COMPARATIVE ANALYSIS OF AMOEBIC SEROPOSITIVITY AMONG KELANTAN CATTLE FARM DWELLERS USING CSA- AND rCL-IgG-ELISA

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by

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

%	Percentage
>	More than
^{0}C	Degree Celsius
CBB	Coomassie brilliant blue
cm	Centimeter
mm	Millimeter
CSA	Crude soluble antigen
ELISA	Enzyme linked immunosorbent assay
et al.	*et alii* – 'and others'
x *g*	Gravity
g	Gram
Ig	Immunoglobulin
kDa	Kilodalton
mA	MiliAmpere
min	Minute
mL	Milliliter
NaCl	Sodium chloride
OD	Optical density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Sec	Second
SD	Standard Deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TMB	3,3’,5,5’-Tetramethylbenzidine
rCL	recombinant c-terminal lectin
rpm	Revolutions per minute

ANALISIS PERBANDINGAN PENEMUAN SEROLOGI AMOEBIK DALAM KALANGAN PENDUDUK LADANG LEMBU KELANTAN DENGAN MENGGUNAKAN CSA- DAN rCL-IgG-ELISA

ABSTRAK

Penduduk ladang lembu Kelantan adalah salah satu kumpulan berisiko tinggi untuk amoebiasis invasif kerana aktiviti penternakan melibatkan penggunaan sumber air yang tidak terawat seperti air bawah tanah dan air perigi. Penyaringan untuk amoebiasis invasif oleh antigen larut kasar (CSA) dan antigen c-terminal rekombinan (rCL) memberikan interpretasi yang berbeza mengenai status pendedahan penyakit. Ujian berdasarkan CSA mempunyai kecenderungan yang lebih tinggi terhadap antibodi anti-ameba umum, manakala rCL lebih spesifik untuk amebiasis invasif. Kajian ini bertujuan untuk mengkaji penemuan serologi ameba dalam kalangan penghuni ladang lembu Kelantan menggunakan CSA dan rCL-IgG-ELISAs yang disesuaikan. Dua antigen yang berbeza itu dihasilkan dan digunakan untuk penyaringan sampel serum berdasarkan parameter yang dioptimumkan sebelum ini. Sampel serum penderma darah digunakan untuk penentuan nilai *cut-off* untuk dua ujian yang disesuaikan itu. RIDASCREEN® *Entamoeba histolytica* IgG digunakan sebagai ujian rujukan. Daripada analisis tersebut, ujian rujukan mengesan 31 (34.83%) kes positif, sementara dua ujian lain CSA dan rCL-IgG-ELISAs masing-masing mengesan 17 (19.10%) dan 41 (46.07%) kes. Kedua-dua ujian yang dibangunkan sendiri menunjukkan persetujuan yang sederhana dengan ujian komersial, namun CSA-ELISA menunjukkan persetujuan yang lebih tinggi dengan penemuan ujian komersial apabila dibandingkan dengan rCL-ELISA. Daripada analisis ROC,

permukaan di bawah cerun bagi CSA-IgG-ELISA juga lebih tinggi daripada rCL-IgG-ELISA. Kesimpulannya, rCL- dan CSA-IgG-ELISA menunjukkan penemuan serologi yang berbeza; penemuan oleh ujian yang menggunakan rCL adalah lebih rendah dan mungkin lebih spesifik untuk amoebiasis invasif.

COMPARATIVE ANALYSIS OF AMOEBIC SEROPOSITIVITY AMONG KELANTAN CATTLE FARM DWELLERS USING CSA- AND rCL-IgG-ELISA

ABSTRACT

Kelantan cattle farm dwellers are one of the high-risk groups for invasive amoebiasis, as the farming activities involve the use of untreated water source such as underground and well water. Screening for invasive amoebiasis by crude soluble antigen (CSA) and recombinant c-terminal lectin (rCL) antigen provide different interpretation on the disease exposure. Assay based on the former antigen has higher affinity towards variety of anti-amoebic antibodies, while the latter is specific for invasive amoebiasis. The present study aimed to study the amoebic seropositivity among Kelantan cattle farm dwellers using customised CSA- and rCL-IgG-ELISA. The two different antigens were produced and used for the screening of the serum samples based on previously optimised parameters. Thirty blood donor serum samples were used for the determination of cut-off values for the two customised assays. RIDASCREEN® *Entamoeba histolytica* IgG was used as the reference test. From the analysis, the reference test detected 31 (34.83%) positive cases, while CSA- and rCL-IgG ELISA detected 41 (46.07%) and 17 (19.10%) cases, respectively. The two customised assays showed moderate agreement with the commercial test, nonetheless, CSA-ELISA showed higher agreement with the findings of commercial test, as compared to rCL-ELISA. From ROC curve analysis, the area under the curve of CSA-IgG-ELISA was higher than that of rCL-IgG-ELISA, as well. In conclusion, CSA- and rCL-IgG-ELISA

showed different seropositivity; findings by the rCL-IgG-ELISA are lower and could be more specific for invasive amoebiasis.

CHAPTER 1

INTRODUCTION

1.1 Research background

Amebiasis is a parasitic infection caused by the protozoan *Entamoeba histolytica*. Intestinal amebiasis is one of the top ten causes of severe diarrhoea in the developing countries (Shirley, Farr, Watanabe, & Moonah, 2018). There are eight different types of human intestinal amoeba namely *E. histolytica, E. polecki, E. gingivalis, E. moshkovskii, E. dispar, E. coli, E. hartmanni and E. Bangladeshi*. The presence of a central karyosome in the nuclei of *Entamoeba histolytica*, *Entamoeba dispar*, and *E. moshkovskii* has been demonstrated by microscopic observations, distinguishing them from other *Entamoeba* species.

