1987; Haque et al., 2001).

Natural history of acute amoebic colitis has a gradual onset, with a 1-2 weeks history of mild-to-moderate abdominal pain and tenderness, tenesmus and watery diarrhoea with five to seven episodes per day with scarce amounts of faeces, abundant mucus with or without blood. About 80% of patients complain of localized abdominal pain; some patients may have only intermittent diarrhoea alternating with constipation. Fever is unusual, occurring in <40% of patients (Adams & MacLeod, 1977). Other associated symptoms are weight loss and anorexia. Microscopically, trophozoites can be detected in submucosal tissue or faecal samples using normal saline temporary staining and the finding can be confirmed by permanent staining technique i.e. trichrome staining. Since *E. histolytica* invades the colonic mucosa, faeces are almost universally positive for occult blood.

This syndrome resolves within a few days following appropriate anti-amoebic treatment. Severe cases of amoebic colitis are characterized by dysenteric stools, diffuse abdominal pain, high fever and severe dehydration; patient usually appears very ill. Differential diagnoses include infection with bacteria such as *Shigella, Salmonella, Campylobacter* and enterohaemorrhagic *Escherichia coli* and noninfectious causes which include inflammatory bowel disease, ischaemic colitis and diverticulitis. Other spectrums of acute intestinal amoebiasis include extensive fulminant necrotizing colitis, toxic megacolon and perianal ulceration. Patient with fulminant amoebic colitis usually presents with profuse bloody diarrhoea, fever, pronounced leukocytosis, and widespread abdominal pain, often with peritoneal signs and extensive involvement of the colon (Takahashi et al., 1997). Although fulminant necrotizing colitis and toxic megacolon are very rare, they are usually associated with a high mortality rate.

Another clinical spectrum of intestinal amoebiasis is chronic intestinal amoebiasis and patient with this condition present with intermittent abdominal pain, diarrhoea and weight loss. Patients who are at increased risk of severe disease include those who are very young, very old, malnourished, pregnant and those who are receiving corticosteroid. Recent studies in non endemic areas of amoebiasis i.e. Japan, Taiwan, Republic of Korea and Australia have reported *E. histolytica* as an emerging pathogen in men who have sex with men (MSM) (Ohnishi et al., 2004; Hung et al., 2005; Tsai et al., 2006; Park et al., 2007; Stark et al., 2008). MSM are also reported to be in the higher risk to develop invasive amoebiasis (Hung et al., 2005).

Acute complications of intestinal disease include bleeding, perforation, peritonitis, perianal skin ulceration and rectovaginal fistulas. Deep ulcer may heal with stricture and adhesion and these will lead to intestinal obstruction. Ameboma results from the formation of annular colonic granulation tissues in the caecum and ascending colon may mimic carcinoma of colon is a rare late complication of intestinal amoebiasis (Adams & MacLeod, 1977). On rare occasions, *E. histolytica* trophozoites enter the bloodstream and disseminate to other body sites. Amoebic liver abscess (ALA) is the most frequent manifestation of extraintestinal amoebiasis (Bruckner, 1992). Abscess complicates amoebiasis in 3 to 9% of patients (Frey et al., 1989). The clinical presentation is highly variable, ranging from weight loss, weakness, and low-grade fever on an acute to febrile illness. Pain may include vague right-upper-quadrant discomfort, point tenderness between ribs on palpation, or pleuritic discomfort, spreading to the right shoulder. Patients, who present acutely with symptoms of less than two weeks of duration,
have more prominent abdominal pain with fevers and rigors (Katzenstein et al., 1982).

Anemia, leukocytosis and elevated alkaline phosphatase are often noted, but jaundice, striking transaminase elevation, and eosinophilia are unusual (Guerrant, 1986). Older patients tend to present with chronic illness, lasting longer than two weeks. They have less fever (only 30%), and may have a wasting disease with significant weight loss (Katzenstein et al., 1982). The number of patients presenting with acute ALA seems to be increasing, possibly reflecting earlier diagnosis and better access to medical care. ALA is characterized by a significant male preponderance and it is a disease seen most commonly in patients who reside in or have emigrated from an endemic area (Hughes & Petri, 2000). Cutaneous amoebiasis is a rare extraintestinal manifestation of *E. histolytica* infection (Mhlanga et al., 1992). In children it always occurs in the anogenital or perineal region as a result of direct inoculation of trophozoites from prolonged contact with infected stools in a child's diaper. Direct inoculation of skin has also been reported from scratching, anal or vaginal intercourse and following surgical drainage or spontaneous rupture of an abscess at a colostomy site or laparotomy incision (Magana-Garcia & Arista-Viveros, 1993a).

