MOLECULAR EPIDEMIOLOGY OF *ENTAMOEBA HISTOLYTICA, ENTAMOEBA DISPAR* AND *ENTAMOEBA MOSHKOVSKII* INFECTIONS IN RURAL YEMEN

MONA ABDULLAH MOHAMMED Al-AREEQI

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA KUALA LUMPUR

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MONA ABDULLAH MOHAMMED Al-AREEQI

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50603 KUALA LUMPUR

ABSTRACT

Intestinal amoebiasis is highly prevalent in Yemen particularly in rural areas; however there is a great scarcity of information on the prevalence of species-specific *Entamoeba* infections in Yemen and many other countries due to the re-description of pathogenic *Entamoeba histolytica* and non-pathogenic *E. dispar* and *E. moshkovskii*. Therefore, this community-based study is the first to provide information on the true prevalence of *E. histolytica*, *E. dispar* and *E. moshkovskii* infections in rural communities in Yemen. The study also aimed to examine the association of these *Entamoeba* infections with some potential risk factors. A total of 605 stool samples from four provinces namely Sana'a, Dhamar, Taiz and Hodiedah were randomly collected and examined by wet mount, formalin-ether sedimentation, trichrome staining and nested multiplex PCR techniques. Demographic, socioeconomic and environmental information was collected by using a pre-tested questionnaire. Overall, 324 (53.6%) of the samples were positive for *Entamoeba* cysts and/or trophozoites by microscopic examination. The prevalence was significantly higher among male participants compared to female (P = 0.008). An age-dependency distribution was also observed (P< 0.001). Using molecular analysis, it was found that 20.2%, 15.7% and 18.2% of the samples were positive for *E. histolytica*, *E. dispar* and *E. moshkovskii*, respectively. Multivariate analysis showed different sets of species-specific risk factors among these communities. Educational level was identified as the significant risk factor for *E. histolytica*; age and gender were the significant risk factors for *E. moshkovskii*; and sources of drinking water and consumption of unwashed vegetables were the significant risk factors for *E. dispar*. Moreover, living in coastal/foothill areas and presence of other infected family members were risk factors for both *E. histolytica* and *E. moshkovskii* infections. The present study provides new insight into the distribution and risk factors of intestinal amoebiasis in Yemen and reveals that *Entamoeba* spp. infection is highly prevalent among these communities

with *E. histolytica, E. dispar* and *E. moshkovskii* differentiated for the first time.Hence, the study emphasizes the need for molecular methods in the diagnosis of infections and for conducting a large-scaled study throughout Yemen to determine the actual species-specific prevalence of *Entamoeba* spp. Moreover, identifying and treating infected family members, providing health education pertinent to good personal and food hygiene practices, and providing clean drinking water should be considered in developing a strategy to control intestinal parasitic infections in these communities, particularly in the coastal/foothill areas of the country.

ABSTRAK

Amoebiasis usus sangat umum di Yaman khususnya di kawasan luar bandar, bagaimanapun terdapat satu kekurangan maklumat yang besar mengenai kelaziman spesies khusus jangkitan *Entamoeba* di Yaman dan negara-negara lain disebabkan oleh penerangan semula daripada *Entamoeba histolytica* patogenik dan bukan patogen *E. dispar* dan *E. moshkovskii*. Oleh itu, kajian berasaskan komuniti ini adalah yang pertama untuk memberi maklumat tentang kelaziman sebenar *E. histolytica*, *E. dispar* dan jangkitan *E. moshkovskii* dalam kalangan masyarakat luar bandar di Yaman. Kajian ini juga bertujuan untuk mengkaji kaitan antara jangkitan *Entamoeba* dengan beberapa faktor risiko yang berpotensi. Sebanyak 605 sampel najis daripada empat wilayah iaitu Sana'a, Dhamar, Taiz dan Hodiedah dikumpulkan secara rawak dan diperiksa melalui wet mount, pemendapan formalin-eter, pewarnaan trichrome dan teknik PCR multipleks bersarang. Maklumat demografi, sosioekonomi dan alam sekitar telah dikumpulkan dengan menggunakan soal selidik pra-diuji. Secara keseluruhan, 324 (53.6%) daripada sampel adalah positif untuk sista *Entamoeba* dan/ atau trophozoites melalui pemeriksaan mikroskopik. Kelaziman adalah jauh lebih tinggi di kalangan peserta lelaki berbanding perempuan (P = 0.008). Pengagihan usia pergantungan juga diperhatikan (P<0.001). Dengan menggunakan analisis molekul, ia telah mendapati bahawa 20.2%, 15.7% dan 18.2% daripada sampel adalah positif untuk *E. histolytica*, *E. dispar* dan *E. moshkovskii*, masing-masing. Analisis multivariat menunjukkan set faktor risiko spesies khusus di kalangan masyarakat ini. Tahap pendidikan telah dikenal pasti sebagai faktor risiko yang penting bagi *E. histolytica*; umur dan jantina merupakan faktor risiko yang signifikan bagi *E. moshkovskii*; dan sumber-sumber air minum dan penggunaan sayur-sayuran yang tidak dibasuh merupakan faktor risiko yang signifikan bagi *E. dispar*. Lebih-lebih lagi, yang tinggal di kawasan pantai/ kaki bukit dan kehadiran ahli keluarga lain yang dijangkiti merupakan faktor risiko untuk kedua-dua *E. histolytica* dan

jangkitan *E moshkovskii*. Kajian ini memberikan pandangan baru ke dalam pengedaran dan risiko faktor amoebiasis usus di Yaman dan mendedahkan bahawa jangkitan *Entamoeba* spp. sangat umum di kalangan komuniti ini dengan *E. histolytica*, *E. dispar* dan *E. moshkovskii* jangkitan untuk kali pertama. Oleh itu, kajian ini menekankan keperluan untuk kaedah molekul dalam diagnosis jangkitan dan juga dalam menjalankan kajian berskala besar di seluruh negara untuk menentukan prevalens spesies khusus sebenar *Entamoeba* spp. Selain itu, mengenal pasti dan merawat ahli keluarga yang dijangkiti, memberikan pendidikan kesihatan berkaitan dengan baik amalan kebersihan diri dan makanan, dan menyediakan air minuman yang bersih perlu dipertimbangkan dalam membangunkan strategi untuk mengawal jangkitan parasit usus dalam komuniti ini, terutamanya di kawasan pantai / kaki bukit negara.

Dedication

Every challenging work needs self-efforts as well as guidance of elder especially those who are very close to our heart.

My humble effort I dedicate to my sweethearts and loving,

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& Mother "Maryiam",

Whose affection, love, encouragement and prays of day and night make me able to get such success and honor,

Along with those give me their love and all the support, my lovely sisters & brother, and my best friend ***"Abkar"****…*

May Almighty ALLAH continue to protect, guide and bless them.

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LIST OF SYMBOLS AND ABBERVIATIONS

SPSS	Statistical Package for Social Sciences
AOR	Adjusted Odds Ratio
OR	Odds Ratio
χ^2	Chi-square
CI	Confidence Interval
IQR	Interquartile Range
P	Level of significance
SD	Standard Deviation
%	Percentage
PARF	Population Attributable Risk Fraction
mg	Milligram
g	Gram
μm	Micrometer
mm	Millimeter
mM	Millimole
mL	Millilitre
L	Liter
KDa	Kilo Dalton
km	Kilometer
km^2	Square Kilometer
>	Larger than
<	Less than
≥	Equals or larger than
≤	Equals or less than
ºC	Degree Celsius
E	*Entamoeba*
spp.	Species
Gal/GalNAC	Galactose/N-Acetyl-D-Galactosamine
GIT	Gastrointestinal Tract
ALA	Amoebic Liver Abscess
HIV	Human Immunodeficiency Virus

PVA	Polyvinyl Alcohol
ELISA	Enzyme-Linked Immunosorbent Assay
CIE	Counter Immunoelectrophoresis
IHA	Indirect Haemagglutination
IgG	Immunoglobulin G
bp	Base pair
DNA	Deoxyribonucleic acid
rDNA	Ribosomal DNA
dNTP	Deoxynucleoside Triphosphate
RNA	Ribonucleic Acid
SSU rRNA	Small subunit Ribosomal RNA
PCR	Polymerase Chain Reaction
WHO	World Health Organization
CDC	Center of Disease Control and Prevention
UNICEF	United Nations International Children's Education Fund
NIC	National Informatics Centre
DALYs	Disability-Adjusted Life Years
GDP	Gross Domestic depending Product
RM	Malaysian Ringgit
US$	US Dollar
YER	Yemeni Rials

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

Entamoeba histolytica is a protozoan parasite cause a disease called amoebiasis that is estimated to affect 50 million people annually with about 10% of them develop invasive amoebiasis that leads to about 40,000-110,000 deaths per annum (WHO, 1997; Baxt and Singh, 2008; Fletcher *et al*., 2012). Human gets the infection by ingestion of food or drinks polluted with *Entamoeba* cysts and it might be transmitted among homosexual men frequently due to oral-anal and oral-genital sexual contact (Stanley, 2003; Stark *et al*., 2008).*E. histolytica* infection in its clinical manifestation varies from asymptomatic colonization, which accounts for 90% of all infections, to fatal complications such as amoebic dysentery and invasive extra-intestinal amoebiasis which manifests as amoebic liver abscess (ALA) or spreads to other organs such as the lungs, brain and subcutaneous tissues (Ximénez *et al*., 2009). In developed countries, amoebiasis is commonly reported with a prevalence ranging between 0.2% and 12.5% (Fletcher *et al*., 2012), and the risk is increased among immunocompromised, sexually active homosexuals and institutionalized individuals (Hung *et al*., 2008; Salit *et al*., 2009). In developing countries, a higher prevalence of amoebiasis is associated with poor socioeconomic, environmental, and sanitary and hygiene conditions, with a high severity rate among children and malnourished individuals, and ALA is measured as the third parasitic infection leading to death after malaria and schistosomiasis (Stanley, 2003; Ilikkan *et al*., 2005; Cairncross *et al*., 2010; Anuar *et al*., 2012b; Hegazi *et al*., 2013).

The new classification of human intestinal *Entamoeba* includes eight species namely *E. histolytica, E. dispar, E. moshkovskii, E. coli, E. hartmanni, E. bangladeshi* and *E. poleki* (Nath *et al*., 2015). Of these, three species have an identical morphology -

E. histolytica, E. dispar, and *E. moshkovskii* (together mentioned as *Entamoeba* complex) - and *E. histolytica* is the pathogenic and the causative agent of the symptomatic disease (Ximénez *et al.*, 2009). According to Diamond and Clark, *Entamoeba dispar,* a non-invasive species,was first proposed by Brumpt in 1925 when he differentiated both species (*E. histolytica* and *E. dispar)* depends on their pathogenicity in humans and kittens, and further evidence was provided by Simic in 1931 (Diamond and Clark, 1993). However, the proposed distinction between the two species was only buttressed in 1978 when two groups of *Entamoeba*, isolated from symptomatic and asymptomatic individuals, were distinguished based on their isoenzymatic profile (zymodemes) (Sargeaunt *et al.*, 1987). Subsequently, several biochemical, immunological and molecular assays were developed to successfully distinguish the two species (Tanyuksel and Petri, 2003; Diamond and Clark, 1993).

Another non-pathogenic species is *E. moshkovskii*, which was initially discovered in Moscow sewage by Tshalaiain 1941 (Tshalaia, 1941). It was considered as a free-living environmental amoeba until 1956 when it isolated from a dweller of Laredo, Texas that existing with reduction of weight, epigastric ache, and diarrhea (Clark and Diamond, 1991). Since then, many countries such as Italy (Scaglia *et al.*, 1982), Bangladesh (Ali *et al*., 2003), Thailand (Hamzah *et al*., 2006), Turkey (Tanyuksel *et al*., 2007), Tunisia (Ayed *et al.*, 2008), Australia (Fotedar *et al.*, 2008), Colombia (López *et al*., 2015), Tanzania (Beck *et al.*, 2008), Malaysia (Anuar *et al*., 2012b), Iran (Zebardast *et al*., 2014),and India (Nath *et al.*, 2015) have successfully discriminated *E. moshkovskii* strain in humans.

Despite many previous studies that differentiated *Entamoeba* species in developing diagnostic molecular protocols or reporting purposes, there is still a scarcity of information concerningthe aetiological determinants of *E. histolytica*, *E. dispar,* and *E. moshkovskii* infections between targeted populations.While several studies showed

no pathogenic role for both *E. dispar*, some studies revealed that *E. dispar* has proteolytic activity and can produce significant focal intestinal and hepatic lesions in experimental animals resemble those caused by *E. histolytica* with controversial strain-specific role for the presence of microbiota (Vohra *et al.*, 1989; Diamond and Clark, 1993; Costa *et al.*, 2006; Shibayama *et al.*, 2007; Dolabella *et al.*, 2012). Similarly, previous studies from different countries including Malaysia, Bangladesh, Turkey and Australia showed that individuals infected with *E. moshkovskii* can develop gastrointestinal (GIT) symptoms including abdominal pain, nausea, weight loss, dysentery, diarrhea, and loss of appetite (Anuar *et al.*, 2012a; Ali *et al.*, 2003; Tanyuksel *et al.* 2007; Fotedar *et al.* 2008).

Amoebiasis is diagnosed by a variety of laboratory techniques. Conventionally, finding of *E. histolytica* in a human sample in the laboratory has depended on the microscopic investigation of fixed or fresh faecal specimens (WHO, 1997; Nath *et al.*, 2015). Although microscopic examination is cheap and easy to perform, but it has a number of limitations; the most important being the incapability to discriminate *E. histolytica*, *E. dispar* and *E. moshkovskii*. (Baxt and Singh, 2008). The epidemiology of *Entamoeba* could be further studied through determining isoenzyme patterns using gel electrophoresis and culturing trophozoites (Sargeaunt *et al.*, 1987). However, these methods are time consuming, difficult, luxurious, and are not workable for routine diagnostic laboratories (Fotedar *et al.*, 2007b). Antibodies detection for amoeba in patients' sera has been described to elucidate the infection by *E. histolytica*. On the other hand, with serological analysis, it might be challenging to discriminate previous from current infections in persons who travel from, or recently live in endemic regions (Fotedar *et al.*, 2007b). Currently, many polymerase chain reaction (PCR) protocols were developed; these methods provide a high sensitivity reaching 100% in the

diagnosis of *Entamoeba* spp. and successfully differentiate the three species of *Entamoeba* (Tanyuksel *et al*., 2003).

Yemen is a low-income country in which over half (50%) of the total population of about 25 million individuals survive under the country poverty line (World Bank, 2010). There is a severe lack of proper sanitation and safe drinking water, with rain and ground water being the only source of water and only quarter of the people have access to health care services and safe drinking water (World Bank, 2004; Oxfam, 2015). Many previous studies have been published on the prevalence of intestinal parasitic infections in various parts of Yemen and these have revealed that *Entamoeba* complex infection is highly prevalent in the country (Kopecký *et al*., 1991; Azazy and Raja'a, 2003; Al-Shibani *et al*., 2009a; Al-Shibani *et al.*, 2009b; Al-Haddad and Baswaid, 2010; Alyousefi *et al*., 2011; Bin Mohanna *et al*., 2014). Overall, there is a dearth of information on the risk factors related to the intestinal amoebiasis as well as data on the true prevalence and molecular epidemiology of *E. histolytica*, *E. dispar* and *E. moshkovskii* infections in Yemen are not available.

1.2 STATEMENT OF RESEARCH PROBLEM

Yemen is located the Middle East at the southern part of the Arabian Peninsula and has a total population of about 26 million. It is one of the poorest countries with more than 50% of the population lives below the poverty line. The source of water in the country completely depends on rain and ground water. Yemen suffering a severe water depletion disaster characterized by very quick taking out of groundwater, risky water supply diminish in the main cities, and restricted access to the people to drink safe water (World Bank, 2010).

Amoebiasis remains a significant public health problem in Yemen particularly in villages with a prevalence that ranged from 2.4% to 52.0% (Kopecký *et al*., 1991; Raja'a and Mubarak, 2006; Azazy and Raja'a, 2003; Al-Shibani *et al*., 2009a; Al-

Shibani *et al*., 2009b; Al-Haddad and Baswaid, 2010; Alyousefi *et al*., 2011; Al-Qobati *et al*., 2012; Bin Mohanna *et al*., 2014). A previous study showed that the *E.histolytica*/*dispar* comprised 17.7% among 503 patients looking for medical care in Sana'a City, the capital of Yemen (Alyousefi *et al*., 2011). In southern Yemen (Hadhramout governorate), Al-Haddad and Baswaid (2010) reported a prevalence of 16.8% among 600 children of urban and rural areas. Another study was carried out among 206 patients in an anticancer chemotherapy center in Sana'a City and detected *E. histolytica*/ *dispar* in 2.4% of the patients (Al-Qobati *et al*., 2012).