Many infected persons remain as asymptomatic carriers (90%), and the remaining 10% symptomatic patients might present with colitis, dysentery, and extra intestinal disease. Extraintestinal manifestations of *E. histolytica* infection include amoebic liver abscess (ALA), which is the most frequently reported type. According to the 2013 Global Burden of Disease report, intestinal protozoan infections are the third leading cause of death in the world (Herricks et al., 2017). Despite the recent developments in better sanitation facilities and improvements in water quality, foodborne and waterborne transmission remain the primary reservoirs of amoebiasis infection specifically in endemic countries. Food contamination can occur at various stages of the food production process, including manufacturing, harvesting, and handling (Association, 2006). Farm dwellers, in addition to those who live in endemic

regions, are a relatively higher-risk group for acquiring amoebiasis in the future. A significant contribution to the global economy is made by cattle, which meets the basic needs of low-income people around the world. In area with low socioeconomic status, the farming activities are not based on industrialised settings. Many rely on manpower and non-public water supply for farm management. These increase the risk of farm dwellers to water-borne disease, including amoebiasis as in endemic areas (Budu-Amoako, Greenwood, Dixon, Barkema, & McClure, 2012). Surveillance of amoebiasis high-risk group is important to ensure the termination of the parasite life cycle, otherwise the asymptomatic infected persons will continue passing the cyst containing stools into the environment. A few diagnostic tools could be considered for field surveillance purpose *i.e.* microscopy and amoebic serology. Microscopy remains the routine standard for diagnosis of amoebiasis, but the latter could be easily used for mass screening (Saidin, Othman, & Noordin, 2019).

1.2 Problem statement and rational of the study

High prevalence of anti-amoebic antibody *i.e.* 37.5% was previously reported in Kelantan blood donor. The finding was consistent with the high relative incidence of Kelantan water-borne disease as compared to the national incidence (Zeehaida, Zairi, Tan, Wong, & Lim, 2009). Apart from personal hygiene practice, high incidence of anti-amoebic antibody prevalence could partly associated with insufficient employment of public water supply and prevail usage of untreated underground water which contains high level of coliform counts. (Abdul Shukor, 2014). The latest national statistics also showed that the incidence of the water-borne diseases in Kelantan remain high from year 2015 to year 2018 (DSM, 2019). Farming activities associated with the usage of untreated water remains at risk of being exposed to

amoebiasis. However, there were no detailed study on this aspect yet. Data on the disease prevalence could provide insight in preventing and control of amoebiasis, as well as waterborne disease spreading via the similar route.

Previous study by Fong (2021) showed high seroprevalence of anti-amoebic antibody among Kelantan cattle farm dwellers using CSA-ELISA method (52.22%). However, there were several pros and cons associated with the findings by CSA-IgG-ELISA. Crude soluble antigen (CSA) comprised multiple antigens. It offers high affinity toward heterogenous anti-amoebic antibodies but not all antigens are specific for invasive amoebiasis. Signals from non-specific or cross-reactive antigens could divert or mask the real study finding. As an alternative approach, many defined protein biomarkers were reported to be specific for sero-detection of invasive amoebiasis such as serine-rich *Entamoeba histolytica* proteins (SREHP), pyruvate phospho dikinase (PPDK), and Gal/GalNAc lectin protein. Gal/GalNAc lectin protein is the most well studied biomarker.

Assay using recombinant antigen offers more standardised and reproducible antigenic properties, as compared to CSA (Min et al., 2016). Hence, screening of field samples using the recombinant antigen as a specific biomarker for invasive amoebiasis could strengthen the finding by CSA-IgG-ELISA. A high correlation in findings by the two different assays could strengthen the findings of invasive amoebiasis. The use of defined proteins in serodiagnosis will facilitate standardization of the assays and lead to more consistent results. This study aimed to determine the amoebic seropositivity by rCL- and CSA-IgG-ELISA in relation to occupational exposure of amoebiasis among Kelantan cattle farm dwellers.

1.3 Objective of the study

1.3.1 General objective

To compare the amoebic seropositivity among Kelantan cattle farm dwellers, via CSA and rCL IgG-ELISAs.

1.3.2 Specific objectives

1. To determine the CSA and rCL protein concentrations and their profiles by Bradford assay and SDS-PAGE
2. To determine the seropositivity of amoebiasis among Kelantan cattle farm dwellers by RIDASCREEN® *Entamoeba histolytica* IgG
3. To determine the seropositivity of amoebiasis among Kelantan cattle farm dwellers by CSA- and rCL-IgG ELISAs
4. To compare the seropositivity of amoebiasis between the customised ELISAs (CSA and rCL proteins) and the reference ELISA

1.4 Flow chart of the study

In this study, a commercial assay *i.e.* RIDASCREEN® *Entamoeba histolytica* IgG was used as a reference test. Three main phases involved which comprised: (i) protein profiling of rCL and CSA proteins, (ii) screening of the serum samples, and (iii) comparative analysis.

Figure 1.1 summarizes the experimental work involved in this research.