**Diagnosis**

Microscopic techniques employed in a diagnostic clinical laboratory include wet preparation, concentration, and permanently stained smears for the identification of *E. histolytica/E. dispar/E. moshkovskii* in faeces. Microscopic examination of a direct saline (wet) mount is a very insensitive method (<10%) which is performed on a fresh specimen (Huston et al., 1999). The sample should be examined within 1 hour of collection to search for motile trophozoites which may contain RBCs. However, in patients who do not present with acute dysentery, trophozoites will not contain RBCs. Patients with asymptomatic carriage generally have only cysts in the faecal sample. Although the concentration technique is helpful in demonstrating cysts, the use of permanently stained smears (trichrome or iron hematoxylin) is an important method for recovery and identification of *Entamoeba* species. Microscopy is a less reliable method of identifying *Entamoeba* species than either culture or antigen detection tests (Krogstad et al., 1978; Haque et al., 1995). The sensitivity of microscopy can be poor (60%) and confounded with false-positive due to misidentification of macrophages as trophozoites, PMNs as cysts (especially when lobed nuclei of PMNs break apart), and other *Entamoeba* species (Gonzalez-Ruiz et al., 1994; Haque et al., 1997; Haque et al., 1998; Tanyuksel & Petri, 2003). As *Entamoeba* trophozoites generally degenerate rapidly in unfixed faecal specimens (Proctor, 1991) and refrigeration is not recommended, specimens should be preserved with a fixative which prevents the degradation of the morphology of the parasite and allows concentration and permanent smears to be performed. Fixatives used for the concentration procedure include Schaudinn's fluid, merthiolate iodine-formalin, sodium acetate-acetic acid formalin (SAF), or 5% or 10% formalin. The fixatives for the permanently stained smears include trichrome, iron hematoxylin, Ziehl-Neelsen stains, modified polyvinyl alcohol (PVA) and SAF. Examination for ova and parasites in a minimum of three stool samples over no more than 10 days is recommended, as these organisms may be excreted intermittently or may be unevenly distributed in the stool. This improved the detection rate to 85 to 95% (Li & Stanley, 1996).

Culture techniques for the isolation of *Entamoeba* species have been available for over 80 years. Culture media include xenic (diphasic and monophasic) and axenic systems. Xenic cultivation is defined as the growth of the parasite in the presence of an undefined flora (Clark & Diamond, 2002). The xenic culture of *E. histolytica* was first introduced by Boeck and Drbohlave in 1925 in a diphasic egg slant medium, and a modification of this medium (Locke-egg) is still used today. Different monophasic media that were developed for *E. histolytica* are the egg yolk infusion medium of Balamuth.
(Balamuth, 1946), Jones's medium (Jones, 1946), and TYSGM-9 (Diamond, 1982). Of the different media developed for the xenic cultivation of *E. histolytica*, only three media, diphasic Locke-egg, Robinson's medium (Robinson, 1968), and the monophasic TYSGM-9 (Diamond, 1982) are in common use. Axenic cultivation involves the cultivation of parasites in the absence of any other metabolizing cells (Clark & Diamond, 2002). The axenic cultivation of *E. histolytica* was first achieved by Diamond (1961). The monophasic medium TP-S-1 was developed and used widely for culture of *E. histolytica* (Diamond, 1968; Clark & Diamond, 2002). Currently, TYI-S-33 (Diamond, Harlow & Cunnick, 1978) and YI-S (Diamond, Clark & Cunnick, 1995) are the most widely used media for axenic cultivation of *E. histolytica* (Clark & Diamond, 2002).

Culture of *E. histolytica* can be performed from faecal specimens, rectal biopsy specimens or liver abscess aspirates. As the liver abscess aspirates of ALA patients are usually sterile (98% cases) (Blessmann et al., 2002b), addition of a bacterium or a trypanosomatid is necessary before inoculation of amoebae into xenic culture (Freedman, Maddison & Elsdon-Dew, 1958; Wang, Jen & Cross, 1973; Clark & Diamond, 2002). The success rate for culture of *E. histolytica* is between 50 and 70% in reference laboratories (Clark & Diamond, 2002). As culture of *E. histolytica* from clinical samples such as faeces or liver abscess has a significant false-negative rate and is technically difficult, it has not been adopted as a routine clinical laboratory. *Entamoeba dispar* can be grown in xenic culture; however, most isolates grow poorly in monoaxenic culture and the growth of only a few strains has been reported to be viable in axenic culture, suggesting that *E. dispar* may be less able than *E. histolytica* to obtain nutrients in a particle-free medium (Clark, 1995; Kobayashi et al., 1998). The use of different media for the culture of *E. dispar* has been investigated and these studies indicate that YI-S may not be a suitable medium for the culture of *E. dispers* (Kobayashi et al., 1998). For *E. moshkovskii* strains, culture media employed include TTY-SB-monophasic with the trypanosomatid, TP-S-1-GM monophasic for the axenic culture of amoebae (Diamond, 1968) and the TP-S-1-GM monophasic medium (Diamond & Bartgis, 1970). Other media containing bovine serum used for culture of *E. moshkovskii* include axenic medium TYI-S-33 with 10% bovine serum at 24°C (Diamond, Harlow & Cunnick, 1978) or xenic medium TYSGM-9 with 5% bovine serum at either 24°C or 37°C (Diamond, 1982).

The pioneering work of Sargeaunt et al. (1978) demonstrated that isoenzyme analysis of cultured amoebae would enable the differentiation of *Entamoeba* species. A zymodeme is defined as a group of amoeba strains that share the same electrophoretic pattern and mobilities for several enzymes. Zymodemes consist of electrophoretic patterns of malic enzyme, hexokinase, glucose phosphate isomerase and phosphoglucomutase isoenzyme (Sargeaunt et al., 1987). A total of 24 different zymodemes have been described, of which 21 are from human isolates (9 of *E. histolytica* and 12 of *E. dispar*). The presence of starch in the medium influences the most variable zymodeme patterns (Blanc & Sargeaunt, 1991) and many zymodemes “disappear” upon removal of bacterial floras, suggesting that at least some of the bands are of bacterial rather than amoebal origin (Jackson & Supersad, 1997). If the zymodemes defined by stable bands alone are counted, only three remain for *E. histolytica* (II, XIV and XIX) and one for *E. dispers* (I). Isoenzyme (zymodeme) analysis of cultured amoebae enables differentiation of *E. histolytica* from *E. dispers* and was considered the gold standard for diagnosis amoebic infection prior to development of newer DNA-based techniques.