However, there is a scarcity of information on the risk factors concomitant with amoebiasis in the country. Moreover, molecular information on *Entamoeba* spp. is lacking. Correct discrimination of *E. histolytica*from the non-pathogenic species, *E. dispar* and *E. moshkovskii*, is crucial to the clinical management of patients. It is a popular practice in Yemen and other developing countries to prescribe metronidazole (the drug of choice) for invasive amoebiasis but it is not effective for the treatment of non-invasive forms of amoebiasis in the lumen of the bowel (Wolfe, 1973) to *Entamoeba*-microscopy-positive individuals. This practice may add additional economic burden on patients due to unnecessary treatment, as well as the underlying cause of illness, may persist untreated if the detected *Entamoeba* is the non-pathogenic species. Thus, the present study aimed to investigate the molecular epidemiology of *E. histolytica*/ *E. dispar*/ *E. moshkovskii* infections and to identify the potential risk factors associated with these infections among rural communities in Yemen.

1.3 OBJECTIVES OF THE STUDY

1.3.1 General objective

The aim of this study was to investigate the molecular epidemiology of *Entamoeba histolytica, E. dispar* and *E. moshkovskii* infections among rural communities in Yemen.

1.3.2 Specific objectives

1. To determine the prevalence and distribution of *Entamoeba* complex infection.
2. To identify the risk factors of *Entamoeba* complex infection.
3. To differentiate *Entamoeba histolytica, E. dispar* and *E. moshkovskii* in stool samples collected from the study population in the study area using Nested Multiplex PCR.
4. To determine the species-specific prevalence and distribution of *Entamoeba histolytica, E. dispar* and *E. moshkovskii* infections.
5. To identify the species-specific risk factors of *Entamoeba histolytica, E.dispar* and *E. moshkovskii* infections.

1.4 HYPOTHESES

1. The prevalence of *Entamoeba* complex infection is high in rural Yemen.
2. There are significant associations between the high prevalence of *Entamoeba* complex infection and some demographic, socioeconomic, behavioural and environmental factors.
3. The three *Entamoeba* species (*Entamoeba histolytica, E. dispar* and *E. moshkovskii*) exist among the study population from Yemen.
4. The prevalence of *Entamoeba histolytica, E. dispar* and *E. moshkovskii* infections is high in rural Yemen.
5. There are significant associations between the high prevalence of *Entamoeba histolytica, E. dispar* and *E. moshkovskii* and some demographic, socioeconomic, behavioural and environmental factors of the study population.

1.4 SIGNIFICANCE OF THE STUDY

Entamoeba histolytica plays an important role as a pathogen with an important effect on human health especially in developing countries including Yemen, one of the poorest countries in the world. In Yemen, several epidemiological studies provided information

on the prevalence of intestinal parasites, however, none of the previous studies differentiated *Entamoeba* species (Farag, 1985; Kopecký *et al.*, 1991; Azazy and Al-Tiar, 1999; Raja'a and Mubarak, 2006; Azazy and Raja'a, 2003; Al-Shibani *et al*., 2009a; Al-Shibani *et al*., 2009b; Al-Haddad and Baswaid, 2010; Alyousefi *et al*., 2011; Al-Qobati *et al*., 2012; Bin Mohanna *et al*., 2014). Clinically, all *Entamoeba*-microscopy-positive individuals in Yemen and some other developing countries are treated by metronidazole, a practice that may add additional economic burden on patients due to unnecessary treatment, as well as the underlying cause of illness, may persist untreated if the detected *Entamoeba* is the non-pathogenic species.

Within this context, the current study is the first to give information on the species-specific epidemiology of *E. histolytica*, *E. dispar* and *E. moshkovskii* in Yemen. It is hoped that the findings of the present study will assist public health authorities to identify integrated effective measures to control intestinal parasitic infections including amoebiasis in the targeted communities. Moreover, the findings will serve as a baseline data for further studies on the prevalence of the *Entamoeba* species in Yemen and will help in correcting the overestimation of amoebiasis global burden.

CHAPTER 2: LITERATURE REVIEW

2.1 Entamoeba

2.1.1 Entamoeba histolytica

In 1875, *Entamoeba histolytica* was discovered by FedorLo¨sch, who recognized and described the parasite in faeces of a patient suffering from dysentery in St. Petersburg, Russia. In 1903, Fritz Schaudinn was the first who gave the *E. histolytica* its name. Later, in 1890, Osler reported a case of a young man with dysentery that he then died from the liver abscess. Moreover, the role of amoeba in tissue invasion was proven by Councilman and Lafleur in 1891 and they presented the amoebic liver abscess and amoebic dysentery. Up till now, the only pathogenic species of *Entamoeba* is *E. histolytica*.

2.1.2 Entamoeba dispar

Emile Brumpt was the first who described *Entamoeba dispar* in 1925, and he suggested that *E. histolytica* and *E. dispar* were diverse and recommended that they have to name as pathogenic (*E. histolytica*) and nonpathogenic (*E. dispar*) species but his theory was promptly dismissed at that time. Later, many scientists confirmed his theory and indicating that *E. histolytica* and *E. dispar* are different biochemically, immunologically, and genetically.

Though *E. dispar* is considered as a non-pathogenic species and commensal, but some studies revealed the presence of symptoms of GIT with patients have this species only. A study carried out in India by Parija and Khairnar showed that 11 samples out of 68 were positive for *E. moshkovskii* and *E. dispar* and had gentle abdominal distress (Parija and Khairnar, 2005). However, the review did not exclude the existence of other parasites or viral or bacterial pathogens among the 11 positive cases. In addition, it has been noticed that *Entamoeba dispar* can cause variable crucial intestinal injuries in animals and can destroy epithelial cell monolayers in vitro (Costa *et al.*, 2006). There

are similarly a few confirmations that *E. dispar* may cause obsessive intestinal changes in some humans (Oliveira *et al*., 2015).

2.1.3 Entamoeba moshkovskii

Entamoeba moshkovskii is mainly a free-living amoeba and is difficult to discriminate it in its cyst and trophozoite forms from *E. histolytica* (the pathogenic species) and *E. dispar* (a non-pathogenic species), excluding cases of *E. histolytica* trophozoites that might possibly hold ingested red blood cells. First isolation of *E. moshkovskii* was by Tshalaia in 1941 from sewage in Moscow. In 1956, a dweller of Laredo, TX, who found with diarrhoea, epigastric pain, and loss of weight noticed with an *E. histolytica*-like strain. At that time, the *E. histolytica*-like strain was called *E. histolytica* Laredo strain, and has many characteristics with *E. moshkovskii.* Later, they reported that this strain is a type of *E. moshkovskii*.

In addition, the free-living amoeba (*E. moshkovskii*) can exist in various environments fluctuating from clean riverine deposits to brackish coastline ponds. *E. moshkovskii* has characteristics that can differentiate it from *E. histolytica* and *E. dispar* including, being osmotolerant, is unaffected by emetine, and able to grow at room temperature. Some studies in different countries reported that *E. moshkovskii* is an enteropathogen causing gastrointestinal symptoms, emphasizing the requirement for additional studies to look at the pathogenesis of the organism.

2.2 Classification

The genus *Entamoeba* classifies according to the recent classification system for protists as; Phylum: Rhizopoda, Class: Entamoebidea, Order: Endamoebida, Family: Endamoebidae.

Many species fall within the genus *Entamoeba*, seven of them can live inside human intestine; these are *Entamoeba histolytica*, *Entamoeba dispar*, *Entamoeba moshkovskii*, *Entamoebacoli*, *Entamoeba hartmanni, Entamoeba polecki*, and

Entamoeba Bangladeshi (Table 2.1). The first three species have identical morphology however they are different in genetic and biochemical features, which lead to the re-classification of the three species as *Entamoeba* complex (Ali *et al*., 2008; Pritt and Clark, 2011; Hemmati *et al*., 2015; Lopez *et al*., 2015). *E. histolytica* is the only pathogenic species, while the non-pathogenic *E. dispar* can be found as a commensal of the human intestine and *E. moshkovskii* is a free-living amoeba exist in anoxic residues (Tanyuksel and Petri, 2003; Fotedar *et al*., 2008; Anuar *et al*., 2012c; Lau *et al*., 2013; Hemmati *et al*., 2015; Nath *et al.*, 2015;).

2.3 Morphology

Entamoeba species has three forms namely trophozoite, precyst and cyst (Figure 2.1).

2.3.1 Trophozoite

The trophozoite is also known as an active vegetative stage, or feeding stage. The trophozoite doesn't have fixed shape. It is highly variable in size from 5 µm to 60 µm. This stage is motile and moves by cytoplasmic protrusions called pseudopodia. The cytoplasm of trophozoite form is separable in two parts, a granular endoplasm, and clear transparent ectoplasm, and they aren't clearly separated. *E. histolytica* cytoplasm is opulent in glycogen and extremely vacuolated, and may occasionally have red blood cells, white blood cells, cellular remains, and bacteria. Trophozoite has only one nucleus with 4-6 µm in size, and it is a spherical shape. Nucleus has clearly defined nuclear membrane. It contains karyosome that is a small, dense structure and an external "beaded" membrane. Nucleus considered as a marking feature of the genus *Entamoeba.*

2.3.2 Precyst

This form is colourless, round or oval in shape. It is smaller than trophozoite but larger than cyst. It ranges from 10-2 µm in size. The endoplasm is free of blood cells and other food particles. Pseudopodial activity is sluggish and there is no progressive movement. The nucleus in precyst is same as in trophozoite.

TABLE 2.1: The seven species of *Entamoeba* infecting human intestine (Paniker and Ghosh, 2013).

Year	Species	Discovered by
1875	*Entamoeba histolytica*	Losch
1925	*Entamoeba dispar*	Brumpt
1941	*Entamoeba moshkovskii*	Tshalaia
1870	*Entamoeba coli*	Lewis
1912	*Entamoeba hartmanni*	Von Prowazek
1912	*Entamoeba polecki*	Von Prowazek
2012	*Entamoeba Bangladeshi*	Tricia L. Royer

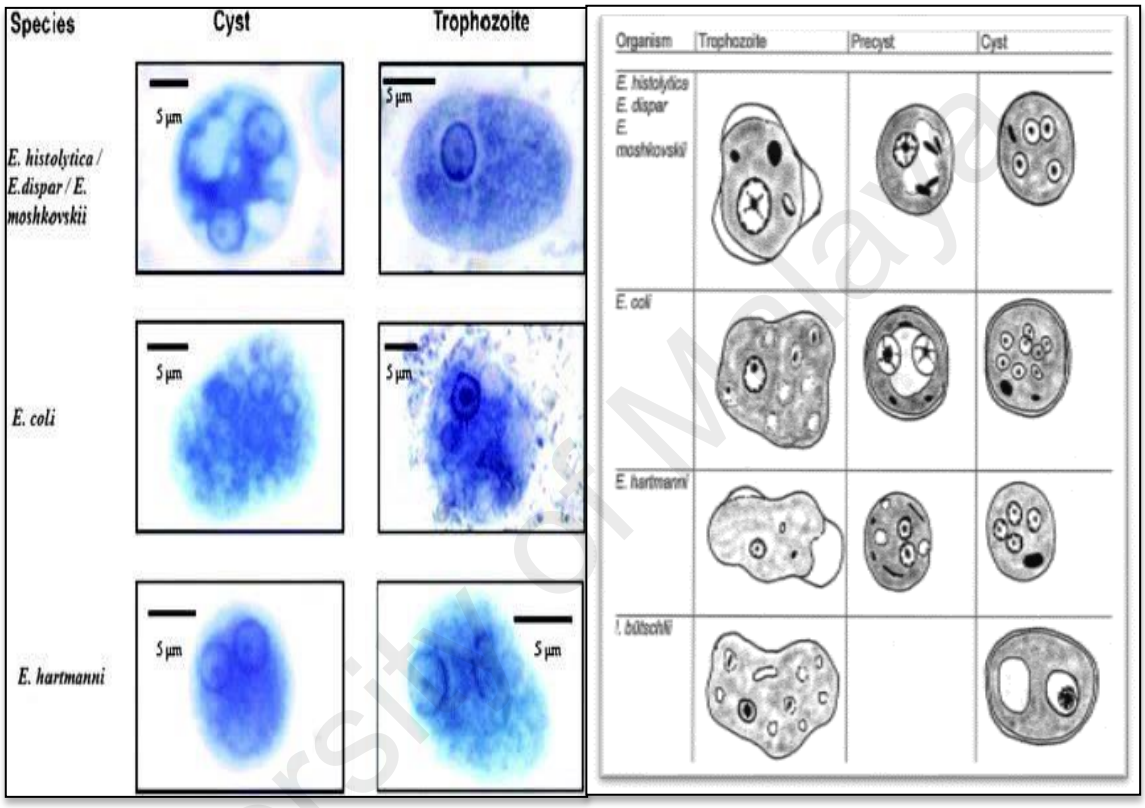

FIGURE2.1: Morphology of trophozoites and cysts of *Entamoeba* species

(Source: Tanyuksel and Petri, 2003; Fotedar *et al*., 2007b)

2.3.3 Cyst

Cysts are round in shape. The size of cyst varies in diameter from 3.8 µm (as in *E. hartmanni*) to 33 µm (as in *E. coli*). Cysts of *E. histolytica* usually range from 10 µm to 16 µm. Early cysts contain 1-4 chromatoid bodies that are cigar-shaped refractile bars which stain black with iron haematoxylin stain and a glycogen mass that stains brown with iodine. Chromatoid bodies are regularly trademark for specific species, for example *Entamoeba* complex *(E. histolytica/ E. dispar/ E. moshkovskii*) has chromatodial bars with squared or rounded ends, *E. coli* contains chromatodial bars with pointed or angular ends and *E. polecki* has silver shaped or irregular chromatoidals. (Tanyuksel and Petri, 2003).

The cyst contains up to eight nuclei and their appearance is analogous to nuclei of the trophozoite. Table 2.2 shows characteristics of trophozoites and cysts of common intestinal *Entamoeba* species.

2.4 Life cycle

Entamoeba species pass frequently in two phases in their life cycle, trophozoite, and cyst (Figure 2.2). The first phase is the trophozoite, which is the feeding, motile and reproductive phase. The second phase is the cyst that is considered the infective and diagnostic stage of human and is resistant to environmental changes. Both stages passed out through human faeces. Human gets the infection via swallowing or intake of mature cysts in faecally polluted drinks, food, or hands. Due to the presence of the thick cell wall structure, cysts can resist the stomach acids. They easily go through the lumen of the gut toward the small intestine, where each excysts producing eight daughter trophozoites. The daughter trophozoites are stick to the epithelial cells of the small intestine which outlines the GIT, invading them and feeding on bacteria. Moreover, these small trophozoites are motile. Motility and feeding process is easy due to the presence of projections from trophozoite's cytoplasm called pseudopodia.

TABLE 2.2: Characteristics of trophozoites and cysts of common intestinal *Entamoeba* species. (Fotedar *et al*., 2007b; Paniker and Ghosh, 2013).

Characteristics	*E. histolytica/E. dispar/E. moshkovskii*	*E. hartmani*	*E. coli*	*E. polecki*
Trophozoite:				
Size	12-60 µm	4-12 µm	20-50 µm	15-20 µm
Nucleus	Not clearly seen in unstained preparation	Not visible in unstained preparation	Visible in unstained preparation	Rarely seen in wet preparation
Nuclear membrane	Delicate with fine chromatin dots.	Coarse chromatin granules	Thick, with coarse chromatin granules	Chromatin with fine granular
Karyosome	Small, central.	Small, eccentric	Large, eccentric	Small, usually central
Motility	Active	Usually unprogressive but may be progressive occasionally	Sluggish	Sluggish
Pseudopodia	Finger-shaped, rapidly extruded	Finger-shaped, rapidly extruded	Short, blunt, slowly extruded	Finger-shaped, rapidly extruded
Inclusions	RBCs present, no bacteria. Non invasive organisms may have bacteria	Bacteria and other particles, no RBCs	Bacteria and other particles, no RBCs	No bacteria
Cyst:				
Size	10-15 µm	5-10 µm	10-30 µm	10-15 µm
Nuclei in mature cyst	4	4	8	1 nuclei Rarely with 2 or 4
Glycogen mass	Seen in uninucleate, but not in quadrinucleate phase	Seen in uninucleate, but not in quadrinucleate	Seen up to quadrinucleate	Seen in immature cysts
Chromatoid bodies	1-4 with rounded ends	Many with irregular shape	Splinter like with angular ends	Many with various shapes and sizes