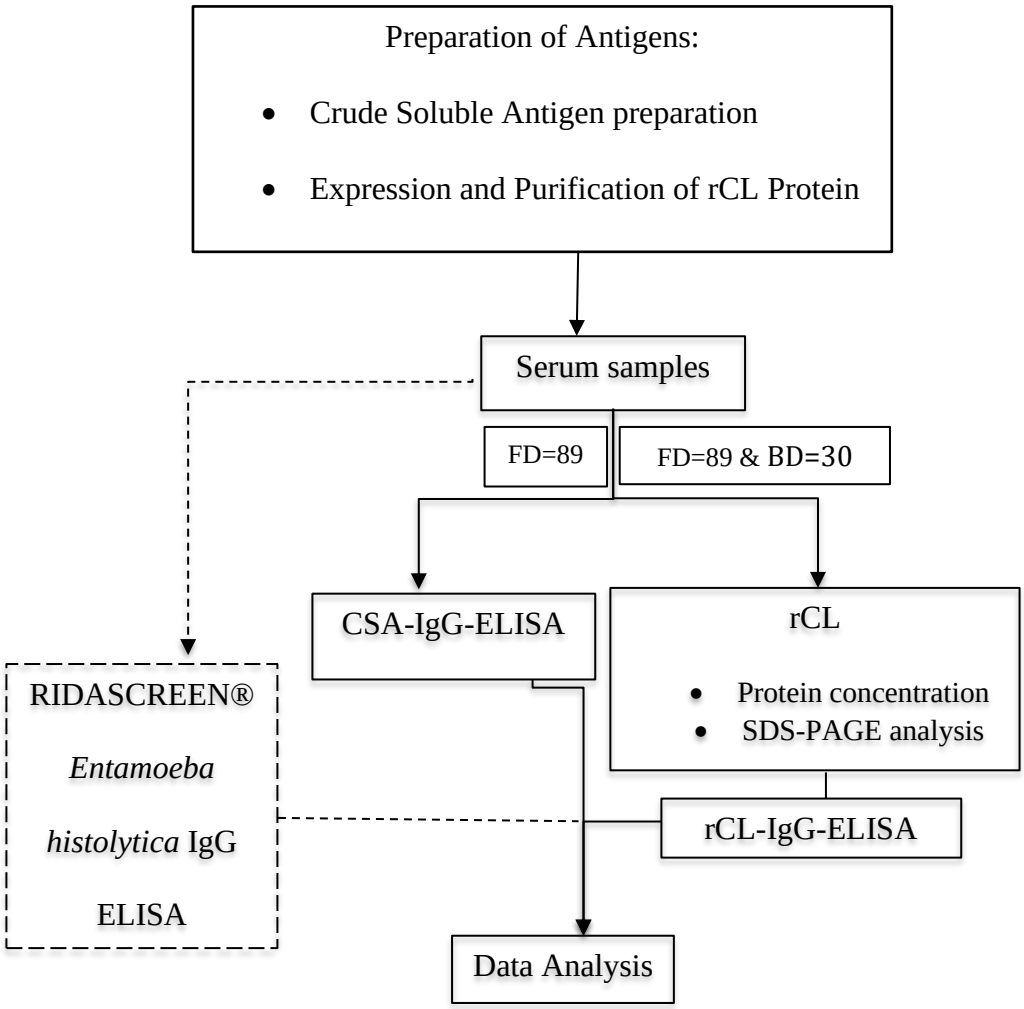

Figure 1.1 Flowchart of the study.

Note: FD: Farm dwellers; BD: Blood Donors; rCL: recombinant c-terminal lectin; CSA: crude soluble antigen

CHAPTER 2

LITERATURE REVIEW

2.1 *Entamoeba histolytica*

Entamoeba histolytica protozoan; the cause of amoebiasis, was first identified in 1875 by Fedor Aleksandrovich Lösch who detected amoebic trophozoites in the stools of a patient experiencing severe diarrhea in St. Petersburg (Ravdin, 1995). Kock and Gaffky in 1887 and Kartulis in 1889 provided a definite correlation between amoeba and disease when they reported amoebae in dysenteric patients' intestinal lesions. Fritz Richard Schaudinn was a German zoologist who distinguished *E. histolytica* from *E. coli*. Because of its ability to produce tissue lysis, Schaudinn named it *E. histolytica*. It is pathogenic, as its name implies (*histo–lytic* = tissue destroying) (Pinilla, López, & Viasus, 2008).

2.2 Taxonomy

E. histolytica is defined as a unicellular eukaryote that belong to the kingdom of *Protista*, phylum of *Sarcomastigophora* (with pseudopodia and/or flagella), and the subphylum of *Sarcodina,* order *Amoebida,* family *Endamoebidae* and the genus *Entamoeba* (Lee, Leedale, & Bradbury, 2000).

2.3 Morphological description

There are eight different types of human intestinal amoeba (*E. histolytica, E. polecki, E. gingivalis, E. moshkovskii, E. dispar, E. coli, E. hartmanni and E. Bangladeshi*). However, *Entamoeba histolytica* is the only pathogenic species found to cause amoebic dysentery and produce a number of severe symptoms such as amoebic liver

abscess, purulent pericarditis, and pneumonia. Other species are considered non-pathogenic and cause disease in humans only in rare cases (Verweij et al., 2003).

E. histolytica can be found in five different forms: trophozoites, precysts, cysts; which is the infective form, metacysts, and metacystic trophozoites. The trophozoite is a motile amorphous shape phagocyte that moves with the help of pseudopodia, usually measures 10–60 μm in diameter, and has a transparent coarse granular cytoplasm and a vesicular nucleus with a central endosome, peripheral chromatin and radial achromatic fibrils, with chromatin beads aggregating at the nuclear membrane. The nucleus and food vacuoles are found in the endoplasm, which may also include bacteria or red blood cells. The trophozoite of *E. histolytica* has proteolytic enzymes like proteinase and hyaluronidase which can lyse cells and tissues when crossing intestinal barriers to spread to various vital organs, especially the liver, as well as the lungs, and brain. Figure 1

The cysts are round in shape, 10-20 μm in size. Mature cysts have four nuclei, each one with central karyosomes and fine distributed peripheral chromatin (Figure 2.1 & Figure 2.2).

E. moshkovskii cannot be differentiated easily from *E. histolytica* and *E. dispar*, and its cyst and trophozoite structures are microscopically similar to them. A variety of non-pathogenic species can invade the human gastrointestinal tract, which can lead to misdiagnosis. They include *Entamoeba gingivalis, Entamoeba coli,* and *Entamoeba hartmanni.* Morphological comparison among *Entamoeba* species is listed in Table 1.