Many different assays have been developed for the detection of antibodies, including indirect hemagglutination (IHA), latex agglutination, immunoelectrophoresis, counterimmunoelectrophoresis (CIE), the amoebic gel diffusion test, immunodiffusion, complement fixation, indirect immunofluorescence assay (IFA), and enzyme-linked immunosorbent assay (ELISA). A variety of antibody assays for detection of *E.
histolytica antibodies in human serum are also commercially available (Table 2). Complement fixation tests appear to be less sensitive than others, cost more to perform and are not used by most laboratories. IHA is simple to perform and has been shown to be a highly specific (99.1%) diagnostic tool in human immunodeficiency virus-infected patients presenting with gastrointestinal symptoms (Hung et al., 1999). However, the lower sensitivity may lead to false-negative results compared to ELISA. The latex agglutination test appears to detect the same antibody as IHA. Commercial kits are available and the test can be performed in 10 min. However, due to nonspecific reactions, the specificity of this test appears to be disappointing (Sanchez-Giillen et al., 2000). Immuno-electrophoresis, CIE and immunodiffusion use the property of antibody and antigen precipitation in agar gel membrane. Sheehan et al. (1979) reported that detection of antibody to extraintestinal E. histolytica by CIE is time-consuming but has a high sensitivity (100%) in patients with invasive amoebiasis. Detection of antibodies using the IFA test was shown to be rapid, reliable and reproducible and helps to differentiate ALA from other nonamoebic etiologies. In addition to this, IFA tests has been shown to differentiate between past (treated) and present disease (Garcia et al., 1982). A study conducted by Jackson et al. (1984) indicated that monitoring of immunoglobulin M (IgM) levels using the IFA can be of clinical value in a short period of time after infection, with more than half of the subjects having negative results at 6 months or 100% becoming negative by 46 weeks after treatment. In ALA the sensitivity of the IFA is reported to be 93.6%, with a specificity of 96.7%, making it more sensitive than the

<table>
<thead>
<tr>
<th>Antibody assay</th>
<th>Sensitivity (%) &amp; Reference</th>
<th>Specificity (%) &amp; Reference</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellognost-</td>
<td>100&lt;sup&gt;a&lt;/sup&gt; (Pillai et al., 1999)</td>
<td>90.9-100&lt;sup&gt;a&lt;/sup&gt; (Pillai et al., 1999)</td>
<td>Dade Behring Marburg GmbH, Marburg, Germany</td>
</tr>
<tr>
<td>Amoebiasis (IHA)</td>
<td>99 (Hira et al., 2001)</td>
<td>99.8 (Hira et al., 2001)</td>
<td></td>
</tr>
<tr>
<td>Novagnost</td>
<td>&gt; 95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt; 95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NovaTec Immunodiagnostica GmbH, Dietzenbach, Germany</td>
</tr>
<tr>
<td>Entamoeba IgG</td>
<td>&gt; 95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt; 95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NovaTec Immunodiagnostica GmbH, Dietzenbach, Germany</td>
</tr>
<tr>
<td>Bichro-Latex Amibe</td>
<td>93.3 (Van Doorn et al., 2005)</td>
<td>95.5 (Van Doorn et al., 2005)</td>
<td>Fumoze Diagnostics, Levallois-Perret Cedex, France</td>
</tr>
<tr>
<td>I.H.A Amoebiasis</td>
<td>93.4 (Robert et al., 1990)</td>
<td>97.5 (Robert et al., 1990)</td>
<td>Fumoze Diagnostics, Levallois-Perret Cedex, France</td>
</tr>
<tr>
<td>Amoeba-Spot IF</td>
<td>NA&lt;sup&gt;c&lt;/sup&gt; (Gatti et al., 2002)</td>
<td>NA (Gatti et al., 2002)</td>
<td>bioMerieux, Marcy-l’Etoile, France</td>
</tr>
<tr>
<td>Amoebiasis Serology</td>
<td>95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>97&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Light Diagnostics</td>
</tr>
<tr>
<td>microplate ELISA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amoebiasis Serology</td>
<td>97.9 (Hira et al., 2001)</td>
<td>94.8 (Hira et al., 2001)</td>
<td>LMD Laboratories, Inc., Carlsbad, CA</td>
</tr>
<tr>
<td>microwel EIA (HK-9 antigen, axenic)</td>
<td>92.5 (Shenai et al., 1996)</td>
<td>91.3 (Shenai et al., 1996)</td>
<td></td>
</tr>
<tr>
<td>RIDASCREEN</td>
<td>100&lt;sup&gt;b&lt;/sup&gt;</td>
<td>95.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>R-Biopharma AG, Darmstadt, Germany</td>
</tr>
<tr>
<td>Entamoeba</td>
<td>97.7-100&lt;sup&gt;c&lt;/sup&gt; (Knappik et al., 2005)</td>
<td>97.4 (Knappik et al., 2005)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> For the titer of ≥1:64, 100% sensitive and 90.9% specific; for the titer of ≥1:512, 100% sensitive and 100% specific.
<sup>b</sup> As recommended by the manufacturer.
<sup>c</sup> NA, not available.
ELISA (Shamsuzzaman et al., 2000). A negative test therefore indicates that a patient never had invasive amoebiasis. However, this test requires skills in culture and subsequent antigen preparation, making it difficult to undertake in a routine clinical laboratory (Patterson & Schoppe, 1982). ELISA is the most popular assay in diagnostic laboratories throughout the world and has been used to study the epidemiology of asymptomatic disease (Gonzalez et al., 1995) and the diagnosis of symptomatic amoebiasis after faecal examination. This method is widely thought to be sufficient for clinical purposes, particularly for diagnosis of patients with ALA and can be easily performed in a clinical laboratory. It may also be useful in the evaluation of intestinal and extraintestinal infections where amoebiasis is suspected but organisms cannot be detected in faeces (Rosenblatt, Sloane & Bestrom, 1995).