E: Entamoeba

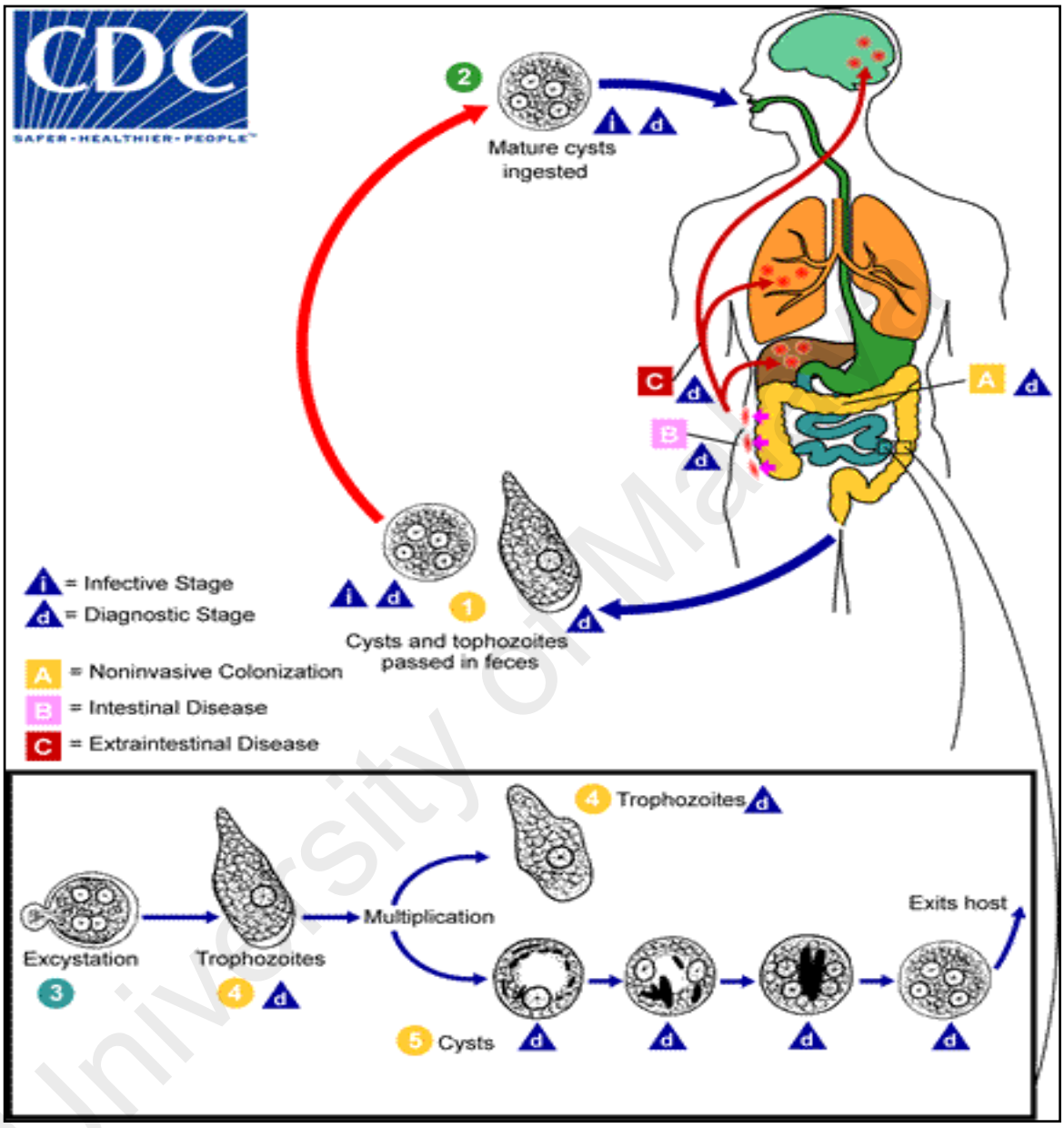

FIGURE 2.2: Life cycle of *Entamoeba* species.

(Source: https://www.cdc.gov/dpdx/amebiasis/)

The cytoplasm of invasive amoeba often ingests the red blood cells (RBCs). The trophozoites of *E. histolytica* contain only one nucleus. The trophozoite can transform to a precyst shape that also contains a single nucleus (two nuclei can found in *E. coli* precyst), then the precyst develops into a tetranucleated mature cyst which travels down and out of the colon.

The precyst has chromatoid bodies which are aggregates of ribosomes, in addition to nourishment vacuoles that are thrown out as the cell contracts to grow into a mature cyst. Trophozoites are usually occurred in diarrheal faeces, while cysts typically exist in formed faeces. Different environmental conditions will activate trophozoites to encyst and go out into the environment via stool to continue the infectious life cycle, and cysts can stay alive for weeks or months outside the host, particularly under moist circumstances, but are quickly damaged at temperatures over 40°C and under 5°C.

2.5 Mode of transmission

A human can get *Entamoeba* infection via oral ingestion of contaminated food and/or drinks with infective mature cysts. The principal well-known reservoir for *E.histolytica* is humans, therefore the carrier considered as the main source of infection. The faecal-oral transmission might be by way of direct from one person to another transmission or from polluted hands of food handlers. Oral-anal sexual contact among homosexual men is one of the transmission modes especially in developed countries (Hung *et al*., 2008). Additionally, another way for transmission that has not been proven is a zoonotic transmission.

2.6 EPIDEMIOLOGY OF AMOEBIASIS

2.6.1 Global prevalence

Amoebiasis is an infection with *Entamoeba histolytica* with or without clinical manifestations (WHO, 1997). The World Health Organization (WHO) revealed that approximately half a billion persons worldwide are diseased with *Entamoeba* species,

and around 50 million develop symptoms of invasive amoebiasis (WHO, 1997). After the fruitful discrimination of *E. histolytica* from the other two indistinguishable species, the high number of infected people was rectifying to 50 million (Fletcher *et al*., 2012). *Entamoeba histolytica* infection is a worldwide medical issue as it is responsible for 40,000 to 100,000 deaths annually and it is considered as the second cause of worldwide decease caused by protozoa after malaria, and third responsible for mortality after malaria and schistsomiasis in parasitic disease around the world (Simonishvili *et al*., 2005). Extra-amoebic consequences are the main cause of mortality from amoebiasis, of which ALA is the most widely recognized (Haque *et al*., 2010). Figure 2.3 shows the global prevalence of amoebiasis. Generally, around 90% of the cases (45 million) are asymptomatic as a result of infection with *E. histolytica (*pathogenic amoeba) or *E. dispar* and *E. moshkovskii* (non-pathogenic species), while just 10% of them (5 million) produce invasive infection of *E. histolytica*, the only pathogenic species.

Amoebiasis is highly prevalent in developing countries and this may be attributed to the poverty, overloaded housing, high population density, low personal hygienic standards, contaminated food and water (human faeces have not been suitably separated from food and water supplies), and the hot and humid environments (Stanley, 2003). Infection with *E. histolytica* is widespread in many countries including, India, South Africa, Mexico, Asian Pacific countries, and some Central and South American countries (Ximenez *et al*., 2009).

The distribution of species appears to vary according to the areas considered. A high prevalence of *E. dispar* comparing to *E. histolytica* have been described in Nicaragua, Latin American countries, Ecuador and Brazil (Tellez *et al.,* 1997; Calegar *et al.,* 2016). Nevertheless, countries as Mexico and Venezuela have stated that *E. dispar* is less common than *E. histolytica* (Lopez *et al.*, 2015).

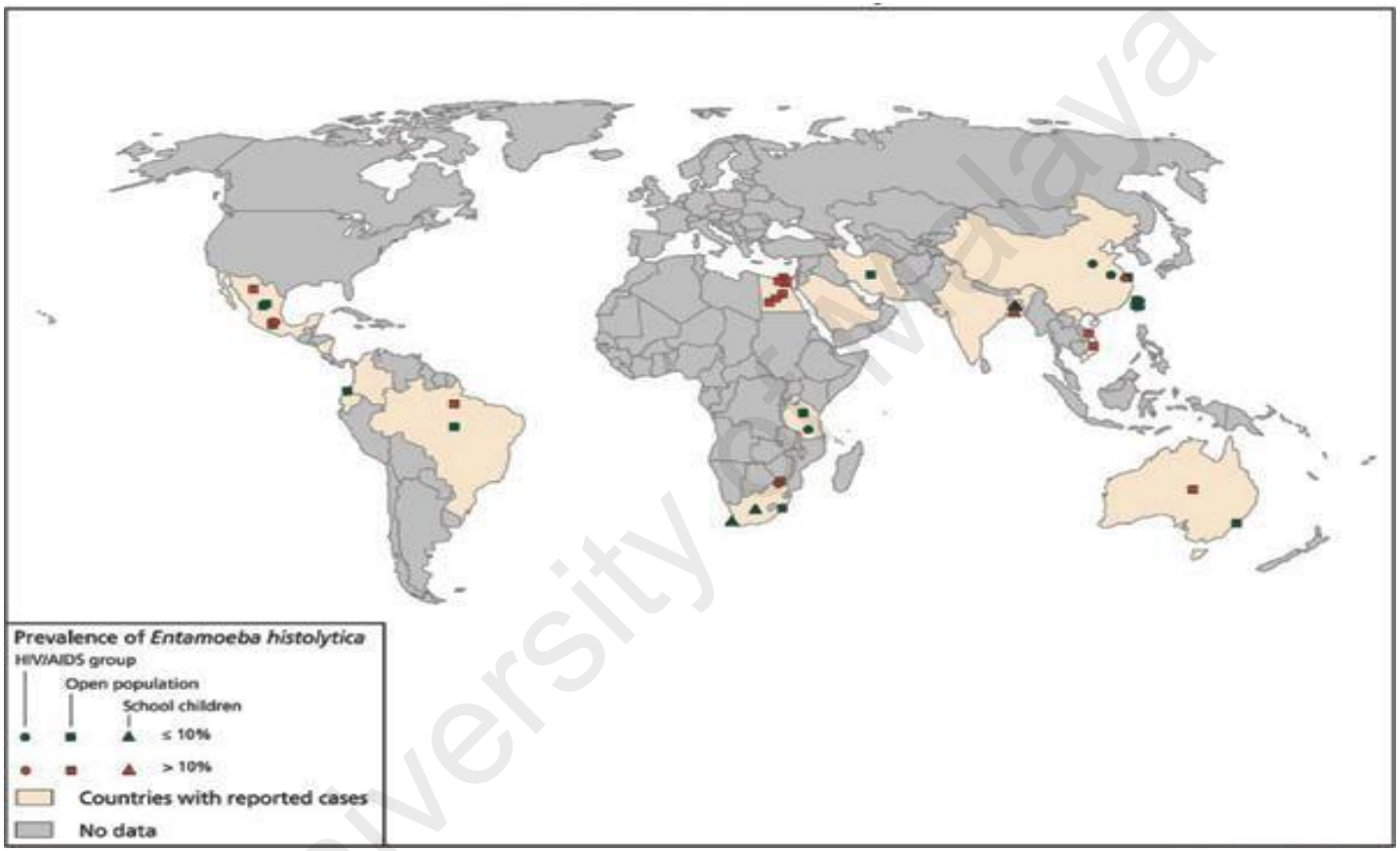

FIGURE 2.3: Global prevalence of amoebiasis in 2011.
(Source: http://www.emedmd.com/content/amoebic-infections

Additionally, it has been reported in Dhaka, Bangladesh that in urban slum areas 39% of children got the *E. histolytica* infection throughout one year of study (Blessmann *et al*., 2002). Moreover, several epidemiological studies from Iran have demonstrated *Entamoeba* spp. infection percentage nearby 2.2 to 30 percent (Solaymani-Mohammadi *et al*., 2006; Zebardast *et al*., 2014; Sharif *et al*., 2015). In Malaysia, *Entamoeba* complex prevalence varies from 1% to 61% (Anuar *et al*., 2012b).

Entamoeba histolytica and *E. moshkovskii* are considered as a potent cause of diarrheal infection among kids (Royer *et al*., 2012). Many countries have been reported the human infection with *E. moshkovskii* include, Bangladesh, India, Iran, Turkey, Malaysia, Australia, America, Italy, South Africa, and Tanzania, and in overall, they were not correlated with serious illness (Haque *et al*., 1998; Parija and Khairnar, 2005; Solaymani Mohammadi *et al*., 2006; Ali *et al*., 2008; Anuar *et al.* 2012).In developing countries, it has been observed that the population below 15 years of age are the most frequently affected, especially those aged 5-9 years old (Ximenez *et al*., 2009). A study carried out in Mozambique among children and youth aged from 7 to 22 years revealed aprevalence of 31.2% (Oliveira *et al*., 2015).

In developed countries, amoebiasis occurs in immigrants, tourists who travel to endemic areas, institutionalized persons, human immunodeficiency virus HIV-positive individuals, and sexually active homosexual men (Blessmann *et al*., 2002; Hung *et al*., 2008). On the other hand, in industrialized countries, the general prevalence of *E. histolytica* infection has been expected to reach 4% yearly, regardless of the existence of some high-risk groups. In Australia, it has been accounted for that the incidence of *Entamoeba* species changes from 4 to 1% in rural and urban populations, respectively (Fotedar *et al*., 2007a; Fotedar *et al*., 2008). Another study revealed a 37% of men who have sex with men have an infection with *Entamoeba* (Stark *et al*., 2008).

Table 2.3 summarize the findings of many previous studies from different countries on amoebiasis.

2.6.2 Prevalence of amoebiasis in Yemen

Yemen is one of the developing countries and situated in the Middle East on the southern part of the Arabian Peninsula. About 26 million people dwell the country. It is a poor country listed among the low-income countries in which the income per capita is $490 (World Bank, 2010). Due to poverty, a wide range of food-borne and waterborne infections, including E. histolytica, are widely spread in Yemen.

The prevalence of *E. histolytica/ E. dispar* has been reported in various governorates in Yemen by different researchers. An earlier study carried out in Sana'a governorate by Farag (1985) during the period 1980-1982 included more than 37,000 faecal samples revealed that 53% of the subjects were infected with intestinal parasites including *E. histolytica* in high prevalence. In 1991, two pilot studies were carried out in Aden governorate (south of Yemen) covering the villages in the highland and lowland areas and were including 104 children aged from 6 to 15 years old, and presented that the prevalence of *E. histolytica* in highlands and lowland were 36.8% and 42.3%, respectively (Kopecký *et al*., 1991). Azazy and Al-Tiar (1999) showed that the prevalence of *E. histolytica* was 3.5% and 3% in rural and urban areas, respectively among 958 stool specimens of school children aged 6-13 years, by using normal saline and formal ethyl-acetate sedimentation techniques. Another study among school children in seven rural communities of Assahul valley of Ibb governorate carried out by Raja'a *et al.* (2000) and found that the prevalence of *E. histolytica* was 14%.

Subsequently, in 2001, Raja'a *et al.* carried out a study on 897 pupils, randomly selected from Al-Mahweet city and from neighboring countrysides, and showed a high prevalence of *E. histolytica* (36%).

TABLE 2.3: A summary of previous studies on amoebiasis from different countries.

Country	Prevalence (%)				Population studied	References
	E.h	***E.d***	***E.m***	***Entamoeba* spp**		
Angola	0.3	13.1	-	-	School-Aged Children	Oliveira *et al*., 2015.
Australia (Sydney)	5.6	70.8	61.8	-	Hospital-based; all ages	Fotedar *et al*., 2007a
Brazil	-	74.2	-	-	Community-based study; all ages	Pinheiro *et al*., 2004
Colombia	0.55	23.2	25.4	-	Asymptomatic children under16 years old from the hamlet LaVírgen, Cundinamarca	Lopez *et al*., 2015.
Ghana	-	82.8	-	-	Community-based study; all ages	Verweij *et al*., 2003
India	11.1	11.8	7.8	-	Northeast population	Nath *et al*., 2015
India	3.5	9.3	1.9	-	Hospital-based; all ages	Khairnar *et al*., 2007
India	1.7	8.8	2.2	-	Hospital-based study; all ages	Parija&Khairnar, 2005
Iran	2.04	-	-	8.16	Water samples including 18 rivers and 6 wetlands	Hemmati *et al*., 2015.
Iran	Children in community : 4.2 In rural: 4.3 In urban: 1.0	-	-	-	Hospital-based study; all ages	Haque *et al*., 2006
Iran	3.45	91.4	3.45 *E.d+E.m:* 1.7	-	Stool samples from Iranian patients infected with gastrointestinal disorders	Mojarad *et al*, 2010

Table 2.3: Continued

Country	Prevalence (%)				Population studied	References
	E.h	***E.d***	***E.m***	***Entamoeba* spp**		
Malaysia	-	-	-	19.5	Human stool samples were collected from different orangAsli settlements	Lau *et al.*, 2013
Malaysia	-	-	-	18.6	Faecal specimen obtained from Orang Asli communities	Anuar *et al.*, 2013.
Malaysia	75	30.8	5.8	17.6	Participants in five rural villages	Ngui *et al.*, 2012
Malaysia	-	-	12.3	18.6	Orang Asli communities in 3 different states	Anuar *et al.*, 2012a.
Malaysia	3.2	13.4	1.0	18.6	Orang Asli tribes	Anuar *et al.*, 2012c.
Malaysia	-	-	-	33.4	Community study; all ages	Zurainee *et al.*, 2003
Malaysia	Child:79, adult:87	-	-	-	Community study – Orang Asli	Gilman *et al.*, 1976
Malaysia	13.2	25.6	-	-	Community-based, Orang Asli	Azian *et al.*, 2006
Mexico	8.4	-	-	-	Community study; all ages	Caballero-Salcedo *et al.*, 1994
Nigeria	-	0.67	-	-	Hospital-based; all ages	Fadeyi*et al.*, 2009
Palestine	69.6 *E.h*+*E.d*: 7.6	22.8	-	-	Hospital-based; children Gaza Strip	Al-Hindi *et al.*, 2005
Philippine	1.0	7.1	-	-	Community-based study; all ages	Rivera *et al.*, 1998
Saudi Arabia	2.7	-	-	-	Hospital-based; all ages	Barnawi *et al.*, 2007
Saudi Arabia	4.3	95.7	-	-	Hospital study; all ages	Al-Harthi *et al.*, 2007

Table 2.3: Continued

Country	Prevalence (%)				Population studied	References
	E.h	***E.d***	***E.m***	***Entamoeba* spp.**		
South Africa	12.5	87.5	*E.d*+ *E.m*: 3.12	-	HIV patients	Hamzah *et al*, 2010
South Africa	1.0	9.0	-	-	Community study; all ages	Gathiram & Jackson, 1985
South Africa	4.0	5.0	13.0	-	HIV patients	Beck *et al*., 2008
South Africa	All ages: 18.8, Children: 2.1	25.3 8.5	-	-	Hospital-based; all ages	Samie *et al*., 2006
South Africa	12.5	87.5	*E.d*+ *E.m*: 3.12	-	HIV patients	Hamzah *et al*, 2010
Sweden	4.8	79.7	-	-	Hospital-based study; all ages	Lebbad & Svard, 2005
Thailand	13.3	20.0	0.0	33.3	Hospital-based; all ages	Hamzah *et al*., 2006
Turkey	2.6	7.4	-	-	Stool specimens of individuals having diarrhea/ dysentery and individuals who were asymptomatic	Araz *et al.*, 2012
Unite kingdom (London)	0.0	20.0	-	-	UK; male kingdom homosexual	Jones *et al.*, 1986.
Vietnam	11.2	-	-	-	Community-based study; adults	Blessmann *et al*., 2002

A retrospective study was carried out by Azazy *et al*.(2003) in which they revised the results of 9014 faecal specimens from Yemeni kids in the Paediatric Health Centre in Sana'a governorate in the period from January 1998 to December 2000. The study detected that the *E. histolytica* prevalence was 1.7% - 36%. A previous study on school children in Sahar district of Sa'dah governorate found a prevalence of 6.4% (Raja'a1 *et al*., 2006). Moreover, a previous study was conducted to identify intestinal parasites among restaurant workers in Mukalla, Yemen showed that the *E. histolytica* prevalence was 14.8% (Baswaid and AL-Haddad, 2008). Furthermore, two different studies were carried out in 2009 by Al-Shibani and her colleagues on the prevalence of *E. histolytica*. The first study was conducted among three orphanages in Sana'a governorate (North of Yemen), and involved subjects aged 4-20 years old (Al-Shibani *et al*. 2009a). The study reported that the prevalence of *E. histolytica/ E. dispar* and *E. coli* were 13% and 18.5% respectively. The second study was executed among apparently healthy workers aged between 12 to 70 years from 58 restaurants in Sana'a town, in which the prevalence of *E. histolytica/ E. dispar* was 48.9% (Al-Shibani *et al*. 2009b). In Hadhramout governorate, Al-Haddad and Baswaid (2010) carried out a study among children and showed the percentage of *E. histolytica/ E. dispar* was 16.8%. Similarly, another study was carried out in Sana'a city and found a prevalence of 17.1% (Alyousefi *et al*. 2011).