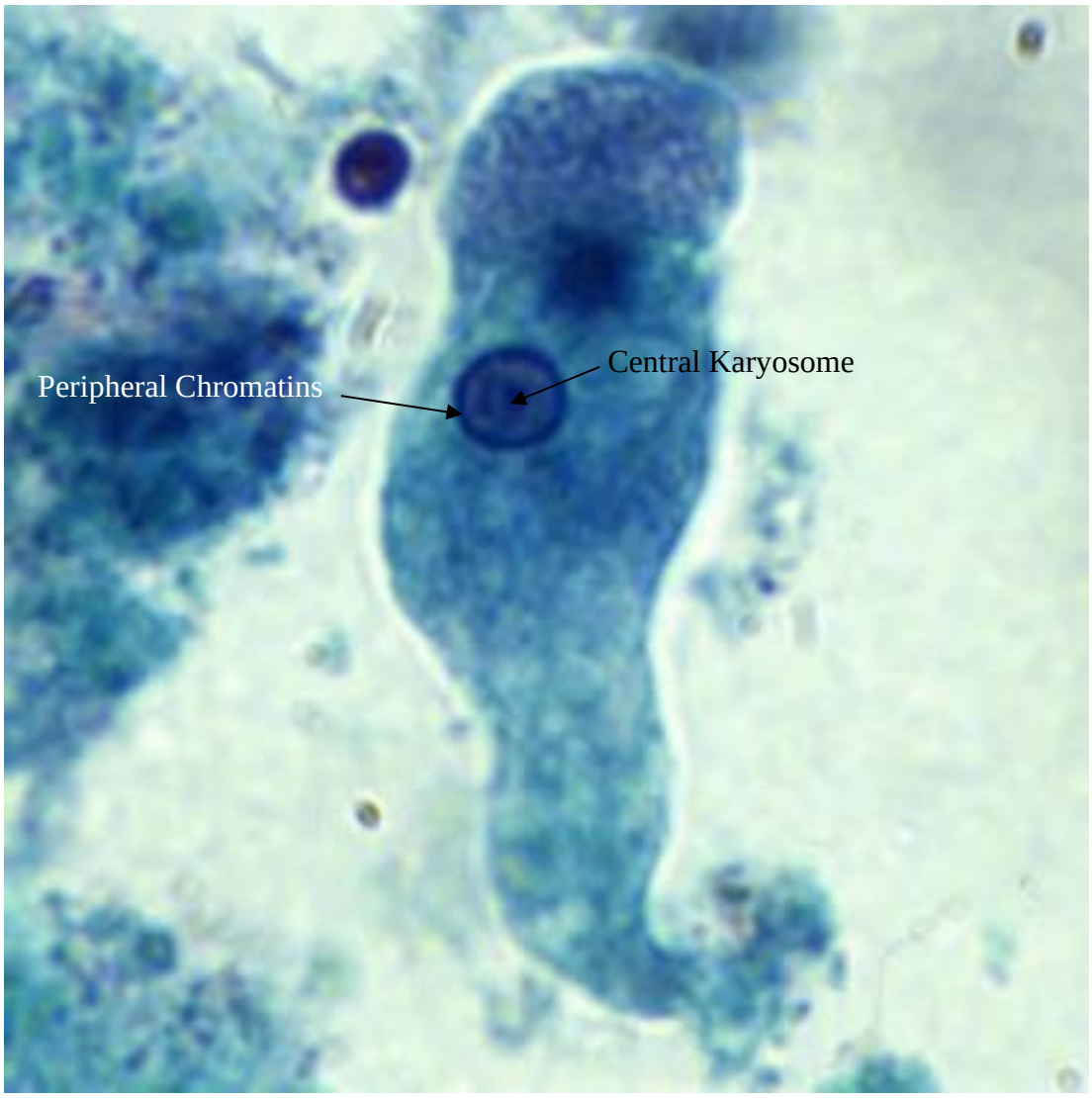

Figure 2.1 Trophozoite of *E. histolytica/E. dispar* stained with trichrome (CDC, 2015).

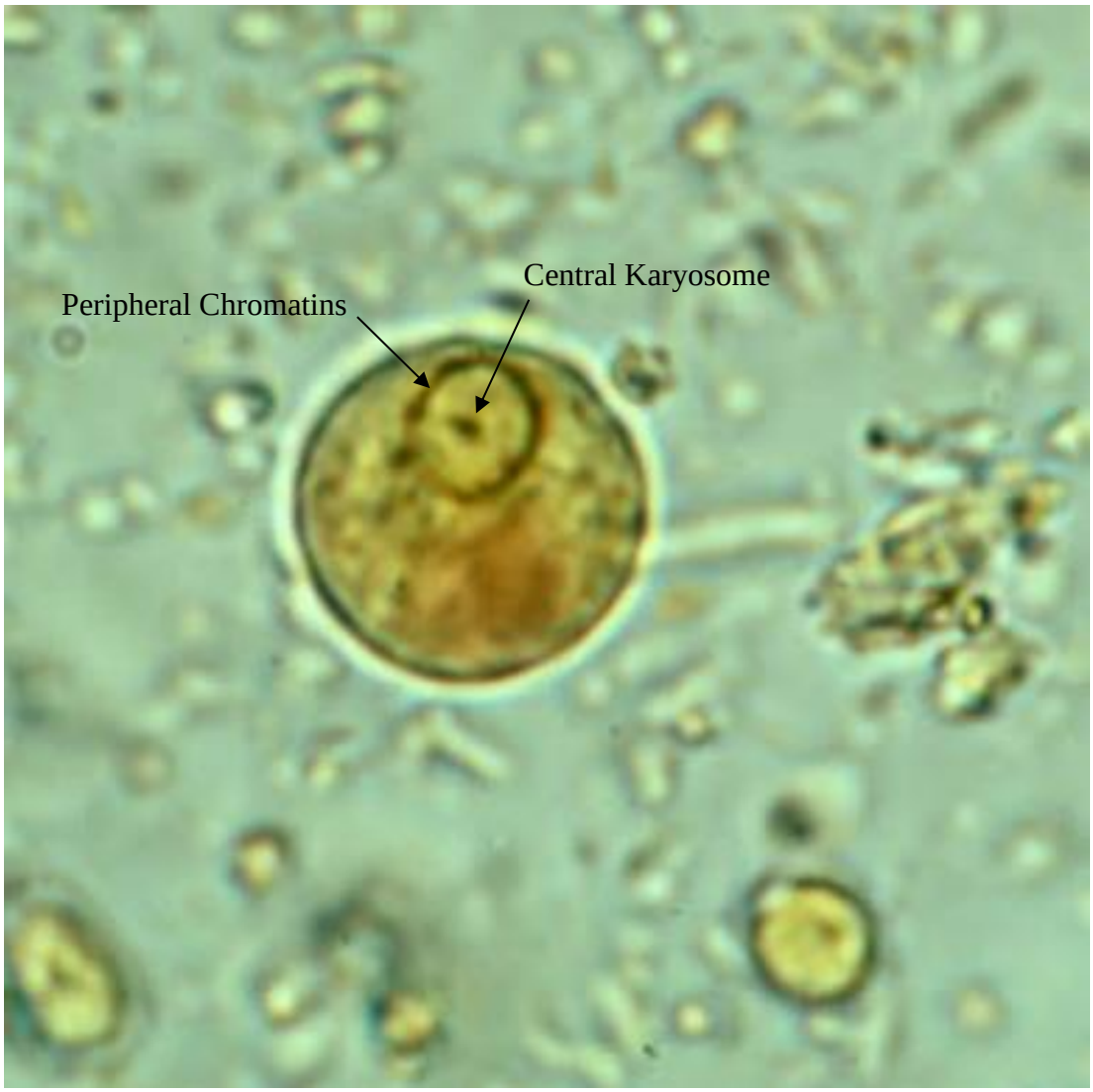

Figure 2.2 Cyst of *E. histolytica/E. dispar* in a concentrated wet mount stained with iodine (CDC, 2015).