Several investigators have developed ELISAs for the detection of antigens in faecal samples. These antigen detection tests have a sensitivity approaching that of stool culture and are rapid to perform. Antigen-based ELISA kits that are specific for *E. histolytica* use monoclonal antibodies against the Gal/GalNAc-specific lectin of *E. histolytica* (II; TechLab, Blacksburg, VA) or monoclonal antibodies against serine-rich antigen of *E. histolytica* (Optimum S kit; Merlin Diagnostika, Bornheim-Hersel Germany). Other ELISA kits for antigen detection include the *Entamoeba* CELISA PATH kit (Cellabs, Brookvale, Australia), which uses a monoclonal antibody specific for lectin of *E. histolytica*, and the ProSpecT EIA (Remel Inc.; previously manufactured by Alexon-Trend, Inc., Sunnyvale, CA), which detects *E. histolytica*-specific antigen in faecal specimens (Table 3). In addition to the above mentioned clinical assays, research-based detection tests have included the use of monoclonal antibodies against a lectin-rich surface antigen (Petri & Singh, 1999), a lipophosphoglycan (Mirelman et al., 1997), a 170-kDa-adherence lectin amoebic antigen detected in saliva (Abd-Alla et al., 2000) and an uncharacterized antigen (Wonsit et al., 1992). The *E. histolytica* TechLab kit was designed in 1993 to detect specifically *E. histolytica* in faeces (Haque et al., 1997; Haque et al., 1998). This antigen detection test captures and detects the parasite’s Gal/GalNAc lectin in stool samples. The lectin is conserved and is highly immunogenic and because of the antigenic differences in the lectins of *E. histolytica* and *E. dispar*, the test enables specific identification of the disease-causing *E. histolytica*. The level of detection of amoebic antigens is quite high, requiring approximately 1,000 trophozoites per well (Haque et al., 1993; Mirelman et al., 1997). However, this test suffers from the disadvantage that the antigens detected are denatured by fixation of the stool sample, therefore limiting testing to fresh or frozen samples. Nevertheless, this test has demonstrated good sensitivity and specificity for detection of *E. histolytica* antigen in stool specimens of people suffering from amoebic colitis and asymptomatic intestinal infection (Haque et al., 1995, 1997, 1998). The ProSpecT EIA (Remel Inc.) is a microplate EIA which detects both *E. histolytica* and *E. dispar*. However, this assay cannot differentiate between *E. histolytica* and *E. dispar*. The advantage of this test is that it can be performed on fresh, frozen or Cary-Blair specimens but not on formalin-fixed faecal samples. The sensitivity of the ProSpecT EIA was compared with that of conventional microscopy (using wet mounts and concentration methods) for the diagnosis of *E. histolytica*/*E. dispar*, and a sensitivity of 78% and specificity of 99% were reported (Ong et al., 1996). In another study, by Gatii et al. (2002), the reported sensitivity and specificity of ProSpecT ELISA were 54.5% and 94%, respectively compared to culture and zymodeme identification for *E. histolytica*/*E. dispar*.

The Triage parasite panel (TPP) (Biosite Diagnostic Ins., San Diego, CA) is the first immunochromatographic assay for the simultaneous detection of antigens specific for *G. lamblia*, *E. histolytica*/*E. dispar* and
C. parvum. The immunochromatographic strip used in this assay is coated with monoclonal antibodies specific for the 29-kDa surface antigen (E. histolytica/E. dispar), alpha-1-giardin (G. lamblia), and protein disulfide isomerase (C. parvum). By using specific antibodies, antigen specific for these organisms from the stool samples are captured and immobilized on a membrane. A high sensitivity (90% to 100%) and specificity (99.1% to 100%) of the TPP kit compared to microscopy (stool ova and parasite examination) for E. histolytica/E. dispar were reported (Garcia, Shimizu & Bernard, 2000; Sharp et al., 2001). In another study, although the specificity of the Triage kit was high (100%), the sensitivity was low (68.3%) compared to that of the ProSpecT test (Pillai & Kain, 1999). A recent study from Sweden has compared the TPP test with PCR and demonstrated a low sensitivity for TPP assay (Leiva et al., 2006). The advantage of the TPP method is that it can be performed in approximately 15 min with fresh or frozen, unfixed human faecal specimens.

There is now a wide variety of PCR methods, targeting different genes, which have been described for detection and differentiation of the three Entamoeba species. The consistent genetic diversity

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity (%) &amp; Specificity (%)</th>
<th>Manufacturer</th>
<th>Detection limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>TechLab</td>
<td>96.9-100 &amp; 94.7-100</td>
<td>TechLab, Blacksburg, VA</td>
<td>0.2-0.4 ng of adhesion per well</td>
</tr>
<tr>
<td>E. histolytica II</td>
<td>14.2 (Gatti et al., 2002) &amp; 100 (Gatti et al., 1997)</td>
<td>TechLab, Blacksburg, VA</td>
<td>0.2-0.4 ng of adhesion per well</td>
</tr>
<tr>
<td>Entamoeba CELISA-PATH</td>
<td>95-100 &amp; 93.100</td>
<td>Cellabs Pty Ltd, Brookvale, Australia</td>
<td>Not given</td>
</tr>
<tr>
<td>Optimum S E. histolytica antigen ELISA</td>
<td>100 (Pillai et al., 1999) &amp; NP</td>
<td>Merlin Diagnostika, Berheim-Hersel, Germany</td>
<td>Not given</td>
</tr>
<tr>
<td>Triage parasite panel</td>
<td>99 (Garcia et al., 2000) &amp; 99 (Garcia et al., 2000)</td>
<td>BIOSITE Diagnostics, San Diego, CA</td>
<td>Not given</td>
</tr>
<tr>
<td>ProSpecT E. histolytica microplate assay</td>
<td>87 (Gatti et al., 2002) &amp; 99 (Gatti et al., 2002)</td>
<td>REMEL Inc., Lenexa, KS</td>
<td>40 ng/ml of E. histolytica-specific antigen</td>
</tr>
</tbody>
</table>