Al-Qobati *et al*. (2012) carried out a study in Sana'a governorate on Yemeni patients with cancer and found the prevalence of intestinal parasitosis was 2.4%. A recent 3-year study (2011-2013) was done at the Specialized Sam Pediatric Center in Sana'a city among subjects aged 3-15 years stated that the prevalence of *E. histolytica*/ *E. dispar* was 25% (Bin Mohanna *et al.* 2014). In addition, a prevalence rate of 52% was reported among school children from urban areas in Sana'a and Al-Mahweet governorates (Azazy *et al.*, 2002).

However, all these previous studies that have been done in different governorates in Yemen did not discriminate the pathogenic *E. histolytica* from non-pathogenic *E. dispar* or *E. moshkovskii*. In general, all the above studies just provided prevalence rates of *Entamoeba* complex infection (i.e. *E. histolytica*/ *E. dispar*/ *E. moshkovskii*). Hence, a study to discriminate the three species of *Entamoeba* is highly required in order to help in the reassessment of Yemen's epidemiology of amoebiasis and the actual load of disease as well as to assess the effectiveness cost of the application of specific control measures among the populations in danger. Table 2.4 shows the prevalence of *Entamoeba* spp. according to several previous studies in Yemen.

2.7 CLINICAL PRESENTATION

Entamoeba histolytica infection, with or without clinical manifestations, is called amoebiasis. About 10%of infected persons are suffering from symptoms. These symptoms might be mild colitis or severe or could reach the extra-intestinal organs causing severe illnesses. Essentially, symptoms take days to years to appear from the first onset of the disease.

Colitis or amoebic inflammation is uncommon, however, it is considered as a fatal disease. The most noticeable symptoms of colitis are severe abdominal discomfort, profuse bloody diarrhoea, and fever. The mortality rate in this infection can reach 40% as result of that patients may develop colonic peritonitis and perforation (Aristizábal *etal.*, 1991; Lucas and Upcroft, 2001).

The most common form of extra-intestinal amoebiasis is amoebic liver abscesses (ALA). Patients with ALA presents with pain in the right upper quadrant abdomen, fever, and weight loss, without simultaneous jaundice. Also *E. histolytica* can cause amoebams that are tumor-like inflammatory masses that cause intestinal obstruction and might be misdiagnosed with cancer. High-risk patients include children, elderly, pregnant women and immunocompromised patients (Petri and Singh, 1999).

TABLE 2.4: A summary of some previous studies on the prevalence of *Entamoeba* spp. in Yemen.

Population studied	**Prevalence of *Entamoeba* spp. (%)**	**References**
Specialized Sam Pediatric Center in Sana'a, including Ages (3-15 years) for 3 years (2011-2013)	25.0	Bin Mohanna *et al.*, 2014
Patients on anticancer chemotherapy in Sana'a city	2.4	Al-Qobati *et al*., 2012
Hospital-based study in Sana'a city	17.1	Alyousefi *et al*., 2011
Study in Hadhramout governorate among Children from urban and rural regions	16.8	Al-Haddad &Baswaid, 2010
In Sana'a City among healthy workers in 58 restaurants aging from 12 to 70 years	48.9	Al-Shibani *et al*., 2009b.
Among food handler workers in cafeterias, restaurants, and other food shops	14.0	Wakid&Hamdi, 2009
Include restaurant workers in Hadhramout University	14.8	Baswaid& Al-Haddad , 2008
Paediatric Health Centre kids in Sana'a (January 1998 to December 2000)	1.7-36.0	Azazy&Raja'a, 2003
Schoolchildren from urban areas in Sana'a and Al-Mahweet provinces	52.0	Azazy *et al.*, 2002
Schoolchildren from Al-Mahweet city and neighboring countrysides	36.0	Raja'a *et al*., 2001
All children of 14th October Primary School	14.0	Raja'a *et al*., 2000
Urban and rural schoolchildren aged between 1-13years	3.0- 3.5	Azazy& Al-Tiar, 1999.
6-15 years old children from the rural community in the lowland and highland areas in Aden	36.8-42.3	Kopecký *et al*., 1991

2.8 LABORATORY DIAGNOSIS

Diagnosis of amoebiasis in the lab mainly depends on microscopical examination and serological techniques such as enzyme-linked immunosorbent assay (ELISA), indirect haemagglutination assay (IHA). Nowadays, molecular methods are widely applied and are the best method for distinguishing the pathogenic *E. histolytica* from others with high sensitivity and specificity reaching 100%.

2.8.1 Microscopy

Traditionally, amoebiasis is diagnosed by examination of specimens under the microscope, using direct, concentrated, temporarily or permanently stained procedures. Microscopic methods have around 60% sensitivity and might because false-positives due to the difficulty in discriminating *E. dispar* and *E. moshkovskii* (non-pathogenic) from *E. histolytica* (pathogenic). Only the presence of ingested RBCs in trophozoites (erythrophagocytosis) can emphasize the existence of *E. histolytica*, the pathogen form (WHO, 1997; Pritt and Clark, 2011).

Different types of stains can be used to help inidentifying *Entamoeba* spp. under the microscope such as Lugol's or D'Antoni's iodine, methylene blue, Giemsa, Wheatley's trichrome, and Chorazole black E, Wright's. If stool specimens not processed directly using fixatives is very important to avoid degeneration of trophozoites, as well as saving the morphology of cysts and ova in faecal samples. These preservatives include 5% or 10% formalin, sodium acetate-acetic acid-formalin (SAF) and modified polyvinyl alcohol (PVA). Due to the sporadic and unequally excretion of parasites in stool, therefore it is recommended to collect the samples at three different times from the patient within 10 days to get an accurate and precise examination (Fotedar, 2007b). Furthermore, misdiagnosis can occur due to an inadequate training and diagnostic testing, so correct investigation needs great levels of skill and experience (Walsh, 1986; Tanyuksel and Petri, 2003).

2.8.2 Culture

Culture techniques for the *Entamoeba* species isolation have been existing more than eighty years ago. In 1925, *E. histolytica* cultured for the first time inside a diphasic egg slant medium by Boeck and Drbohlav, and a modification of this medium (Locke-egg) is still utilized until today. A variety of monophasic media were used for this purpose such as, TYSGM-9, egg yolk infusion medium of Balamuth, and Jones's medium Specimens taken for amoebic culture include stool samples, rectal biopsy, or liver abscess aspirates.In general, media that is used in amoeba cultivation could be axenic and xenic (diphasic and monophasic) methods. The growth of the *Entamoeba* spp. in the existence of an undefined flora is named xenic culture. *E. histolytica* axenic cultivation was initially developed by Clark and Diamond in 1991.The culture of *E. histolytica* has success degree, ranging from 50 to 70% in medical reference laboratories. Generally, culture technique is not appropriate as a routine diagnostic tool, which is considered as costly, difficult and labor-intensive, as well as the hazard of overgrowth of other protozoans, fungi, or bacteria throughout culture that results from mixed infections.

2.8.3 Serology

Serological tests for *E. histolytica* identification are useful in industrialized countries, where infection with *E. histolytica* is unusual. Nevertheless, in areas where the infection is endemic areas, serological tests are unable to discriminate the previous infection from present one makes the diagnosis of them difficult. Usually, using both of serological tests with finding of the parasite, for example, PCR or antigen detection will provide the best way for diagnosis; and these may include;

2.8.3.1 Enzyme-linked immunosorbent assay (ELISA)

ELISA used in medical diagnostic laboratories all over the world and considered as the most popular methods. Antibodies detection is very useful in the case of ALA where the

parasites are difficult to find in patient's sample. It has been reported that the detection of specific antibodies to *E. histolytica* in serum has a sensitivity of almost 100%, which makes it capable of diagnosing ALA. Serum anti-lectin immunoglobulin G (IgG) antibodies might exist within one week after the beginning of symptoms of amebic colitis and ALA patients, with a rate of 95%. Occasionally, false positive results could be found in serological test, so the test must be repeated if the end result is uncertain. *E. histolytica* IgG antibodies in serum remain after infection till years, while the occurrence of IgM antibodies is short-term and can be discovered within one week after the beginning of symptoms. The sensitivity of ELISA can reach 95% in patients with high antibody titer in serum and parasites present in their stool specimens. ELISA assays which based on antigen detection have many substantial advantages, including; (1) they have high sensitivity and specificity; (2) some of the assays discriminate *E. dispar* from *E. histolytica*;(3) Antigen detection based-assays are easy to perform even by non-skilled laboratory staffs; (4) very helpful in epidemiological studies as they possess 96-well in one plate.

The antigens that are specific for *E. histolytica* and widely used in ELISA kits against monoclonal antibodies are the serine-rich antigen of *E. histolytica* (Optimum S kit; Merlin Diagnostika, Bornheim-Hersel, Germany), the Gal/GalNAc-specific lectin of *E. histolytica* (*E. histolytica* test II; TechLab, Blacksburg, Va.), a salivary 170-kDa adherence lectin antigen, and lysine-rich surface antigen. Stool antigen detection tests are specific, sensitive, and useful, however, there is a limitation that fixation of the faecal samples can damage the antigens detected. Therefore, in antigen-based ELISA test, fresh or frozen specimens are recommended to use.

2.8.3.2 Indirect haemagglutination (IHA) test

IHA is an easy test to carry out and very useful in diagnosis of amoebiasis in HIV-infected patients with gastrointestinal symptoms. In ALA patients, the sensitivity of

IHA technique was reported as 72.4% in the first and second week of the infection, and then it reaches 86.9% at the end of the third week. After six months from the onset of the disease, the antibody titer started to decrease. Lower sensitivity of IHA test could be brought about false-negative results in comparison with ELISA.

2.8.3.3 Counter immunoelectrophoresis (CIE) test

Previously, CIE was the most commonly used method in diagnosis of amoebiasis. In this technique, *E. histolytica* HK-9 antigen reacts at 25ºC against inactivated serum in 1% agarose plates at 20 mA for 1 h. The visualization of a white precipitin band(s) between the antigen and antibody will be interpreted as a positive reaction (Sheehan *et al*., 1979). Although, CIE technique has a high sensitivity (100%) in cases with invasive amoebiasis, but it is time-consuming.

2.8.4 Molecular diagnosis

PCR-based techniques are the best method of choice for medical and epidemiological studies, as they have higher sensitivity and specificity than antigen detection. However, it might not be suitable for routine use in developing countries where amoebiasis is widespread due to the expensive equipment and expert skills needed to perform the test. In addition, these techniques are complex and time-consuming. Moreover, cross-reactions (cross-contamination) or false negative may occur using PCR, especially while using stool specimens. That, the stool specimen is one of the most complicated samples for molecular analysis due to the presence of PCR inhibitors, such as bile salts, heme, and complex carbohydrates (Fotedar *et al.*, 2007b).

PCR is a powerful tool in laboratory diagnosis, and many researchers described its higher sensitivity value in comparing with other available techniques (Tanyuksel *et al.*, 2003; Khairnar*et al.*, 2007). PCR is also the appropriate test for ALA diagnosis when aspirated pus is obtainable. One of the most important steps in PCR is DNA extraction from faecal samples, and the use of specific and well-designed primers are

keys to effective PCR diagnosis. The commercial isolation kits are available for DNA extraction. Multiplex PCR amplification has been described by Núñez *et al.* (2001) for the detection of *E. histolytica* and *E. dispar* in faecal specimens (with 94% sensitivity and 100% specificity) through performing a single reaction mixture with two pairs of specific primers simultaneously.

Riboprinting is a process that uses restriction fragment length polymorphism to investigate the small- and large-subunit rDNA after its amplification. It is a very valuable tool to evaluate and understand the *Entamoeba* species epidemiology and detecting the outbreaks of *Entamoeba* species. However, the procedure of ribotyping is time consuming and complex. XbaI, RsaI, TaqI, DdeI, and Sau96I are the common restriction enzymes that used to distinguish *E. histolytica* from *E. dispar*. Riboprinting also can help in studying the polymorphism of the *E. moshkovskii* positive samples.

2.9 TREATMENT

In 1997, WHO and Pan American Health Organization proposed that only those infected with *E. histolytica* should be treated regardless of symptomatic or non-symptomatic (WHO/PAHO/ UNESCO, 1997). In addition, these guidelines recommended that no treatment will be needed for *E. dispar* or *E. moshkovskii* infections, excluding particular conditions. Different therapies are used according to the site affected. In asymptomatic patients and intestinal colitis, the luminal amoebicides such as oral paromomycin, diloxanidefuroate and iodoquinol are commonly used, which they do not penetrate intestinal tissues (Ravdin and Stauffer, 2005; Abramowicz, 2010). Derivatives of nitroimidazoles like metronidazole, ornidazole, and tinidazole are the treatment of the choice against extra-intestinal or invasive amoebiasis, but not useful for luminal amoeba. Treatment for an asymptomatic carrier is valuable because they are responsible for a high percentage of reinfection.

Patients with invasive or extraintestinal amoebiasis are controlled by medical treatment. Procedures as open surgical drainage and percutaneous drainage are not usually suggested. However, those procedures could be helpful in patients with bacterial sepsis, and a ruptured abscess (Salles, 2003; Farthing, 2006; Pritt and Clark, 2011).

2.10 PREVENTION AND CONTROLOF AMOEBIASIS

To prevent and control *E. histolytica* infection along with other enteric infections, many preventive measures should be considered. These include promoting better public health practices and personal hygiene, giving adequate potable safe water and adequate sanitation, and provide proper and adequate health facilities to ensure timely diagnosis and treatment. One of the United Nations Millennium Development aims is to decrease by half the number of individuals without access to potable water and proper sanitation by 2015 (United Nations, 2010). Even though several developing countries have made high progress in these measures, apart (quarter) of the targeted people in these countries are still using unsafe water and about 1.1 billion individuals do not own latrine facilities (United Nations, 2010; Campbell *et al*., 2014). Unluckily, reaching these targets requires huge expenses of money that are not readily available in the countries where the amoebic infection is predominant. Overall, prevention and control programmes against amoebiasis and other intestinal protozoan and helminth infections should be implemented in two-dimensional aspects; short-term and long-term elements. For short-term measures, the target is to curtail the morbidity by these infections and chemotherapy is the main pillar for this aspect. On the other hand, comprehensive long-term measures are required to reduce transmission below the level needed to sustain the infection (WHO, 1997; Campbell *et al*., 2014). According to WHO, wherever the overall prevalence rate is more than 10%, a long-term objective should be planned to reduce the number of cases by non-specific hygienic measures (WHO, 1987).