Table 2.1 Morphological comparison between human intestinal lumen amoebas. (Baron, 1996)

	Size		Trophozoite		Cyst	
Species	**Trophozoite**	**Cyst**	**Motility**	**Nuclei morphology**	**Nuclei Chromatoids**	**Remarks**
Entamoeba histolytica	10-60 μm	10-20 μm round	Active	Karyosome small and central. Chromatin fine and peripheral.	Rounded ends.	Pathogenic
Entamoeba hartmanni	4-12 μm	5-10 μm round	Active	Karyosome small and central. Chromatin fine and peripheral.	Rounded ends.	Nonpathogenic.
Entamoeba gingivalis	5-35 μm	-	-	Karyosome small and central. Chromatin fine and peripheral.	Pointed ends.	Nonpathogenic.
Entamoeba polecki	10-20 μm	5-10 μm	Sluggish	Karyosome small and central. Chromatin variable.	Rounded ends.	Nonpathogenic.
Entamoeba moshkovskii	10-60 μm	5-20 μm round	-	Karyosome small and central. Chromatin fine and peripheral.	Rounded ends.	Nonpathogenic. (rare in human.
Entamoeba coli	10-50 μm	10-35 μm	sluggish	Karyosome large. Chromatin clumpy and peripheral.	Jagged ends.	Nonpathogenic.

2.4 Life Cycle

Amoeba in general transmits and replicates only via humans and some animals such as dogs, cats, and monkeys. However, animals can host *E.histolytica*, yet none of them excrete cysts in their faeces (Regan, Yon, Hossain, & Elsheikha, 2014).

Life cycle of *Entamoeba histolytica* has many stages (Figure 2.3). The infection occurs when the cysts are shed in the host's faeces and eventually infect food and water through direct or indirect contact with contaminated faeces. The cyst's chitin wall protects it from the gastric acidity. The excystation occurs only when cysts reach the ileum to release the trophozoites by lysing the cyst wall due to trypsin enzyme action. Trophozoites migrate to reside in the large intestine, where they feed and compete on ingested nutrients with other organisms such as bacteria and viruses.

In the trophozoite stage the parasite produce galactose and N-acetyl D-galactosamine (Gal/GalNAc)-specific lectin (Gal/GalNAc lectin) which is a biochemical compound that allows the parasite to adhere to the epithelial cells in the digestive tract and penetrate the mucous layer (Bansal et al., 2009).This damage in the intestinal wall results in amoebiasis dysenteric and enables the trophozoites to enter the bloodstream, whereby they may spread to the lung, liver, and brain and give rise to extra intestinal diseases, which account for almost 10 to 20% of cases (Rashidul Haque, Huston, Hughes, Houpt, & Petri Jr, 2003). However, in most cases; trophozoites gather in the intestinal layer, making new cysts and leading to asymptomatic infection (Hategekimana, Saha, & Chaturvedi, 2016).

Later, both forms are shed through the faeces: trophozoites are typically found in loose stool, and cysts are typically found in hard stools. Mature cysts can tolerate harsh environment conditions for several days or even weeks due to the protection provided by their walls (EICHINGER, 2001). Figure 2.3 shows the *E. histolytica* life cycle after the cyst is ingested for both types of disease: asymptomatic (non-invasive) infection which represents 80% to 90% of cases, while invasive infection represents 10% to 20% of cases.