*Specific for E. histolytica
*Sensitivity and specificity compared to culture/zyomodeme, as cited by the manufacturer
*Sensitivity and specificity compared to culture and microscopy
*Compared to isoenzyme analysis
*Compared to culture
*Compared to culture and microscopy
*Compared to real-time PCR
*NP, not published
*Cannot distinguish between E. histolytica and E. dispar
*Compared to permanent staining with trichrome and modified acid-fast stains
*Compared to ProSpecT Entamoeba histolytica microplate assay
*Compared to ovum and parasite examination
*As mentioned by the manufacturer, related to ovum and parasite identifications
*Compared to microscopy (wet mounts and concentration)
*Previously manufactured by Alexon-Trend, Inc., Sunnyvale, CA

Table 3: Commercially available antigen assays for the diagnosis of amoebiasis
detected between the 18S rDNAs of *E. histolytica* and *E. dispar* initiated the use of 18S rDNA as a target for differentiation of the two species (Clark & Diamond, 1991, 1992; Cruz-Reyes et al., 1992). DNA extracted from laboratory-cultured trophozoites and DNA recovered directly from microscopy-positive faecal samples using the manual and automated methods were tested, and the PCR methods proved to be highly sensitive and specific for detecting *Entamoeba* DNA (Clark & Diamond, 1993, 1997; Troll, Marti & Weiss, 1997; Ramos et al., 2000; Heckendorn et al., 2002; Moran et al., 2005). PCR assays targeting 18S rDNA are widely used for the detection and differentiation of *Entamoeba* species, as these targets are present in the genome, extrachromosomal plasmids in the amoeba (Bhattacharya et al., 1989), making the 18S rDNA more easily detected than a DNA fragment of a single-copy gene. The successful use of PCR in studying the epidemiology of *Entamoeba* infection was first reported by Acuna-Soto et al. (1993). They used DNA extracted directly from faeces, avoiding the need to culture trophozoites and the primers were targeted to amplify the extrachromosomal circular DNA. This gene target was subsequently used by other researchers (Aguirre et al., 1995; Britten et al., 1997). This PCR target, with colorimetric detection of the product was also used with DNA extracted from faecal samples, using a modification of the QIAGEN kit (Aguirre et al., 1995; Verweij et al., 2000). Primers for the 29-kDa/30-kDa antigen gene have been used for distinguishing among pathogenic and non-pathogenic species of *Entamoeba* using conventional PCR (Tachibana et al., 1991). In research laboratories, this target has been used for analyses of microscopy-positive faeces which have been cultured in the laboratory (Tachibana et al., 2000; Pinheiro et al., 2004) as well as formalin-fixed faecal samples (Rivera et al., 1996, 1998, 2006).

Other gene targets for PCR include two protein-encoding genes which have been shown to exhibit polymorphism in the coding region. These are the serine-rich *E. histolytica* protein (SREPH) gene (Stanley et al., 1990) and the chitinase gene (De la Vega et al., 1997). SREPH as a target was reported for the amplification of DNA recovered from laboratory cultures and microscopy-positive faeces concentrated by the zinc-sulfate gradient floatation techniques (Ramos et al., 2005). A nested SREPH PCR approach was recently used to investigate *E. histolytica* diversity in a single human population, using DNA extracted from microscopy-positive faeces (Ayeh-Kumi et al., 2001). PCR using the cysteine proteinase gene and actin genes as targets was also used to study the epidemiology of amoebiasis (Freitas et al., 2004). In addition, a novel PCR assay based on the *E. histolytica* hemolysin gene HLY6 (hemo PCR) was developed for the detection of *E. histolytica* DNA with faecal and ALA samples and was shown to have 100% sensitivity and specificity (Zindrou et al., 2001). PCR for the detection of *E. histolytica* DNA from liver abscess samples was first employed using the gene encoding the 30-kDa antigen and 100% sensitivity was reported. In another study, PCR performed on liver samples demonstrated only 33% sensitivity for the presence of *E. histolytica* using primers specific for 18S rDNA of *E. histolytica*, whereas the second pair, specific for the 30-kDa antigen gene (Tachibana et al., 1992), showed a sensitivity of 100% (Zengzhu et al., 1999). Direct amplification for detection of *E. histolytica* DNA (without the extraction of DNA) from ALA pus was reported using 10 different previously published primer pairs (used for amplification of *E. histolytica* from liver and stool samples) (Zaman et al., 2000). Of the 10 different primer pairs tested, two pairs i.e., P1-P2 targeting extrachromosomal circular DNA of *E. histolytica* (Acuna-Soto et al., 1993) and P11-P12 targeting the 30-kDa antigen gene (Tachibana et al., 1992) gave 100% sensitivity. Another PCR assay (hemo PCR), based on the novel hemolysin gene HLY6 of *E. histolytica* was analyzed for the liver abscess samples. The hemo-PCR gave a positive result for 89% of ALA samples compared to 77% and 28% for the 30-kDa antigen gene and 18S rDNA, respectively (Zindrou et al., 2001). The hemo-PCR was found to be a valuable diagnostic tool for identification of *E. histolytica* in liver and
faecal samples. For the identification of *E. moshkovskii* in faecal specimens, a ribiprinting method was first reported by Haque *et al.* (1998). Subsequently, a PCR for the identification of *E. moshkovskii* in faecal samples was developed as a nested 18S rDNA PCR followed by restriction endonuclease digestion (Ali *et al.*, 2003). This method has a high sensitivity and specificity (100%) with DNA extracted directly from stool samples using the QIAGEN stool extraction kit (Fotedar *et al.*, 2007).