In amoebiasis, prevention and control can be achieved by mass chemotherapy

known as mass drug administration (MDA) in order to reduce the mortality and morbidity attributed to amoebic dysentery and extra-intestinal amoebiasis, and this should be focused on the endemic communities of the population as well as on groups at higher risk of infection such as children, primarily in rural and disadvantaged areas (Strunz *et al.*, 2014). Besides, efforts to improve the sanitation and water system should be considered in order to stop the transmission of pathogens in the targeted communities (Montresor *et al.*, 2008). Meanwhile, improving personal and food hygiene as well as providing proper diagnosis and treatment of individual cases should be implemented (WHO, 1997). Other specific measures should be identified to prevent and control epidemics in population sharing water sources, or in institutions such as day-care centers, orphanages, and mental hospitals.

Humans are the definitive host for the *E. histolytica* and many other intestinal parasites, thus sanitation has a vital role and long-lasting impact on the control of these parasites. Safe disposal of excreta and the use of proper latrines have been associated with a significant decline in the prevalence rates of intestinal parasitic infections. Accessibility to safe water, sanitation, and hygiene (WASH) is essential for a long-term and sustained control and elimination of intestinal parasitic infections (Campbell *et al.*, 2014; Strunz *et al.*, 2014). WASH involves a safe water supply, appropriate and adequate sanitation infrastructure that confirms disposal of human excreta safely, and the establishing the good personal and household hygiene practices (for instance washing of hand before eating and/or after defecation, use of soap, wearing shoes while outdoor, washing fruits/ vegetables before eating, etc).

WASH as an intervention has been revealed to be very effective in decreasing the contamination of the environment and curtail the spread of intestinal parasitic infections (Esrey *et al.*, 1991). However, many challenges limit the implementation of WASH especially in rural areas of developing countries. Examples of these challenges

are the high cost, lack of local government contribution, lack of advocacy and lack of perception among targeted populations about the vital role of improved sanitation (Cairncross, 2010).One of the main components of any control programme against intestinal parasites is the health education. The main objective of health education is to assist people to get control over the factors of health behaviors that affect their health status and that of others (WHO, 1997). Therefore, intervention on health education is recommended as the first option in disadvantaged or low socioeconomic communities in order to make the enabling environment for other strategies to grow well (Ekeh and Adeniyi, 1988). Thus, providing health education pertaining good practices of personal hygiene primarily hand hygiene including hand washing before eating or handling food, after using the toilet and after animals handling are imperative in controlling amoebiasis.

Overall, the WHO recommends 3 main interventions to control and prevent intestinal parasitic infections including amoebiasis; chemotherapy, providing a suitable sanitation and an effective health education (WHO, 2005). With regards to Yemen, there has been no control programme against amoebiasis or intestinal parasitic infections in general, however, a single dose of albendazole is distributed together with praziquantel tablet in schistosomiasis endemic communities. In the country, there are only two programmes against parasitic infections; Malaria National Control Programme and Schistosomiasis National Control Programme.

CHAPTER 3: METHODOLOGY

3.1 COUNTRY PROFILE

Yemen (also known as the Republic of Yemen) is located in the west of the Asian continent. It is one of the Arab countries that found in the southwestern to the southern end of the Arabian Peninsula. Yemen is the second biggest country in the peninsula, covering 527,970 $km^2$with a total population of 26.18 million (World Bank, 2015). It is surrounded by Oman to the East, the Red Sea to the West, Saudi Arabia to the North, and the Gulf of Aden and the Arabian Sea to the South. The coastline extends for about 1,200 mi (2,000 km). Yemen has more than 200 islands; as Socotra in the Indian Ocean, and Kamaran in the Red Sea. Yemen is divided into twenty governorates, plus one municipality called "Amanat Al-Asemah" (the later containing the constitutional capital, Sana'a) (Figure 3.1). The governorates are subdivided into 333 districts, which are subdivided into 2,210 sub-districts, and then into 38,284 villages.

Yemen is considered as one of the least developed countries in the world, positioning 148 out of 174 countries secured by the United Nation's Development Program's 2003 Global Human Development Report. The World Bank has been stated that about 73% of Yemen's residents live in rural areas, and agriculture offers 58% of all occupation but accounts for only 15% of the Gross Domestic depending Product (GDP). The growth rate of population in Yemen is the highest in the world reaching higher than 3% annually. The population is estimated to reach above 40 million in the next two decades (Boucek, 2009).

3.2 STUDY DESIGN

Cross-sectional community-based surveys were executed in rural communities in four provinces in Yemen – Taiz, Hodeidah, Sana'a, and Dhamar– between January and July 2012, and February and April 2014.

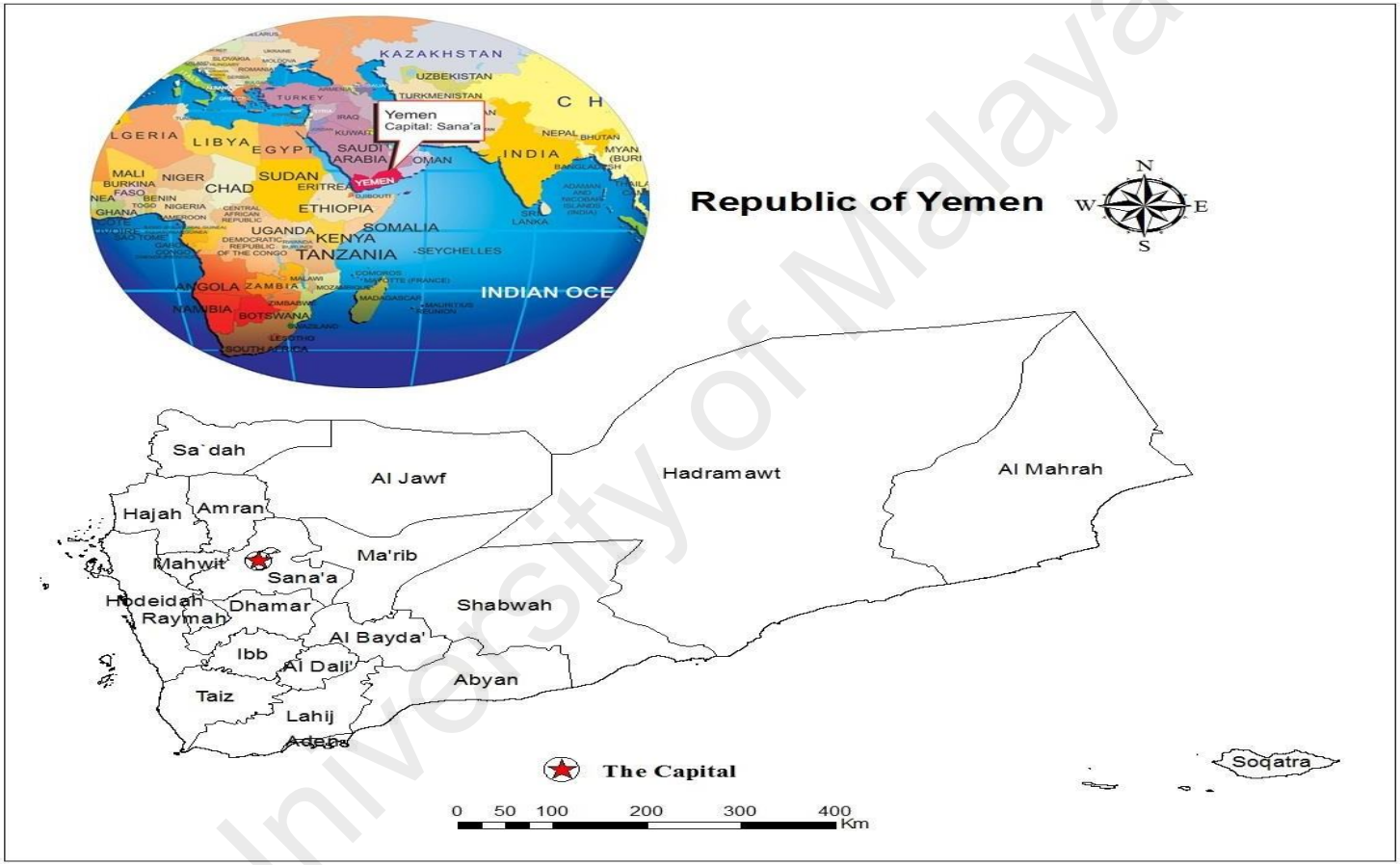

FIGURE 3.1: Republic of Yemen map (The map was created using the EsriArcMap 10.4.1 software).

In each province, two rural districts were nominated randomly from the existing district list and then two villages within the nominated districts were selected for this study. The number of dwellers per household was recorded and all of them were asked to contribute in this study. Distinct reference codes were allocated to each household and study participants. Single faecal samples were collected from the participants and screened for the presence of *Entamoeba* species cysts and/or trophozoites using many diagnostic methods. Moreover, using a pre-tested questionnaire, information about the demographic, socioeconomic, environmental and health status of the contributors and their communities were collected.

3.3 STUDY AREA

The present study was conducted in four provinces in Yemen namely Taiz, Hodeidah, Sana'a, and Dhamar. In each province, two rural districts were randomly selected from the available district lists provided by the respective health office of each province. The nominated districts were Maqbanah and Mawzaa (Taiz), GabalRas and Zabid (Hodiedah) Alhemah Alkharijiah and Manakhah (Sana'a), and Utmah and Gabal Assharq (Dhamar) (Figure 3.2). The villages in these districts were also randomly selected from the provided lists according to the following criteria: the village in a rural area and to have more than 50 houses or ≥ 300 residents.

The provinces of Taiz and Hodeidah, at altitudes of less than 2,000 m, represent coastal plains and foothills. The two provinces have a total population of five million altogether (NIC, 2016). The coastal plains and foothills experience a tropical monsoon in the summer and two rainy seasons (February-April, and July-September); however, the mean annual rainfall is much lower than in the highlands (200 mm/year). The winter in Taiz and Hodeidah is dry and cool, and the mean temperature varies between 24.0°C in the winter and 37.5°C in the summer. These areas have many streams which are considered the major source of water for both drinking and domestic objectives.

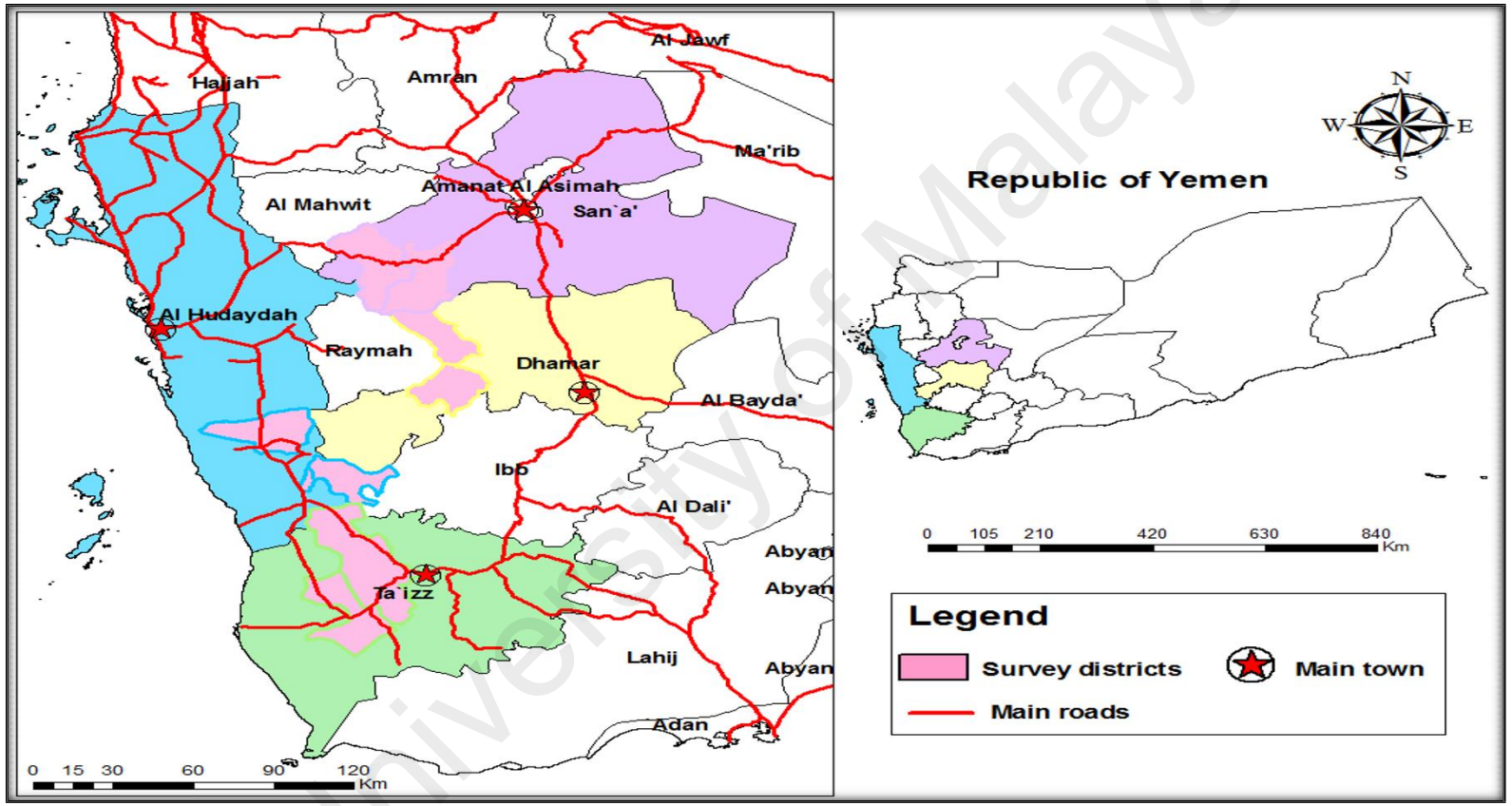

FIGURE 3.2: A geographic map showing the study area in Yemen (eight districts within four provinces).

The map was created using the Esri ArcMap 10.4.1 software.

On the other hand, the provinces of Sana'a and Dhamar, at altitudes of more than 2,000 m above sea level, represent the country's highlands. The combined population of both provinces is 2.2 million (NIC, 2016). The climate in the highlands is moderate in summer and cold and dry in winter, at an annual mean temperature of about 20°C. The mean rainfall per year is 800 mm/year and many dams are constructed to collect water for agricultural irrigation as well as groundwater recharge. Moreover, many traditional small-to-medium uncovered cisterns are scattered throughout every village for domestic water and other daily needs.

3.4 SAMPLE SIZE CALCULATION AND STUDY POPULATION

The minimum sample size wanted for the present study was assessed depends on the WHO practical manual for the determination of sample size in health studies (Lwanga and Lemeshow, 1991). At a 5% level of significance and a 95% confidence level, design effect of 2, the number of participants was valued as being 576, supposing that the average prevalence of *Entamoeba* complex infection in rural Yemen was 30%.

A total of 800 individuals were invited to contribute in the study and those that agreed to participate did so voluntarily. They received proper containers to bring in faecal samples and were interviewed by using a pre-tested questionnaire to obtain information about a range of demographic, socioeconomic and environmental factors. Overall, 605 individuals were involved in the study and they delivered faecal specimens for analysis, while 195 subjects who did not deliver the samples (mostly adult females) were excluded from the study. Table 3.1 shows the definitions of variables were used in this study.

3.5 QUESTIONNAIRE SURVEY

A pre-tested questionnaire was used in the present study. The questionnaire has been pretested and validated by a previous study on schistosomiasis and intestinal parasitic infections among rural communities in Yemen (Sady *et al*., 2013; Sady *et al*., 2015).

Table 3.1: Definition of variables.

VARIABLES	DEFINITION
Age	Age of participants in years (according to birth certificate)
Gender	Male or female
Low fathers' education	Fathers' formal education for less than 6 years
Low mothers' education	Mothers formal education for less than 6 years
Low household monthly income	Total monthly income of the family is below or equal 20,000 Yemeni Riyal (< 100 USD) (Alyousefi *et al*. 2013)
Large family size	The number of the family members is > 7 members, including the parents (Al-Mekhlafi *et al*. 2007)
Fathers' employment status	The employment status of fathers of participants; categorized into working and not working (Norhayati *et al*. 1998)
Presence of toilet in household	Availability of toilet facilities in the house (Ngui *et al*. 2011)
Presence of animals at household	Presence of pets and other domestic animals at the households (Abdulsalam *et al*. 2012)
Water supply status	Sources of drinking water (Abdulsalam *et al*. 2012)
Indiscriminate defecation	Places of defecation (Ngui *et al*. 2011)
Washing hands before eating	Practicing hand washing before eating (Mahdy *et al*. 2008)
Washing vegetables and fruits	Practicing of vegetables and fruits washing before eating (Mahdy *et al.* 2008)

The participants who agreed willingly to contribute were questioned face-to-face in their house settings by two research aides who were trained on how to manage the questionnaire for the goal of this study.

The questionnaire involved information about the demographic (age, gender, and number of family members), socioeconomic (education level, occupation, overall family monthly income), behavioural (personal hygiene practices including washing hands prior eating and beyond using toilets, consuming of raw vegetables and fruits and washing them before consumption, eating by hands, indiscriminate defecation, filtering or boiling drinking water, cutting nails periodically), medical history (whether the participant has taken antiparasitic medicines, history of chronic infections), and environmental factors (housing situation, sanitation and features such as type of water resource, garbage disposal system, latrine system and existence of domestic animals) that were applied to evaluate the possible risk factors of amoebiasis and other intestinal parasitic infections.