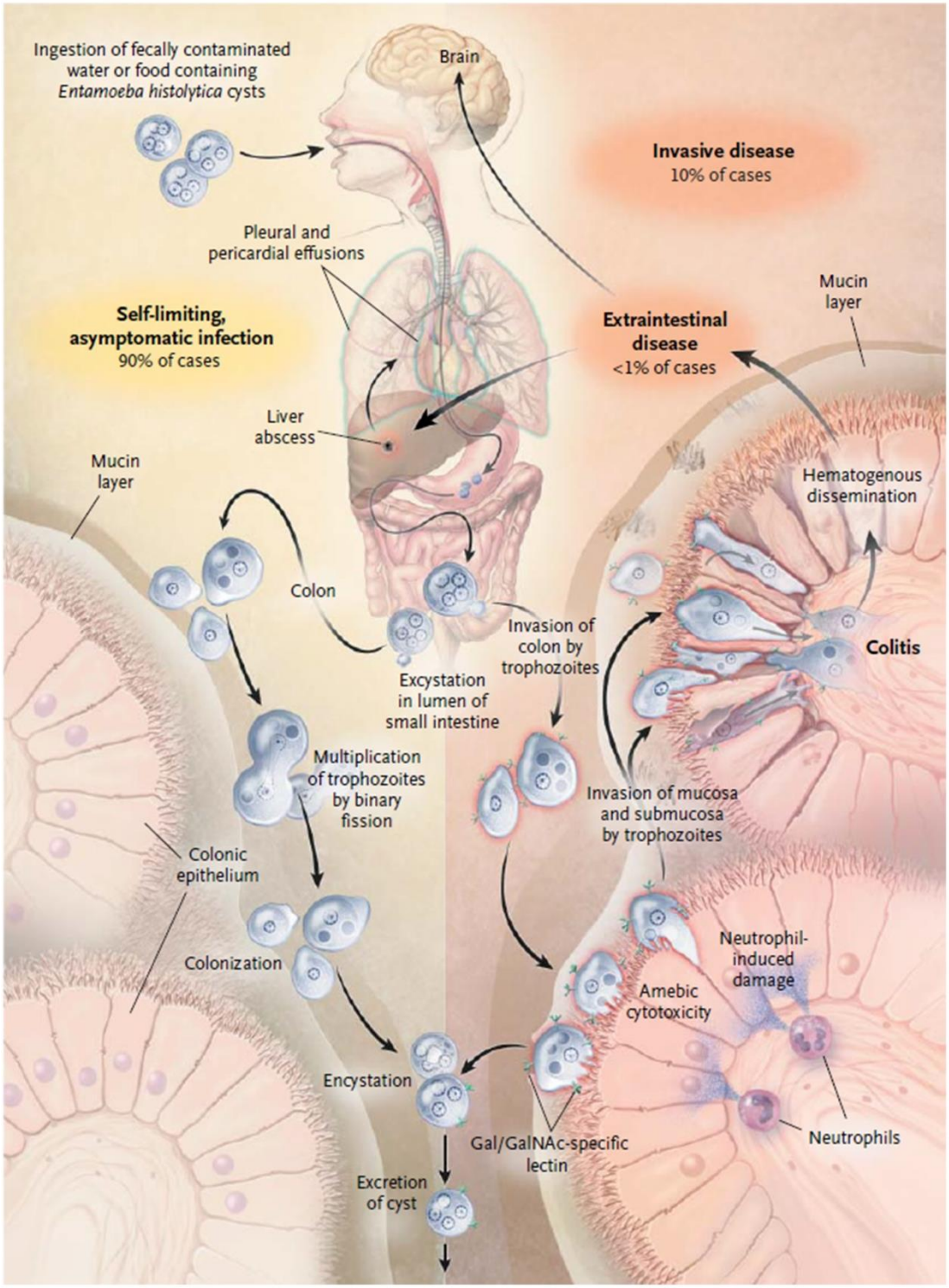

Figure 2.3 Life cycle of *Entamoeba histolytica* (Rashidul Haque et al., 2003).

2.5 Transmission

2.5.1 Routes of transmission and reservoirs

E. histolytica, like most intestinal protozoa, is transmitted through the ingestion of cysts found in contaminated water or food. The trophozoite stage found exclusively in the host and in fresh faeces, while cysts remain alive outside the host in food, water, and moist soils. They can withstand harsh environmental conditions for days to weeks in sewage. Major sources of infection include cattle and domestic pet handling, indirect hand contamination from contaminated surfaces, and rarely anal sexual contact with a asymptomatic carrier (Association, 2006).

Despite continued improvements in better sanitary facilities or urban services, and enhancing water quality, foodborne and waterborne transmission continue to be the main reservoirs of amoebiasis. Food contamination can occur at different phases, including manufacturing, harvesting, handling, shipping, and processing. The infection transmission increases if vegetables is consumed uncooked or undercooked (F. L. Schuster & Visvesvara, 2004).

2.5.2 Risk factors

A study done by (Benetton et al., 2005) in Brazil, reported that people eating raw vegetables had a 1.6 folds higher risk of getting amebiasis than people who did not eat uncooked vegetables. They also identified the sources of contamination and linked them to the water quality being used in washing fruits or vegetables as well as food preparation practices.

Additionally, poor nutrition is one of the important risk factors for amoebiasis due to its impact on immunity. Inadequate hygiene practice, which is remarkably common among children in day-cares and some institutions such as prisons and orphanages, are considered important cause of acquiring and transmitting amoebiasis *E. histolytica*.

In endemic regions, waterborne infections are frequent, due to inadequate chlorine water treatment or no water purification at all (Rashidul Haque et al., 2003). A study conducted in Malaysia among the Orang Asli highlighted that consuming untreated water, bathing in streams and river, those who were less than 15 years old in all studied ethnic groups, poor hygiene practice such as eating with unwashed hands after playing in the soil or gardening, having other protozoan infections, and low socioeconomic status as important risk factors for amoebiasis (Shahrul Anuar et al., 2012).

2.6 Pathogenesis

Complex host-parasite interactions that involve many amoebic and host factors resulting in parasite pathogenicity, triggering host defence responses and parasite tolerance to stress produced by host defences and environmental changes during invasion (Faust & Guillen, 2012). The pathogenesis of *E. histolytica* infection is

expected to be controlled by different mechanisms, the most important of which are: (i) trophozoite adherence to the host cell, (ii) lysis of host cell, and (iii) phagocytosis of host cell (Sehgal, Bhattacharya, & Bhattacharya, 1996).

Trophozoites of *E. histolytica* can cause cell lysis, which in turn causes inflammation and activates the host's immune system (Figure 2.4). Colon epithelium invasion is the consequence of a set of actions, which includes adhesion to host cells, stimulation of host cell lysis, and phagocytosis of dead cells (Ahmad, Mishra, Lata, & Gourinath, 2020).When a trophozoite moves to the large intestine, it proliferates and produces a number of proliferating cells inside the mucosa. Part of the cell group then grows into mature cyst and shed with faeces. The trophozoites that grow in the intestine can penetrate the mucosa and invade the intestinal epithelium through a variety of mechanisms. (Figure2.4) (Frederick & Petri, 2005).

Proteolytic enzymes are of special interest due to their important role in amoebic pathogenicity. In the *E. histolytica* genome, 86 peptidase genes were identified, which include 50 cysteine, four aspartic, ten serine, and twenty metallo peptidases (Clark et al., 2007). Several cysteine proteases have been discovered as cyst specific, but their specific roles in amoebic biology and virulence are unknown. Rhomboid proteases are another type that has been identified lately. They are intramembrane serine proteases able to cleave transmembrane proteins in their transmembrane domains and have been linked to parasite host cell invasion (Urban, 2006).