Real-time PCR is a new and a very attractive methodology for laboratory diagnosis of infectious diseases because of its characteristics that eliminate post-PCR analysis, leading to shorter turnaround times, a reduction in the risk of amplicon contamination of laboratory environments and reduced reagents costs (Klein, 2002). This approach allows specific detection of the amplicon by binding the one or two fluorescence-labeled probes during PCR, thereby enabling continuous monitoring of amplicon (PCR product) formation throughout the reaction. An important aspect of real-time PCR is enhanced sensitivity compared to conventional PCR, with an ability to detect 0.1 cell per gram of faeces (Blessmann *et al.*, 2002a). In addition, real-time PCR is a quantitative method and allows the determination of the number of parasites in various samples. Distinct real-time PCR protocols have recently been published for identification and differentiation of *E. histolytica* from *E. dispar*. These include a Light Cycler assay utilizing hybridization probes to detect amplification of the 18S rDNA from faecal samples (Blessmann *et al.*, 2002a; Calderaro *et al.*, 2006) and two TaqMan assays, one targeting the 18S rDNA (Verweij *et al.*, 2003, 2004; Kebede *et al.*, 2004) and another targeting the episomal repeats using DNA extracted from faecal samples collected from primates and humans (Verweij *et al.*, 2003). A molecular beacon-based real-time PCR targeting 18S rDNA of *E. histolytica* for use on faecal and ALA specimens was described (Roy *et al.*, 2005). A SYBR green real-time assay targeting the 18S rDNA was described by Qvarnstrom *et al.* (2005). The sequences selected in the majority of these real-time studies have included rDNA as the target for PCR. A recent evaluation of three real-time PCR assays, focusing on the weakness and strengths of each assay and their usefulness for clinical laboratory diagnosis, was published by Qvarnstrom *et al.* (2005). This study highlighted major differences in detection limits and assay performance that were observed among the evaluated tests. Two of the assays in this study could not reliably distinguish *E. histolytica* from *E. dispar*, including the Light Cycler assay (Blessmann *et al.*, 2003) and the TaqMan assay targeting episomal repeats (Verweij *et al.*, 2003). A multiplex real-time assay was subsequently developed for detection of different intestinal parasites with 100% sensitivity and specificity (Verweij *et al.*, 2004). This assay allows detection of *E. histolytica*, *G. lambia* and *C. parvum* and offers the possibility of introducing DNA detection in the routine diagnosis of intestinal parasitic infections. The implementation of such multiplex assays and the development of automated DNA isolation procedures could have a tremendous impact on routine parasitology practice. Accurate diagnosis necessitates that the same reaction conditions are used for a standard and for the sample. Duplex or multiplex approaches with internal standardization provide a solution for this problem. A real-time PCR for detection of *E. moshkovskii* in clinical samples has not yet been reported. Further research is therefore required to develop these methods for the detection of *E. moshkovskii*.

Recently, a novel nucleic acid amplification method termed loop-mediated isothermal amplification (LAMP) has been developed (Liang *et al.*, 2009). The loop-mediated isothermal amplification (LAMP) assay was originally developed by Notomi *et al.* (2000), Mori *et al.* (2001) and Nagamine *et al.* (2002) (Eiken Chemical Co., Ltd., Japan). LAMP employs a DNA polymerase with strand displacement activity and four primers that recognize six sequences on the target DNA. This method amplifies DNA with high specificity, sensitivity, and rapidity under isothermal conditions. Since this reaction is performed under isothermal conditions (60
to 65°C), simple incubators such as a water bath or heat block are adequate for the DNA amplification (Notomi et al., 2000). Moreover, a large amount of white precipitate of magnesium pyrophosphate is produced as a byproduct, which enables the visual judgment of amplification by the naked eye (Mori et al., 2001). Considering these advantages, the LAMP assay could become a valuable diagnostic tool in developing countries or hospital laboratories. With the LAMP assay, *E. histolytica* in faecal samples can be detected rapidly via the naked eye under UV light without any other sophisticated equipment (Mori et al., 2001) and LAMP outperforms microscopy in its ability to discriminate *E. histolytica* from *E. dispar* and *E. moshkovskii*. Since commercial kits such as the TechLab II kit detect the antigens of *E. histolytica*, they were not compared with the LAMP assay which is based on DNA detection. The LAMP assay developed by Liang et al. (2009) has levels of sensitivity and specificity similar to those of nested PCR and can be useful for clinical detection and active surveillance of *E. histolytica* parasites in countries where amoebiasis is endemic. The LAMP assay requires minimal laboratory facilities and can differentiate DNA samples from *E. histolytica* and *E. dispar*. Moreover, compared to the requirements for performing nested PCR, the simplicity and affordability of the LAMP assay allow easy identification of *E. histolytica*.

**Epidemiology of amoebiasis in Malaysia**

*Entamoeba histolytica* is an enteric anaerobic protozoan parasite that causes about 50 million infections with a death rate of over 100,000 worldwide annually. Although the parasite has a worldwide distribution, high prevalence of more than 10% of the population have been reported varies with the population of individuals affected, different between countries and areas with different socioeconomic conditions. Areas of highest incidence (due to inadequate sanitation and crowding) include most developing countries in the tropics, particularly Mexico, Indian and nations of Central and South America, tropical Asia, and Africa. In Malaysia, foodborne and waterborne diseases which are closely associated with environmental and personal hygiene practices are still among the major health problems in Malaysia; intestinal protozoan infections for example are still a public health concern in Malaysia, although are well controlled and only sporadically limited to specific areas or within certain population groups i.e. the aboriginal settlements and amongst people living in remote area (Ministry of Health Malaysia, 2008).