3.6 SAMPLE COLLECTION

3.6.1 Stool collection, transport, and processing

Following the administration of the questionnaire, each participant was given a 60-ml labelled, wide-mouth, screw-capped container and was taught to bring a proper size of the faecal sample that was not contaminated with urine or water or soil. Each participant was taught to measure a thumb sized stool sample, using a provided scoop, into the container. After that, he/ she put the container in a zip-locked plastic bag. During the sample collection, children were monitored by their parents and guardians as they were instructed to make sure that the children placed their faecal specimens into the right containers. All participants were requested to deliver enough quantity of faecal specimen to be sufficient for both microscopic methods and the molecular technique could be applied. The samples were placed in zip-locked plastic bags, kept in a suitable

cool box (4-6ºC) and transported (within 5 hours of collection) for examination at the nearest health centre equipped with laboratory standard facilities, according to prior arrangements. The samples were processed immediately for microscopic examination by different standard methods including wet mount, formalin-ether sedimentation, and trichrome staining techniques. Almost 1 g of each faecal specimen was conserved in 70% ethanol (DNA-friendly) (Verweij *et al.*, 2007) before being refrigerated and shipped to the Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur for genomic DNA extraction and molecular analysis.

For quality control, 20% of the trichrome-stained slides as well as duplicate slides for the wet-mount and formalin-ether sedimentation techniques were read by another microscopist. Overall, a sample was considered positive if cysts and/or trophozoites of *Entamoeba* spp. were investigated by using any one of the three methods and/or the nested multiplex PCR assay. Figure 3.3 shows the flow chart of the study.

3.6.2 Stool examination by microscopy

The specimens were treated immediately via wet mount and formalin-ether sedimentation techniques following Cheesbrough (2005) and examined for the existence of cysts and/or trophozoites of *Entamoeba* spp. as well as diagnostic stages of other intestinal parasites. Moreover, about 5 g of each sample was placed into a 15-ml screw-cap centrifuge tube containing 3 ml of polyvinyl alcohol (PVA) as a preservative; this portion was processed by trichrome staining technique (WHO, 1998).

3.6.2.1. Direct smear

A drop of normal saline was placed on a glass slide. A small amount of sample was picked up using a wooden applicator stick and mixed with normal saline. A coverslip was placed gently on the preparation with avoiding the formation of air bubbles. The entire coverslip area was then examined under the light microscope using low power

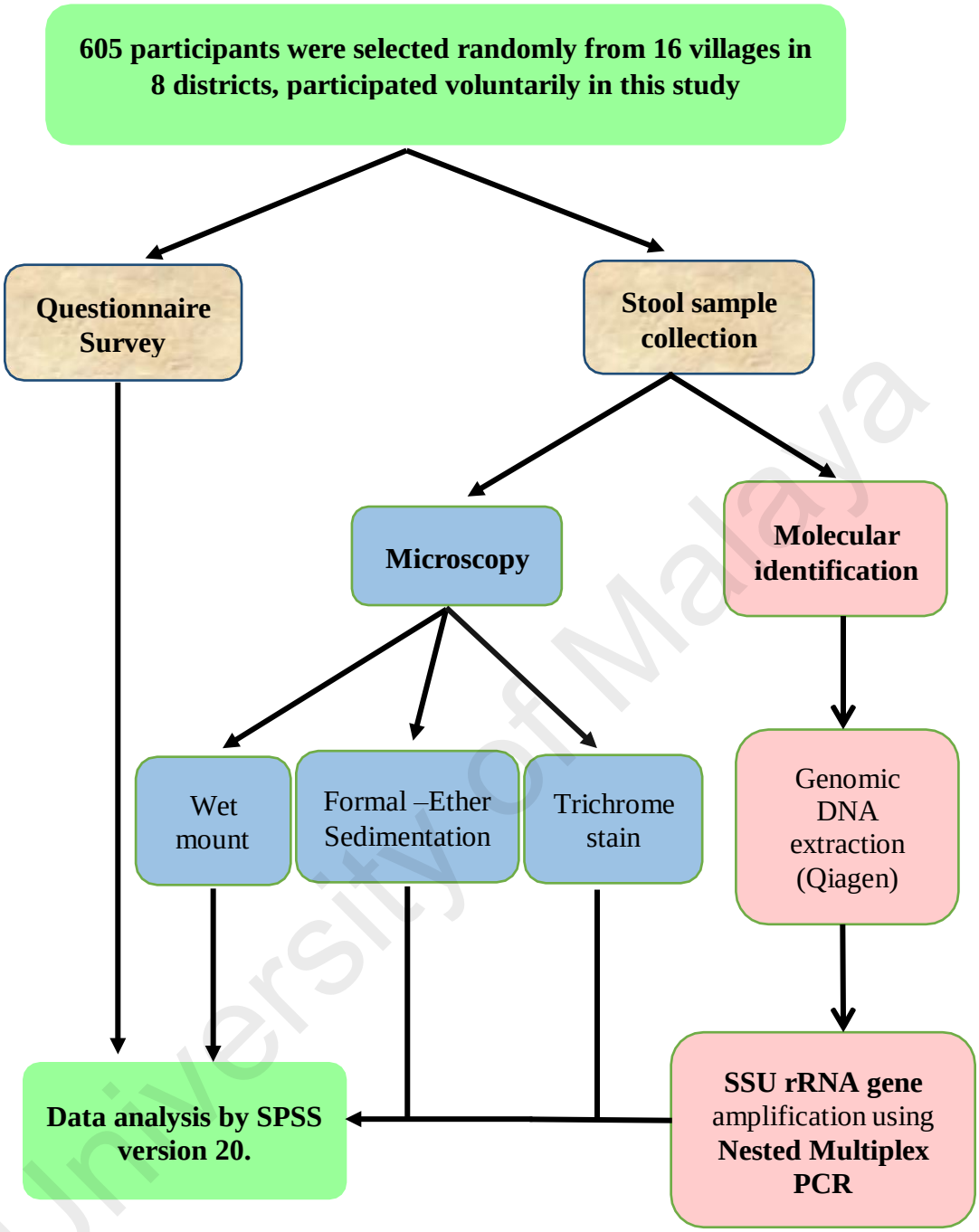

FIGURE 3.3: Flow chart of the study

(10X) then high power objectives (40X). A drop of iodine was added to the edge of the coverslip in order to be absorbed under the coverslip when identifying cysts was required (Cheesbrough, 2005).

3.6.2.2. Formalin-ether sedimentation technique

Formalin-ether sedimentation technique is the method of choice to detect intestinal parasites (Cheesbrough, 2005). This method used to concentrate parasitic elements through sedimentation to enhance recovery.

Briefly, about 1-2 g of each faecal sample was emulsified in 7 mL of 10 % formalin. The resulting emulsion was filtered through two layers of gauze into a conical centrifuge tube. Then, 3 mL of ethyl acetate was added to the filtrate; the tube was locked with a stopper and shacked vigorously, and centrifuged at 3000 rpm for 1 min. After centrifugation, four layers were separated; ethyl acetate as the top layer, a plug of faecal debris, formalin, and parasites-containing sediment as the bottom precipitate. The plug of faecal debris was detached from the side of the tube by using a wooden applicator stick and then top three layers were poured off leaving a small quantity of formalin for suspension of the sediment. The remaining sediment was examined as a direct smear (iodine may be used) for the detection of protozoa, eggs, and larvae of intestinal parasites.

3.6.2.3. Trichrome staining technique

The faecal sample in PVA was centrifuged at 1500 rpm (3 min) and the PVA was poured out. With a wooden applicator stick or a plastic Pasteur pipette, some of the sediment of stool material was transferred and spread to the slide evenly to form a fine smear. The slide was allowed to dry for an hour at 35-37^{o}C or overnight at room temperature. Then, the slide was processed for trichrome staining as the following: placed the slide in Wheatley trichrome stain for 10 min, the slide was placed in 90% acid ethanol for 1-3 seconds. Then, the slide should be processed immediately and

dipped several times in 95% ethanol. The slide was then placed in two changes of 95% ethanol for 3 min each. After that, the slide was placed in two changes of xylene for 5-10 min each. Then, the mounting medium was applied to the smear and covered with a No. 1 thickness coverslip. The smear was allowed to dry overnight at room temperature or for 1 hour at 35-37 ºC and then examined microscopically with the 100X objective (WHO, 1998; Anuar *et al*., 2013).

3.7 MOLECULAR ANALYSIS

3.7.1 Genomic DNA samples and extraction

Before DNA extraction, all ethanol-fixed stool samples were washed three times in MilliQ H2O buffer. Afterward, the samples were centrifuged for 5 min at 2000 rpm to get rid of the ethanol. Then, extraction was treated using the QIAamp Fast DNA Stool Mini Extraction Kit (QIAGEN, Hilden, Germany). In the first step, lysis buffer from the kit was added to each stool sample. Then, DNA was extracted from all faecal specimens (n = 605) as stated by the manufacturer's instructions (QIAamp Fast DNA Stool Mini Extraction Kit).

The extracted DNA was then kept at -20°C until further use. DNA isolated from axenically grown *E.histolytica* HM-1: IMSS was a gift from Dr. Lim Boon Huat (Universiti Sains Malaysia), while DNA isolated from axenically grown *E. dispar* SAW760 and *E. moshkovskii* Laredo were gifts from Dr. Graham Clark (London School of Hygiene and Tropical Medicine) were used as positive controls in this study.

3.7.2 Nested-multiplex PCR amplification

Nested-multiplex PCR investigation was depended on the amplification of the small subunit ribosomal RNA (SSU rRNA) gene of *Entamoeba* sppaccording to an earlier protocol described by Khairnar and Parija (2007). In the first round of PCR, primers specific for the genus of *Entamoeba* species was used, including E-1 (5'-TAAGATGCACGAGAGCGAAA-3') as a forward primer, and E-2 (5'-

GTACAAAGGGCAGGGACGTA-3') as a reverse primer. The secondary PCR was designed for distinguishing the three different species of *Entamoeba* using species-specific primer pairs for each species; a forward EH-1 (5'-AAG CAT TGT TTC TAG ATC TGA G-3') and reverse EH-2 (5'-AAG CAT TGT TTC TAG ATC TGA G-3') primers for *E. histolytica*;ED-1 (5'-TCT AAT TTC GAT TAG AAC TCT-3') forward primer and ED-2 (5'-TCC CTA CCTATT AGA CAT AGC-3') reverse primer for *E. dispar* andMos-1 (5'-GAAACCAAGAGTTTCACAAC-3') forward primer and Mos-2 (5'-CAATATAAGGCTTGGATGAT-3') reverse one for *E. moshkovskii*.

Briefly, the amplification in nested multiplex PCR was executed in a final volume of 25 µl, including 2.5 µl of 10X PCR buffer (Biogene), 1.4 µl of deoxynucleoside triphosphate mix (5 mM each dNTP, ABgene), 1.5 µl of 25 mM $MgCl_2$ (Bangalore genei), 0.3 µl (5 IU/µl) of Taq polymerase (Biogene), 0.3 µM of each primer (IDT) and 2.5 µl of template DNA was added in genus specific and species specific PCR. Finally, the PCR tubes were placed in an Eppendorf Thermal cycler (Master cycler gradient). The PCR mix was exposed to an initial denaturation at 96°C for 2 min, followed by 30 cycles of 92°C for 60 seconds, 56°C for 60 seconds and 72°C for 90 seconds. Lastly, one cycle of extension at 72°C for 7 min was performed. In the species-specific nested-multiplex PCR (which had multiple primer sets in the same tube), only the annealing temperature was changed to 48°C, leaving the other parameters of the amplification cycles unchanged. Ten microliters of the amplification products were separated by electrophoresis through 2% agarose gel (Agarose Low EEO, Bangalore genie products, Bangalore, India) in 0.5 × Tris-Acetate EDTA at 100 V for 45 min, then were stained with SYBR® safe DNA gel stain (Invitrogen, USA) and visualized under UV documenting system for the suitable size of DNA bands compared to 100 or 50 bp DNA ladder. Positive and negative control reactions were included with each batch of nested multiplex PCR. The species-specific product size for *E. histolytica*,

E. moshkovskii and *E. dispar* was 439, 553 and 174 bp, respectively.

3.7.3 Sequencing of PCR product

To confirm the species description, 12 specimens were randomly submitted to DNA sequencing of the partial region of the SSU rRNA gene for the confirmation of the diagnosis illustrated by PCR. Sequence chromatograms were viewed using Sequence Scanner version 1.0 program (Applied Biosystem, USA). Forward and reverse sequences were edited, manually aligned and the consensus sequence was created for each sample using the BioEdit software, and compared with the National Centre for Biotechnology Information (NCBI) reference sequences using the Sequence Basic Local Alignment Search Tool (BLAST) (Hall, 1999).

The following reference sequences were used in the analyses as reference sequences: GenBank accession number X56991 for *E. histolytica*, GenBank accession number Z49256 for *E. dispar* and GenBank Accession number AF149906 for *E. moshkovskii*.

3.8 DATA MANAGEMENT AND STATISTICAL ANALYSIS

Data was entered, reviewed and cleaned via two different research assistants. Afterward, a third researcher crosschecked the two data sets for accuracy and created a single data set for data analysis. Data analysis was done by using IBM SPSS Statistics, version 20 (IBM Corporation, NY, USA). Pearson's Chi Square test was applied to estimate the association of *Entamoeba* spp. infection as the main outcome with some socio-demographic, behavioural and environmental factors as the explanatory variables. Except for age, all the variables included in the present study were coded as binary dummy variables (i.e., 0 and 1). For instance, *Entamoeba* infection (positive = 1, negative = 0); gender (male = 1, female = 0); presence of toilet in the house (no = 1, yes = 0); washing hands before eating (no = 1, yes = 0); and family size was (> 7 members = 1, ≤ 7 members = 0). Similarly, family size (number of people living in the same house) was classified into two groups (< 8 and ≥ 8 members) according to a previous

study conducted among rural populations in the studied governorates (Youssef *et al*., 2000). Moreover, multiple logistic regression analysis was used to identify the risk factors significantly associated with the infection. Odd ratios (OR) and their corresponding 95% confidence intervals (CI) were calculated using univariate and multivariable logistic regression analyses. Also, the population attributable risk fraction (PARF) was calculated for risk factors significantly associated with *Entamoeba* complex infection (WHO, 2016). The level of statistical significance was set at $P < 0.05$.

3.9 ETHICAL CONSIDERATION

Ethical approval was gained from the Medical Ethics Committee of the University of Malaya Medical Centre (Ref. no: 968.4). It was also permitted by the Hodeidah University (Ref. no: 2174), Yemen, and the governorate health offices in the provinces (Ref. no: 228 and 887). All the participants were informed about the aims and techniques of the study as well as about the nature of their participation. Afterward, written and signed or thumb-printed informed approvals were gathered from the contributors before the commencement of the survey. For children, the related permissions were obtained from their parents or carers, and this procedure was also approved by the ethics committee.

CHAPTER4: RESULTS

4.1 GENERAL CHARACTERISTICS OF STUDY POPULATION

A total of 605 individuals (64% male, 36% female) aged between 1 and 80 years with median age of 11 years (interquartile range (IQR) = 8, 18) participated in the current study.

Overall, poverty dominates in these rural communities: more than half of the participants (68.4%) were on a low monthly household income (< 20,000 Yemeni Rials (YER) equivalent to US$93), and most of them were unemployed. Moreover, only 22.3% of the participants had at least a primary education, and about half of the families (52.4%) had more than seven members. Most of the houses were built of stone and mud (traditional houses) and some were built of bricks and concrete; only 26.0% of the houses had a piped water supply, 49.8% had toilets and 24.1% had electricity (during night time). The general characteristics of the participants are shown in Table 4.1.

4.2 PREVALENCE AND DISTRIBUTION OF *Entamoeba* COMPLEX INFECTION (MICROSCOPY-BASED RESULTS)

The overall prevalence of *Entamoeba* complex infection was 53.6% (324/605). An age-dependency distribution was observed with the prevalence of infection increasing until the 21-30 years of age group and then reducing among older participants, thus displaying a convex pattern of distribution (χ^2 = 39.373, P< 0.001). Also, male participants had a significantly higher prevalence rate in comparison with female (57.6% vs 46.3%, χ^2 = 7.149, P = 0.008). The distribution of infection in relation to age and gender is presented in Figure 4.1.

Figure 4.2 displays the distribution of signs and symptoms among the infected participants. Overall, loss of appetite, abdominal discomfort and fatigue were the most common symptoms followed by diarrhoea and nausea.