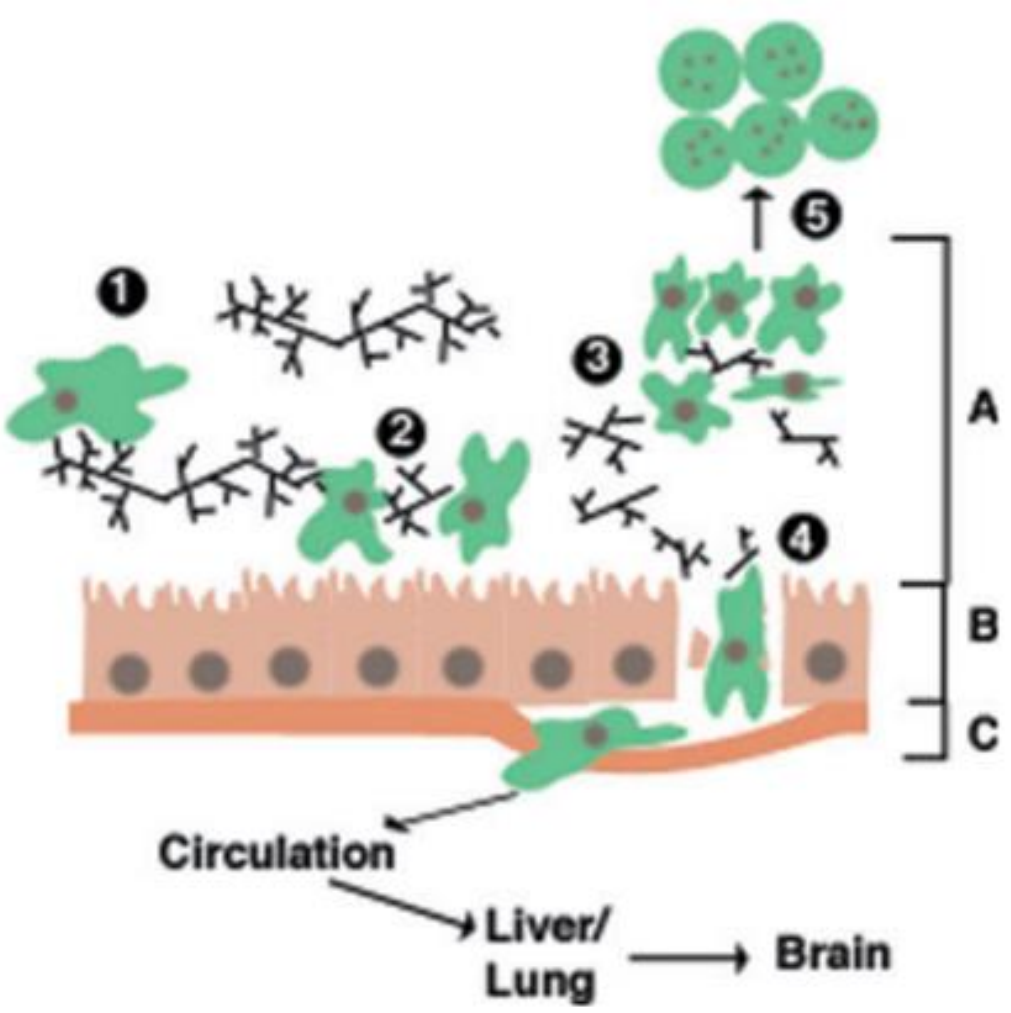

Figure 2.4 Colonization of *Entamoeba histolytica* in large intestine. (A) Mucus layer, (B) epithelium, and (C) basal lamina.

Note: Trophozoites travel from the small intestine into the large intestine where they interact with bacteria and the intestinal mucin layer. (1) Proteolytic enzymes such as glycosidase and protease dissolve the mucosa layer. (2) Once crucial ratios of mucin glycosylation and cell number is achieved, (3) trophozoites combine together and produce mature cysts. (4) Trophozoites can also destroy host cells after the protecting mucous layer has been dissolved. (5) This results in invasion and may lead to extra intestinal disease (Frederick & Petri, 2005).

Parasites can engulf dead host cells in order to limit immune reactions by the host due to accumulation of dead cells and immune system signalling. Invasive *E. histolytica* infection is characterised by cytotoxicity and phagocytosis of host cells. *E. histolytica* triggers apoptosis before phagocytosis via a process that needs interaction with an amoebic galactose-specific lectin. However, lectin blockage can partially prevent phagocytosis of already dead host cells, this indicates that phagocytosis involves at least one more receptor. The serine-rich *E. histolytica* protein, which is an immunogenic surface protein, found to be involved in the phagocytosis of dead host cells (Teixeira & Huston, 2008).

2.7 Epidemiology

According to the World Health Organization, *E. histolytica* infects roughly 500 million people globally; leading to symptomatic infection in 50 million and death in 100,000 patients due to serious complications caused by invasive disease, with the majority of cases occurring in tropical and subtropical regions with insufficient health care, and inappropriate water supply and sanitation technologies (Bryce, Boschi-Pinto, Shibuya, & Black, 2005). Parasite infections play a significant role to the burden of the disease, often leading to death, and affects individuals in both the developed and developing countries (WHO, 1997).

Many epidemics caused by the presence of *E. histolytica* have been linked to water contamination. In the United States in the last 30 years, six waterborne and foodborne outbreaks reported between year 1946 and 1980. The most severe outbreak in the United States occurred in 1933, with 1000 cases and 58 deaths; it was related to inadequate infrastructure that led to contaminate drinking water with sewage (Howard, 1997).

Amoebiasis infection is the fifth or sixth leading cause of mortality in Mexico, for example prevalence and morbidity data showed that between the years 2002 to 2006; the prevalence rate ranged from 615.85 to 1228.8 cases per 100,000 population (Ximénez, Morán, Rojas, Valadez, & Gómez, 2009).

A study done in New Delhi slums in India using a PCR assay reported 8% prevalence in people living in slum areas. Another study conducted in the same place reported higher rates; the prevalence rate of intestinal amoebiasis was shown to be about 19.9 %. The studies linked rural residence, poor personal hygiene habits, and low socio-economic status to increased risk of acquiring *E. histolytica* infection (Nath, Singha, Paul, & Ghosh, 2018).

Amoebiasis has been found to affect up to 50% of the African population (Al-harthi & Jamjoom, 2007). However, a previous study in Nigeria reported that 27% of preschool children were infected with *E. histolytica*. These findings are confirmed by a research conducted in west Kenya among people attending local hospital, in which 21% of patients tested positive for *E. histolytica* (Kinuthia, Afolayan, Ngure, & Anjili, 2012).