The prevalence of *E. histolytica/E. dispar* in Malaysia has been reported by many researchers, from way back in the sixties (Table 4); the prevalence ranged from 1% to 83%. The prevalence varied with the population studied, whether preservative were used for faeces collection, frequency of faeces collected for examination and technique used in the detection of the protozoa. Desowitz et al. (1961) reported a prevalence rate of 5-10% in the earliest study carried out among population living in various villages in Singapore and medical students of Singapore University. Following that, many studies reported on the prevalence of *E. histolytica/E. dispar* especially among Orang Asli, estate and rural village communities (Heyneman et al., 1967; Balasingam et al., 1969; Bisseru & Aziz, 1970). Except the study by Bolton (1968), all studies in the sixties and seventies were community studies. In the early eighties Noor Hayati et al. (1981) and Hamimah et al. (1982) reported 3.4% and 2.3% of children admitted with gastroenteritis symptoms excreted cysts of *E. histolytica/E. dispar* respectively. The prevalences were in agreement with finding reported earlier by Bolton (1968). A recent study carried out among population living in tropical highland and mountainous area in Sabah showed a high prevalence of *E. histolytica/E. dispar* (21.0%) (Nor Aza et al., 2003). Only one faecal specimen was collected from each subject and the faeces were not fixed. If faeces were collected more than once and fixed in preservative a higher prevalence of *E. histolytica/E. dispar* would be expected in their study. A later study carried out among school children in Sabah confirmed that *E.
**Table 4. Intestinal E. histolytica/E. dispar in Malaysia**

<table>
<thead>
<tr>
<th>Infection &amp; method of diagnosis</th>
<th>Population studied</th>
<th>Prevalence (%)</th>
<th>Author &amp; Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestinal</td>
<td>Community – village and medical students</td>
<td>5-10</td>
<td>Desowitz et al. (1961)</td>
</tr>
<tr>
<td></td>
<td>Hospital – Orang Asli; all age</td>
<td>3.1-10.3</td>
<td>Bolton (1968)</td>
</tr>
<tr>
<td></td>
<td>Community – Orang Asli; children</td>
<td>1.5</td>
<td>Bissaru &amp; Aziz (1970)</td>
</tr>
<tr>
<td></td>
<td>Community – Orang Asli; all age</td>
<td>5.1</td>
<td>Dunn (1972)</td>
</tr>
<tr>
<td></td>
<td>Community – Orang Asli; all age</td>
<td>8.7</td>
<td>Dissanaik et al. (1977)</td>
</tr>
<tr>
<td></td>
<td>Community – estate workers; adult</td>
<td>1.3</td>
<td>Sinniah et al. (1978)</td>
</tr>
<tr>
<td></td>
<td>Community – village people; all age</td>
<td>1.2</td>
<td>Nawalinski &amp; Roundy (1978)</td>
</tr>
<tr>
<td></td>
<td>Hospital – children</td>
<td>3.4</td>
<td>Noor Hayati et al. (1981)</td>
</tr>
<tr>
<td></td>
<td>Hospital – school children</td>
<td>2.3</td>
<td>Hamimah et al. (1982)</td>
</tr>
<tr>
<td></td>
<td>Community – Orang Asli and medical students</td>
<td>14.4</td>
<td>Che Ghani et al. (1987)</td>
</tr>
<tr>
<td></td>
<td>Community – Orang Asli; all age</td>
<td>8.6</td>
<td>Lai (1992)</td>
</tr>
<tr>
<td></td>
<td>Community – Orang Asli children</td>
<td>61.5</td>
<td>Karim et al. (1995)</td>
</tr>
<tr>
<td></td>
<td>Community – Orang Asli; children</td>
<td>9.0</td>
<td>Rahmah et al. (1997)</td>
</tr>
<tr>
<td></td>
<td>Community – Orang Asli; all age</td>
<td>11.5</td>
<td>Noor Hayati et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>Community – Orang Asli; all age</td>
<td>6.9</td>
<td>Kamel et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>Community – Interior population; all age</td>
<td>21.0</td>
<td>Nor Aza et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>Community – Urban population; all age</td>
<td>0.4</td>
<td>Jamaiah &amp; Rohela (2005)</td>
</tr>
<tr>
<td></td>
<td>Community – Orang Asli; children</td>
<td>8.9</td>
<td>Norhayati et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>Community – Orang Asli; all age</td>
<td>18.5</td>
<td>Noor Azian et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>Community &amp; hospitalized – Orang Asli; all age</td>
<td>9.4</td>
<td>Lokman et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>Community – school children</td>
<td>83.9</td>
<td>Mahsoli et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>Community – Orang Asli; children</td>
<td>22.5</td>
<td>Hartini &amp; Mohamed Kamel (2009)</td>
</tr>
<tr>
<td></td>
<td>Community – Orang Asli; all age</td>
<td>5.6</td>
<td>Ed</td>
</tr>
</tbody>
</table>