TABLE 4.1: General characteristics of the participants (n= 605)

Characteristics	**Number (%)**
Age groups (years):	
≤ 10	274 (45.3)
11 – 17	158 (26.1)
≥ 18	173 (28.6)
Gender:	
Males	387 (64.0)
Females	218 (36.0)
Residency:	
Sana'a	149 (24.6)
Dhamar	150 (24.8)
Taiz	159 (26.3)
Hodeidah	147 (24.3)
Socioeconomic status:	
Education level (at least 6 years)	135 (22.3)
Occupational status (working)	136 (22.5)
Low household monthly income (< YER 20,000)	414 (68.4)
Large family size (> 7 members)	317 (52.4)
Piped water supply	157 (26.0)
Presence of toilet in house	301 (49.8)
Electricity	146 (24.1)

YER, Yemen Rial; (US$1 = YER216)

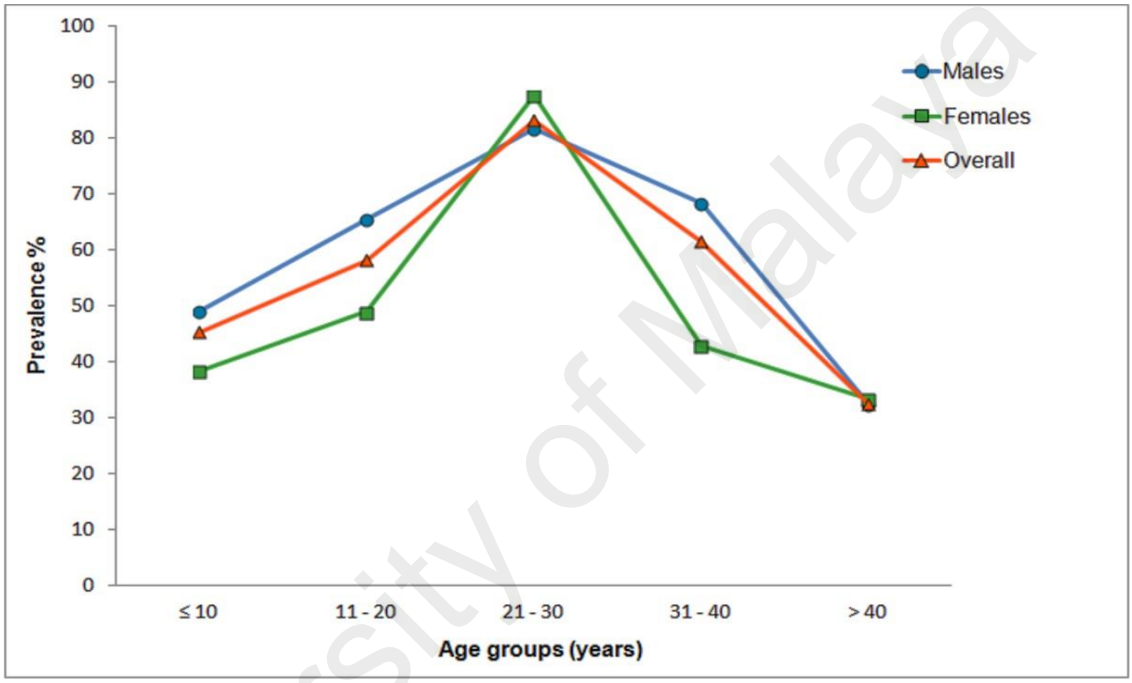

FIGURE 4.1: Prevalence and distribution of *Entamoeba*complexinfection among the participants according to age and gender.

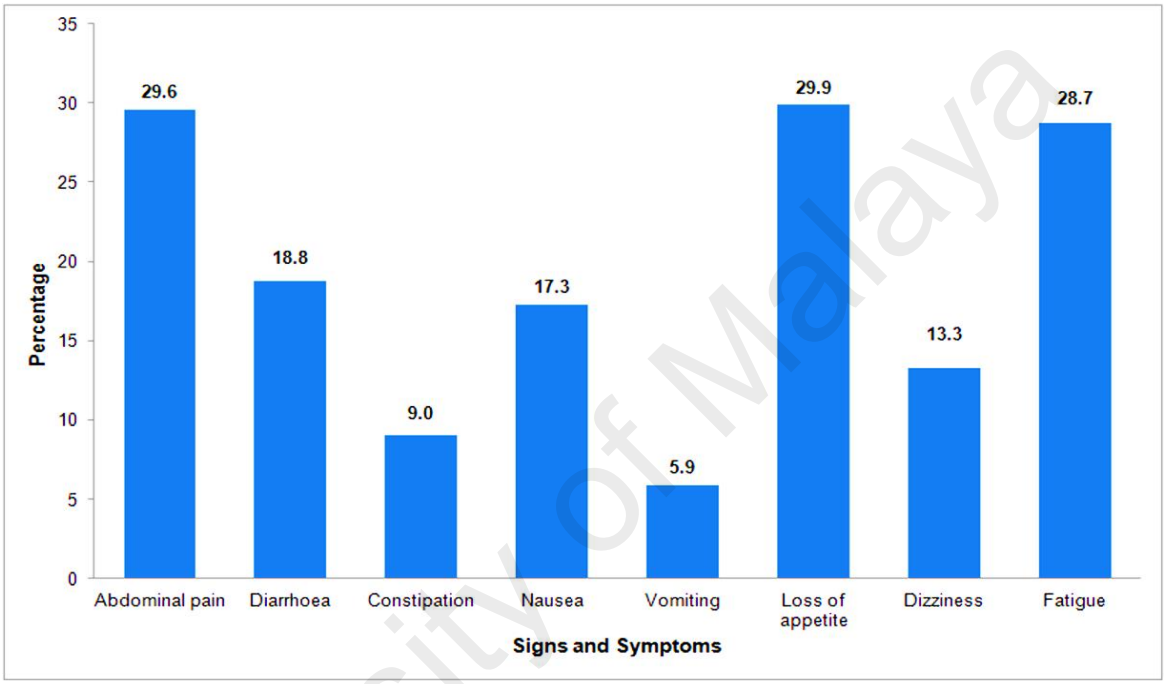

FIGURE 4.2: Distribution of signs and symptoms among *Entamoeba* complex-infected participants.

The prevalence of *Entamoeba* complex among the participants who had abdominal pain (61.1% vs. 50.9%; $\chi^2 = 4.914$; $P = 0.027$) and fatigue (62.0% vs. 50.8%; $\chi^2 = 5.720$; $P = 0.027$) was significantly high when compared with the non-infected.

4.3 ASSOCIATED FACTORS WITH *Entamoeba* COMPLEX INFECTION

4.3.1 Univariate analysis

Table 4.2 shows the association of *Entamoeba* infection with some demographic, socioeconomic and environmental factors. Besides the effect of the age and gender of the participants on the prevalence of the infection, it was also significantly higher among those who had a low educational level compared to those who had at least a primary education (55.7% vs 45.9%; $P = 0.044$) as well as among working participants compared to their unemployed peers (61.0% vs 51.4%; $P = 0.047$), and those who lived in coastal/foothill areas compared to those from the highlands (66.7% vs 40.1%; $P< 0.001$). Moreover, it was found that the presence of other family members infected with *Entamoeba* spp. showed a significant association with a higher prevalence of infection ($P< 0.001$). Likewise, those who used unsafe sources for drinking water had a higher prevalence when compared to those who used piped water (59.2% vs 37.6%; $P< 0.001$). Similarly, those who lived in houses without toilet facilities were found to have a higher prevalence of infection as compared with their counterparts (59.5%vs 47.5%; $P = 0.003$).

In the same vein, significant associations between infection and personal hygiene practices were also reported as participants who did not wash vegetables before consumption had a significantly higher prevalence of infection when compared with those who washed vegetables (65.6% vs 47.1%; $P< 0.001$). Similarly, the prevalence was found to be higher among those who did not wash their hands after playing with soil or gardening compared to their peers who always washed their hands (58.3% vs50.1%; $P =0.048$).

TABLE 4.2: Univariate analysis of potential risk factors associated with *Entamoeba* spp. infection among participants in rural Yemen (n = 605).

Variables	No examined	% infected	OR (95% CI)	*P*
Age group (years):				
≥ 18	173	67.6	2.27 (1.57, 3.29)	<0.001*
< 18	432	47.9	1	
Gender:				
Male	387	57.6	1.58 (1.13, 2.20)	0.008*
Female	218	47.3	1	
Location:				
Coastal/foothill areas	306	66.7	2.98 (2.14, 4.16)	< 0.001*
Highlands	299	40.1	1	
Education level:				
Non-educated	470	55.7	1.48 (1.01, 2.18)	0.044*
Educated	135	45.9	1	
Occupational status:				
Not working	469	51.4	0.68 (0.46, 0.99)	0.047*
Working	136	61.0	1	
Family size				
> 7	317	56.5	1.28 (0.93, 1.76)	0.132
≤ 7	288	50.3	1	
Household monthly: income				
< YER20,000	414	53.9	1.04 (0.74, 1.47)	0.821
≥ YER20,000	191	52.9	1	
Presence of other family members infected with *Entamoeba* spp.:				
Yes	251	70.9	3.47 (2.46, 4.91)	< 0.001*
No	354	41.2	1	
Source of drinking water:				
Unsafe (well, streams, rain, dams);	448	59.2	2.41 (1.67, 3.50)	< 0.001*
Safe (piped water)	157	37.6	1	
Presence of toilet in house:				
No	304	59.5	1.63 (1.18, 2.24)	0.003*
Yes	301	47.5	1	
Washing hands before eating:				
No	244	56.1	1.19 (0.86, 1.65)	0.293
Yes	361	51.8	1	
Washing hands after playing with soil or gardening:				
No	254	58.3	1.39 (1.01, 1.92)	0.048*
Yes	351	50.1	1	

TABLE 4.2: Continued

Variables	No examined	% infected	OR (95% CI)	*P*
Washing hands after defecation:				
No	229	56.8	1.23 (0.89, 1.71)	0.216
Yes	376	51.6	1	
Cutting nails periodically:				
No	352	51.4	0.81 (0.59, 1.13)	0.215
Yes	253	56.5	1	
Close contact with domestic animals:				
Yes	402	50.7	0.71 (0.51, 1.01)	0.051
No	203	59.1	1	
Indiscriminate defecation:				
Yes	332	55.1	1.15 (0.83, 1.59)	0.394
No	273	51.6	1	
Washing vegetables before consumption:				
No	212	65.6	2.14 (1.52, 3.03)	< 0.001*
Yes	393	47.1	1	
Washing fruits before consumption:				
No	182	57.1	1.23 (0.87, 1.75)	0.246
Yes	423	52.0	1	
Boiling drinking water:				
No	554	53.6	1.03 (0.58, 1.83)	0.927
Yes	51	52.9	1	

YER = Yemen Rial; (US$1 = YER216); OR = Odds ratio; CI = Confidence interval; Reference group marked as OR = 1; *Significant association ($P < 0.05$).

4.3.2 Multivariate analysis

Table 4.3 shows that six factors were retained by the multiple logistic regression model analysis. Those aged ≥ 18 years were at about twice as likely to be infected with *Entamoeba* complex (OR = 2.26) when compared with those aged below 18 years (95% CI= 1.82, 4.34). Similarly, the presence of another family member infected with *Entamoeba* spp. increased the individual's odds of having *Entamoeba* complex infection and living in coastal/foothill areas by four (OR = 3.95; 95% CI = 2.71, 5.73) and three times (OR = 2.90; 95% CI = 1.98, 4.26), respectively. Likewise, participants who used unsafe drinking water had 2.52 (95% CI = 1.64, 3.80) times the odds of having *Entamoeba* complex infection compared to those who had a piped water supply in their house.

Moreover, non-educated participants had a 2.53 odds of infection compared to their educated counterparts (95% CI = 1.56, 4.11). Similarly, not washing vegetables before consumption increased the participant's odds of having the disease by 1.63 times (95% CI = 1.09, 2.43) when compared with those who always washed vegetables. The PARF analysis showed that the prevalence of *Entamoeba* complex infection among the study population could be reduced by 29.8% and 23.0% if they had safe drinking water and if all infected family members were diagnosed and treated, respectively. Moreover, 14.2% and 12.1% of the cases could be prevented if the participants had at least a primary education and washed vegetables before consumption, respectively. In addition, 10.5% of the cases could be avoided by focusing control and preventive efforts on people aged 18 years and above.

4.4 PREVALENCE AND DISTRIBUTION OF *E.histolytica, E.dispar* AND *E. moshkovskii* INFECTIONS (PCR BASED RESULTS)

Using nested multiplex PCR, bands were successfully produced with the expected size of the three species, 439 bp, 174 bp, and 553 bp for *E. histolytica*, *E. dispar*, and *E.*

moshkovskii respectively (Figure 4.3. A&B).

The nested multiplex PCR products were detected in 45.6% (276/605) stool samples. *E. histolytica* was detected in 20.2% (122/605) of the samples while *E. dispar*, and *E. moshkovskii* isolates were recovered in 15.7% (95/605) and 18.2% (110/605) of the samples (Figure 4.4). That said, among the 276 *Entamoeba*-PCR-positive isolates, the prevalence of *E. histolytica*, *E. dispar*, and *E. moshkovskii* was 44.2%, 34.4% and 39.9%, respectively. Moreover, single isolations of *E. histolytica*, *E. dispar*, and *E. moshkovskii* were described in 84, 68 and 83 samples, respectively while 14 samples were positive for *E. histolytica* and *E. dispar*, 13 samples were positive for *E. histolytica* and *E. moshkovskii*, and ten samples were positive for the triad *Entamoeba* species (Figure 4.4), (Table 4.4). Overall, 55 microscopy-positive samples were found to be negative by the PCR. These samples were re-tested by PCR and were still found to be negative.

The overall prevalence of *Entamoeba* complex as well as *E. histolytica*, *E. dispar*, and *E. moshkovskii* according to age groups, gender and provinces is showed in Table 4.5.

The results showed significant differences in prevalence of *Entamoeba* complex and *E. Moshkovskii* with all these three factors while the significant differences of *E. histolytica* and *E. dispar* were found with only one variable each; provinces and age groups, respectively. The age-dependency distribution was retained for the three *Entamoeba* species with an obvious difference in the prevalence of *E. moshkovskii* between male and female participants (22.7% vs 10.1%, $\chi^2 = 14.994$, $P < 0.001$). The distribution of *E. histolytica*, *E. dispar* and *E. moshkovskii* infections according to age and gender is presented in Figure 4.5.

TABLE 4.3: Multivariate analysis of risk factors associated with *Entamoeba* complex infection among participants in rural Yemen (n = 605).

Variables	*Entamoeba* spp. Infection		
	Adjusted OR	**95% CI**	***P***
Age groups (≥ 18 years)	2.82	1.82, 4.34	< 0.001*
Gender (male)	1.35	0.91, 2.01	0.142
Location (coastal/foothill areas)	2.90	1.98, 4.26	< 0.001*
Education level (non-educated)	2.53	1.56, 4.11	0.001*
Occupational status (working)	0.69	0.36, 1.28	0.195
Presence of other family members infected with *Entamoeba spp.*	3.95	2.71, 5.73	< 0.001*
Source of drinking water (unsafe)	2.52	1.64, 3.80	< 0.001*
Presence of toilet in house (no)	1.21	0.82, 1.81	0.341
Washing hands after playing with soil or gardening (no)	0.92	0.62, 1.37	0.694
Washing vegetables before consumption (no)	1.63	1.09, 2.43	0.025*

OR, Odds ratio; CI, Confidence interval

*Significant key risk factors ($P < 0.05$).

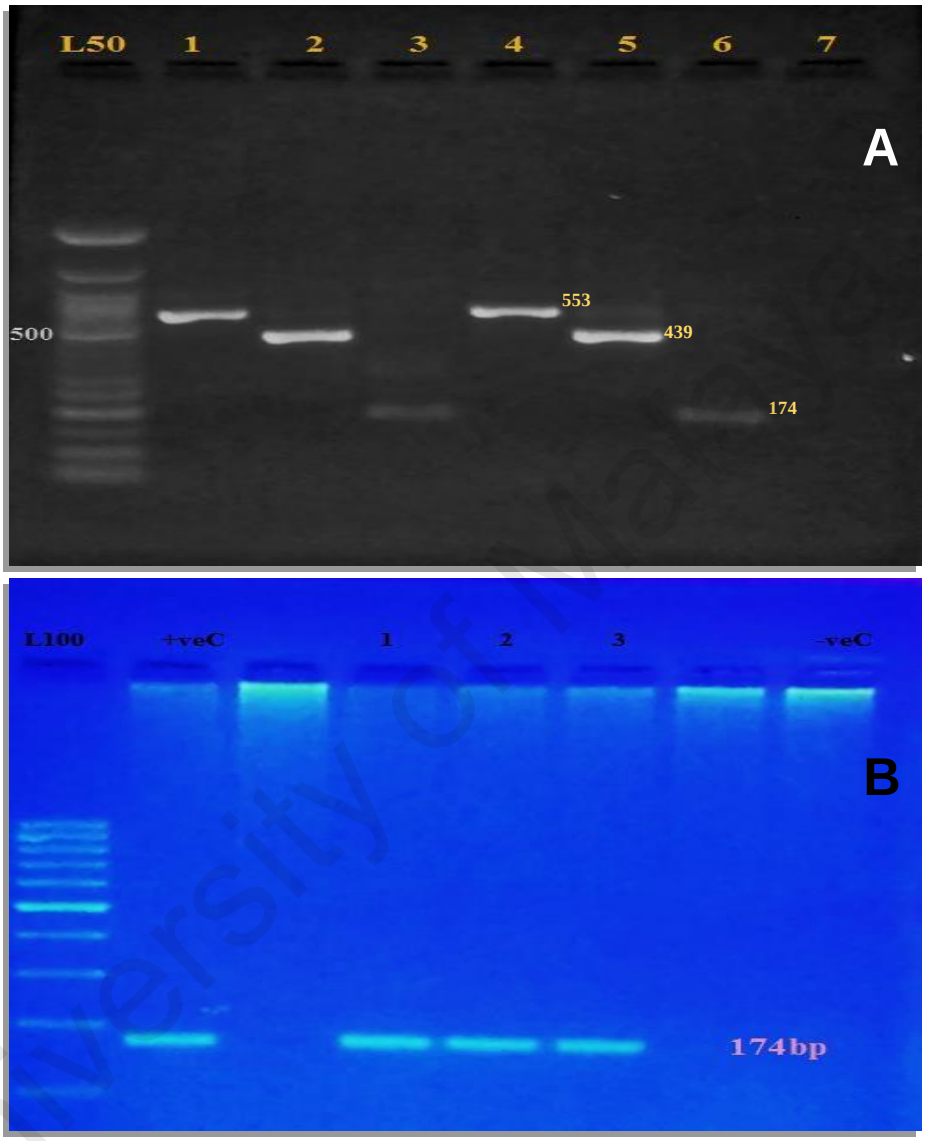

FIGURE 4.3.A: Agarose gel electrophoresis photos

(A) Agarose gel electrophoresis of *Entamoeba* spp. using a Nested multiplex PCR assay. Lane 1, 50 bp ladder DNA marker; lane 2, *Entamoeba moshkovskii* positive control; lane 3, *Entamoeba histolytica* positive control; lane 4, *Entamoeba dispar* positive control; lane 5, *E. moshkovskii* isolate; lane 6, *E. histolytica* isolate; lane 7, *E. dispar* isolates; lane 8, negative Dnase-free water control.