A study in southern Italy involved 1766 patients observed between 2009 and 2010. In the study participants, a broad range of intestinal parasites were detected and indicated that parasite infection was significantly more common in refugees (18–44%) than among natives (9%). *E. histolytica* and *E. dispar* were found in 8% of the refugees and 3% of the natives screened (Belli et al., 2014). In Australia; a retrospective analysis

done by (Domazetovska et al., 2018) in Sydney found that out of 173 patients, 49 were found to be *E. histolytica* positive using serological assays.

A series of community-based questionnaire were conducted among three different native groups to determine the prevalence and risk factors related to *E. histolytica/E. dispar/E. moshkovskii* complex (*Entamoeba* complex) infection in selected villages in several states in Malaysia and faecal samples were examined using staining methods. Out of 500 samples tested positive for *Entamoeba* complex infection, 8.7 % were Malay, 29% percent were Negrito, and 18% percent were Senoi. The prevalence of amoebiasis was shown to be age dependent, with greater rates reported in those under the age of 15 years (Shahrul Anuar et al., 2012).

Microscopy and polymerase chain reaction were used to examine the presence of *Entamoeba* species in 504 faecal samples obtained randomly from 411 individuals in Malaysia. The most prevalent species was *E. dispar* (26.5%), followed by *E. histolytica and E. moshkovskii* (20.4% for each species respectively) (Ngui et al., 2020).

Several observational studies were conducted to determine the contribution of amoebiasis to the prevalence of gastro-intestinal disease in children. Two hundred thirty children were screened in an observational study in Bangladesh and found that asymptomatic infection with *E. histolytica* was 5% and *E. dispar* was 13 % in children less than five years old in refugee camps. Amoebiasis is common in this urban preschool children and they have a higher risk of the future development of invasive disease (Rabiul Haque, Ali, & Petri, 1999).

Another study conducted on preschool-age children in Gaza Strip suffering gastroenteritis found that the incidence of pathogens inducing gastroenteritis was higher in symptomatic patients than among asymptomatic patients (88 and 11%, respectively). *E. histolytica* (28%) and *Giardia lamblia* (26%) were the most common detected intestinal pathogens (Al Laham, Elyazji, Al-Haddad, & Ridwan, 2015).

Farmers are considered to have higher risk of being infected with parasitic infections due to their work duties nature such as dealing with animal excreta and being exposed to contaminated water. According to one study in Vietnam, farming practices that use human and animal excreta and household wastewater as fertilizers were not relevant for the *E. histolytica* transmission. Individuals who never or rarely washed their hands with soap were 3.4 times more likely to become infected than those who always used soap. This indicates that other transmission routes, such as contaminated hands, are more significant in these situations and provides additional evidence that personal hygiene practices, such as hand washing with soap must be encouraged (Duc et al., 2011).

2.8 Laboratory diagnosis of amoebiasis

Accurate diagnosis of amoebiasis is essential in preventing the spread of amoebiasis and avoiding inappropriate treatments of individuals infected with commensal amoebae. Nevertheless, diagnosing amoebiasis is difficult because it is based on clinical manifestations and laboratory tests with limited sensitivity and specificity.

2.8.1 Microscopy

In developing countries, the diagnosis is mainly dependent on the identification of mature quadrinucleate cysts via wet-mount microscopy of faecal samples (Tanyuksel & Petri, 2003). The outcomes of the microscopic observation are affected by several parameters including storage condition, time spent on sample processing, whether the samples are fixed or not, laboratory personnel experience, and parasite density (Goni et al., 2012). As a result, significant research was done on developing a reliable non-microscopic method for diagnosing amoebiasis, which includes stool culture coupled with isoenzyme analysis, antigen detection assays, or molecular-based screening (Saidin et al., 2019).

In 33-50 % of amoebic colitis cases, microscopic examination of stools can detect trophozoites. Several stool samples taken over a period of no more than ten days raises the sensitivity to 85-95 %. However, apart from the trophozoites, leukocytes may be detected, which is common in *E. histolytica* infection (Burchard, Prange, & Mirelman, 1993).

2.8.2 Culture method

Cultured parasites should, in fact, have the same proliferation and pathogenicity characteristics as their counterparts in their natural habitat (F. L. J. C. m. r. Schuster, 2002). Diamond was the first to establish axenic cultivation of *E. histolytica* in 1961, using a diphasic medium consisting of a serum-enriched agar surface coated by a broth supplemented with chicken extract and vitamins (L. S. J. S. Diamond, 1961). Diamond also introduced TPS-1, a new monophasic media, in 1968 (L. S. J. T. J. o. P. Diamond, 1968).

In 50 % to 70 % of patients, cultures can be used to diagnose amoebiasis. Nonetheless, culture is not a standard practice of detection and is less sensitive than microscopic method. *E. histolytica* cultures are generally used as a research tool rather than diagnostic purposes (Dickson-Gonzalez, de Uribe, & Rodriguez-Morales, 2009).

2.8.3 Antibody detection assays

Antigen detection assays of enzyme-linked immunosorbent assay (ELISA) are available commercially. Kits which include monoclonal antibodies against the GAL/GalNAc–specific lectin and the serine-rich antigen of *E. histolytica* have an overall sensitivity of 71% - 100% and a specificity of 93% - 100% (Dickson-Gonzalez et al., 2009). ELISA assay works by detecting IgG anti-lectin antibodies in the serum. It has a high sensitivity for extraintestinal disease (up to 98%) but a low sensitivity for active amoebic infection due to repeated exposure, especially in endemic regions. However, since antibodies remains for years after infection, antibody-based assays often used for diagnoses of active infection (Tanyuksel & Petri, 2003).

ELISA assays have been used to detect anti-amoeba antibodies in serum as well as to identify amoebic antigens in faeces. Because of the difficulties in distinguishing a past infection from a current one, it has been used primarily for sero-epidemiological research (Navarro-García & Valadez-Sánchez, 1991). Nevertheless, cases of invasive amoebiasis without tissue invasion may test negative for serum antibodies, leading to false negatives. Usually, crude soluble antigen (CSA) derived from *E. histolytica* trophozoite is used for serodiagnosis purposes of invasive amoebiasis. Furthermore, in endemic regions, high background seropositivity by CSA-assay hinder the interpretation of positive results in clinical settings (Wong et al., 2017).