In peninsular Malaysia, many recent studies reported high prevalence of *E. histolytica/E. dispar* (Karim et al., 1995; Rahmah et al., 1997; Noor Hayati et al., 1998; Noor Azian et al., 2007; Lokman et al., 2007; Hartini & Mohamed Kamel, 2009). A study carried out among Orang Asli communities in Pos Piah reported 11.5% of population studied was infected with *E. histolytica/E. dispar* (Noor Hayati et al., 1998). In their study, faeces were collected once and fixed in polyvinyl-alcohol. A study by Noor Azian et al. (2007) among aborigines population also reported high prevalence of *E. histolytica/E. dispar* (18.5%). In their study faeces were collected in three consecutive days and fixed in polyvinyl-alcohol upon examination. Cysts were excreted intermittently, thus collection of faeces of more than one and use of polyvinyl-alcohol to fix the faeces contributed to the high prevalence of *E. histolytica/E. dispar* infection detected in this study. High prevalence was also reported by Lokman et al. (2007). A recent study by Hartini & Mohamed Kamel (2009) reported 22.5% of Orang Asli children studied harbored *E. histolytica/E. dispar* cysts. On the other hand, the prevalence of *E. histolytica/E. dispar* was very low in the urban community of Malaysia. A study by Jamaiah & Rohela (2005) among the public in Kuala Lumpur reported a very low prevalence (0.4%).

As almost all surveys previously done relied on stool analysis by microscopy, there is no reliable data on the epidemiology of *E. histolytica* in Malaysia. It is not only in Malaysia, but also in the other parts of the world in general that the current epidemiology of amoebiasis is confusing, mainly because of the recently appreciated distinction between *E. histolytica, E. dispar* and *E. moshkovskii*. Until relatively recently, *E. histolytica* and *E. dispar* were not differentiated, and infection with either
of the two species was referred to as 'amoebiasis', resulting in an overestimation of the true prevalence. In Malaysia, no attempt study is made to differentiate cysts and trophozoites of morphologically identical *E. histolytica*, *E. dispar* and *E. moshkovskii*. This is because all community and hospital studies are based entirely on microscopical examination of fresh faeces specimens for parasite identification. Very little information is available on the prevalence of the two or three *Entamoeba* species. To the best of our knowledge and up to this date, only one community study that has been carried out in Malaysia to differentiate the two species (Nor Azian et al., 2006). In this study the true prevalence of *E. histolytica* and *E. dispar* was determined using Nested PCR and Restriction Enzyme (RE) digestion. The findings showed that out of 31 specimens that were positive for *E. histolytica/E. dispar* microscopically, 13.2% and 5.6% were positive for *E. histolytica* and *E. dispar* respectively. This finding contradicts most studies as reports usually documented higher prevalence of *E. dispar* as compared to *E. histolytica*.

The earliest reports on invasive amoebiasis in Malaysia were by Chellapa & Rangabasham (1977), Vijendran (1977), Balasengaram (1981) and Goh et al., (1987). A case report by Manukaran et al. (1983) reported a rare clinical presentation of intestinal amoebiasis with multiple colonic perforations and ruptured liver abscess in a 43-year-old Indian labourer. Goh et al. (1987) in their study had reviewed 204 cases of liver abscess seen between 1970 and 1985 in University Hospital, Kuala Lumpur; the findings showed 44.1%, 11.8%, and 0.5% of liver abscess were classified as amoebic, pyogenic and tuberculous. The cause of liver abscess in the remaining of 43.6% was not established. This study also reported fever with chills and rigors, right hypochondrial pain and tender hepatomegaly were the most common clinical presentations of amoebic liver abscess (ALA). Almost 87% of ALA seen in this study is a single abscess and mostly located in the right lobe. The patients were predominantly males, Indians and in the 30-60 age group.

A 10-year retrospective study on amoebiasis was also carried out in University Hospital Kuala Lumpur between 1984 to 1994 by Jamaiah & Shekar (1999); of 51 amoebiasis cases traced, 30 (59%), 20 (39%) and 1(2%) were amoebic dysentery, ALA and combination of amoebic dysentery and ALA respectively. Most of the cases were reported in Malays, majority in males and unemployed. The most common clinical presentations were diarrhoea and dysentery. Trophozoites of *E. histolytica* were only identified in 13 (43%) and 9 (30%) of faeces and intestinal biopsy of amoebic dysentery patients respectively. Only one out of 20 ALA cases showed trophozoites in the faeces and biopsy (Jamaiah & Shekar, 1999). These findings showed the difficulty in isolating trophozoites from clinical specimens; thus antigen or antibody test and PCR are very useful to confirm the diseases. A latest 10-year retrospective study in the same hospital showed a decreasing trend of invasive amoebiasis as compared to studies by Goh et al. (1987) and Jamaiah & Shekar (1999); only 34 cases were traced and analysis showed ALA was the commonest presentation with 22 (65%) cases. Amoebic dysentery was seen in 12 (35%) of patients (Farhana et al., 2009). The clinical presentations were almost similar with study carried out by Goh et al. (1987).

Magnitude of invasive amoebiasis can also be in the community by assessing the antibody titers. Gilman et al. (1976) reported 44% of asymptomatic family members of Orang Asli patients diagnosed with acute amoebic dysentery were sero-responders. This study also reported that Orang Asli who lived near towns had significantly more sero-responders (32%) than Orang Asli who lived in deep jungle village (4%). Sero-epidemiological studies of specific antibodies to *E. histolytica* using Indirect Immunofluorescent test among different races in Malaysia by Thomas & Yap (1986), demonstrated low prevalence of antibody in Chinese (3.6%) as compared to Orang Asli (9.7%), Malay (7.2%) and Indians (5.4%).

In conclusion amoebiasis is still a public health and of clinical importance in Malaysia. Since 90% of *E. histolytica* infection is
asymptomatic, determination of the true prevalence of *E. histolytica* infection in the community is very crucial to predict the clinical burden of ameobiasis. Thus, future epidemiological study should give priority to the determination of true prevalence of *E. histolytica* and *E. dispar*.

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