(B) Positive control (Lane +ve C) and positive DNA samples (well No. 1, 2 &3) for *E. dispar* (174bp).

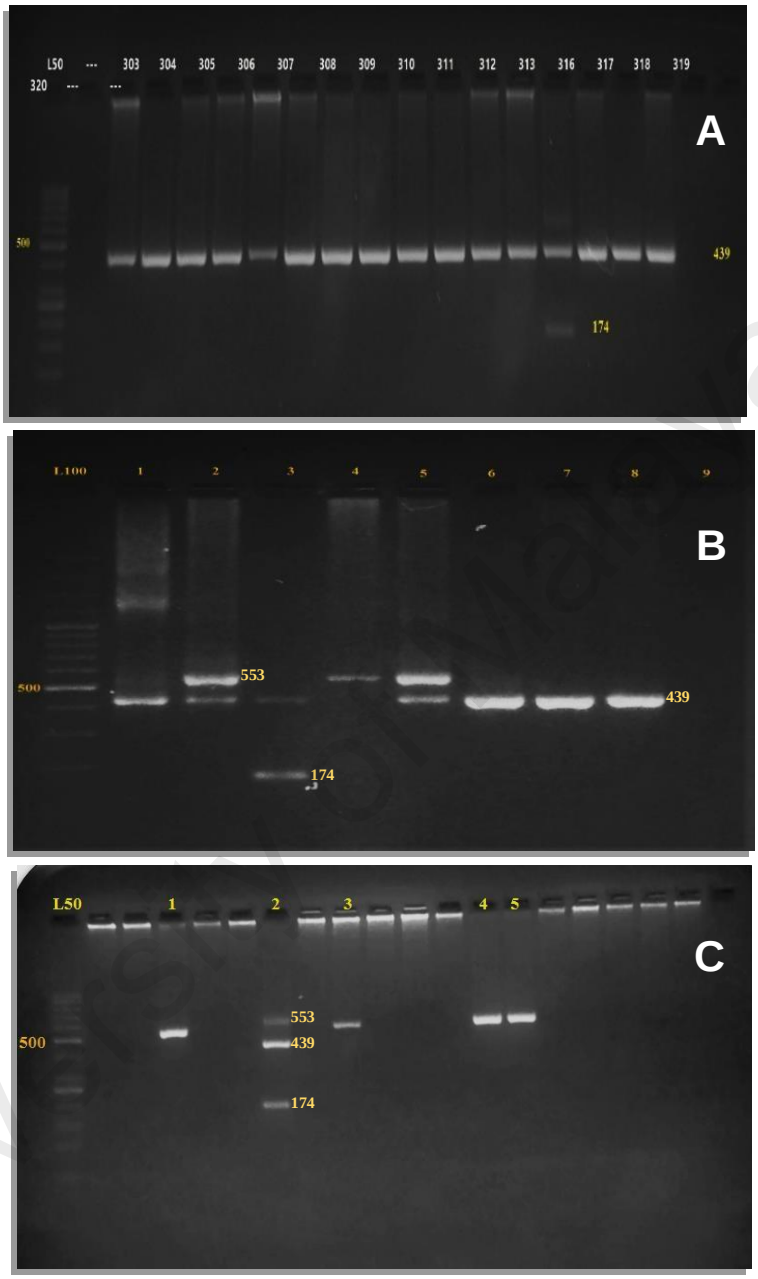

FIGURE 4.3.B: Agarose gel electrophoresis photos

(A) Positive DNA samples of *Entamoeba histolytica* (Well 3-18) and one mixed infection with *E. dispar* (well No. 15).

(B) Shows mixed infections of the *Entamoeba* species: in Lane 2 and 5. (*Entamoeba moshkovskii+ Entamoeba histolytica*), lane 3 (*Entamoeba histolytica+ Entamoeba dispar*).

(C) Shows mixed infections of the three species together in lane 2.

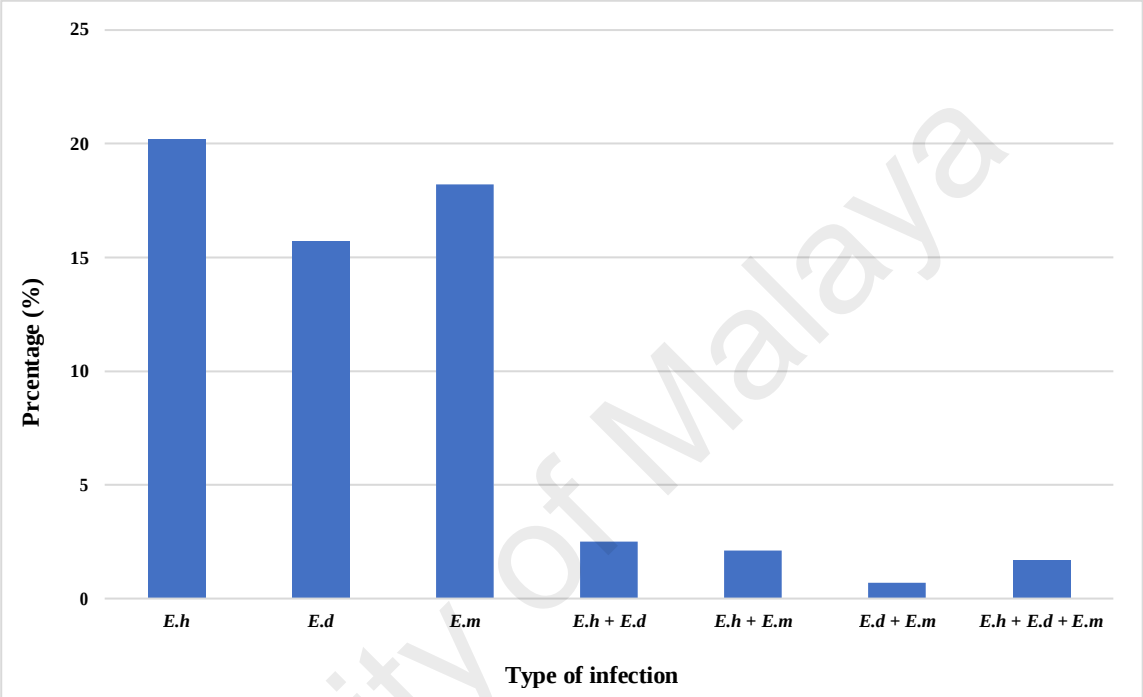

FIGURE 4.4: Prevalence of single and mixed *Entamoeba* infections among participants.

(*E. h: Entamoeba histolytica, E. d: Entamoeba dispar, E.m: Entamoeba moshkovskii).*

TABLE 4.4: Results of microscopic examination (*Entamoeba* complex) and nested multiplex PCR (*E. histolytica*, *E. dispar* and *E. moshkovskii*) performed on 605 stool samples

		Nested-multiplex PCR[b]								**Total**
		E.h	*E.d*	*E.m*	*E.h+E.d*	*E.h+E.m*	*E.d+E.m*	*E.h+E.d+E.m*	Negative	
Microscopic examination[a]	Positive	82	68	78	14	13	4	10	55	324
	Negative	2	0	4	1	0	0	0	274	281
	Total	84	68	82	15	13	4	10	329	605

[a] Species cannot be distinguished.
[b] Species detected by PCR.
E.h,Entamoebahistolytica; *E.d*, *Entamoebadispar; E.m*, *Entamoebamoshkovskii*.

TABLE 4.5: Prevalence and distribution of *Entamoeba histolytica*, *E. dispar* and *E. moshkovskii* infections among the participants according to age, gender and location.

Variable	N	*E. h* n (%)	*E. d* n (%)	*E.m* n (%)	*E.* complex n (%)
Overall	605	122 (20.2)	95 (15.7)	110 (18.2)	324 (53.6)
Age groups (years):					
< 18 (children)	432	84 (19.4)	57 (13.2)	70 (16.2)	207 (47.9)
≥ 18 (adults)	173	38 (22.0)	38 (22.0)	40 (23.1)	117 (67.6)
χ^2	-	0.488	7.179	3.974	19.300
P	-	0.485	0.007*	0.046*	< 0.001*
Gender:					
Male	387	78 (20.2)	64 (16.5)	88 (22.7)	223 (53.6)
Female	218	44 (20.2)	31 (14.2)	22 (10.1)	101 (46.3)
χ^2	-	0.000	0.566	14.994	7.149
P	-	0.993	0.452	< 0.001*	0.008*
Location:					
Sana'a	149	24 (16.1)	31 (20.8)	10 (6.7)	68 (45.6)
Dhamar	150	23 (15.3)	18 (12.0)	15 (10.0)	52 (34.7)
Taiz	159	36 (22.6)	21 (13.2)	50 (31.4)	108 (67.9)
Hodeidah	147	39 (26.5)	25 (17.0)	35 (23.8)	96 (65.3)
χ^2	-	8.005	5.421	41.864	46.630
P	-	0.046*	0.143	< 0.001*	< 0.001*

N, number of individuals examined. n, number of infected individuals.
χ^2, Chi-square test statistic.
E.h.= Entamoeba histolytica, E.d= Entamoeba dispar, E.m= Entamoeba moshkovskii

4.5 ASSOCIATED FACTORS WITH *E. histolytica, E.dispar* AND *E.moshkovskii* INFECTIONS - UNIVARIATE ANALYSIS (PCR BASED RESULTS)

Overall, the prevalence of *Entamoeba* complex infection was significantly higher among participants aged from 18 to 40 years (77.2%) compared to other age groups ($P< 0.001$).

Similarly, the results further revealed that the prevalence rate was significantly higher among those who live in coastal/foothill areas (i.e. Taiz and Hodeidah provinces) compared to those from the highlands (i.e. Sana'a and Dhamar) (66.7% vs. 40.1%; $P< 0.001$). Table 4.6 shows the association of *E. histolytica*, *E. dispar* and *E. moshkovskii* infections with some demographic, socioeconomic and environmental factors.

Moreover, significantly higher prevalence was reported among those who had a low educational level compared to those who had at least a primary education (55.7% vs 45.9%; $P = 0.044$) as well as among working participants compared to their unemployed peers (61.0% vs 51.4%; $P = 0.047$).

Moreover, it was found that the presence of other family members infected with *Entamoeba* spp. showed a significant association with a higher prevalence of infection ($P< 0.001$). Likewise, those who used unsafe sources for drinking water had a higher prevalence when compared to those who used piped water (59.2% vs 37.6%; $P< 0.001$). Similarly, those who lived in houses without toilet facilities were found to have a higher prevalence of infection as compared with their counterparts (59.5%vs 47.5%; $P = 0.003$).

In the same vein, significant associations between *Entamoeba* complex infection and personal hygiene practices were also reported as participants who did not wash vegetables before consumption had a significantly higher prevalence of infection when compared with those who washed vegetables (65.6% vs 47.1%; $P< 0.001$).

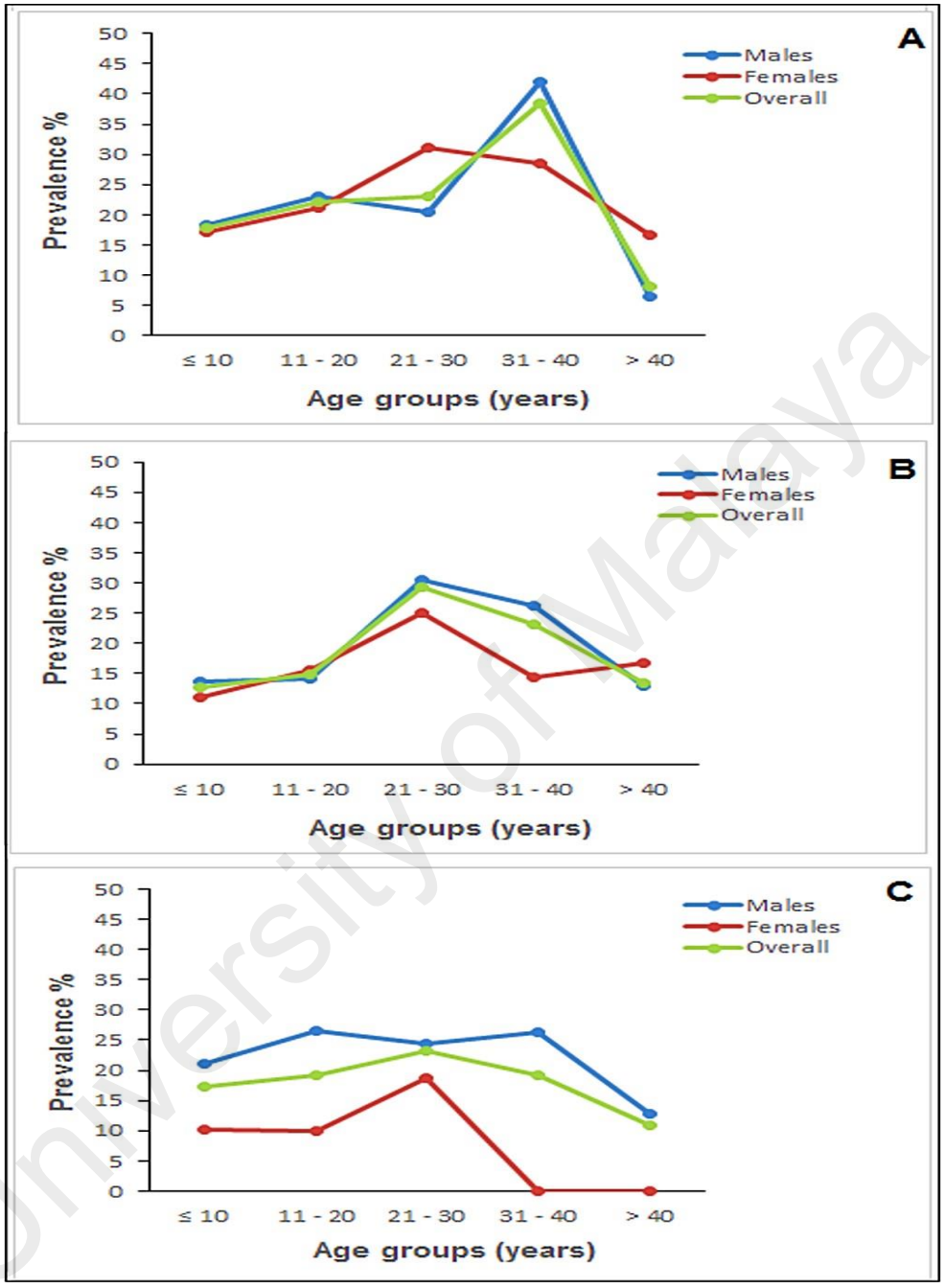

FIGURE 4.5: Prevalence and distribution of *Entamoeba* infections among the participants according to age and gender. A: *Entamoebahistolytica*B: *E.dispar* C: *E.moshkovskii.*

Further species-based analyses reported that the prevalence of *E. histolytica* infection was significantly higher among participants who lived in coastal/foothill areas (24.5% vs 15.7%; *P* = 0.007), those who were not educated (21.9% vs 14.1%; *P* = 0.045), and presence of other family member infected with *E. histolytica* (25.9% vs 16.1%; *P*< 0.003) when compared with their counterparts.

Similarly, the prevalence of *E. dispar* infection was significantly associated with five factors, including age group 18 - 40 years (*P* = 0.003), unsafe source of drinking water as well , dams , rain water or streams (*P*< 0.001), absence of toilet in houses (*P* = 0.006), indiscriminate defecation near to water sources (*P* = 0.004) and not washing vegetables before consumption (*P* < 0.001)**.** Moreover, the prevalence of *E. moshkovskii* infection was significantly higher among males (22.7% vs 10.1%; *P*< 0.001), those aged between 18 and 40 years (*P*< 0.001), those who live in coastal/foothill areas (27.8% vs8.4%; *P*< 0.001), and the presence of other family member infected with *Entamoeba* (25.5% vs 13.0%; *P*< 0.001).

4.6 RISK FACTORS OF *E. histolytica, E. dispar* AND *E. moshkovskii* INFECTIONS - MULTIVARIATE ANALYSIS

Table 4.7 shows that seven variables were retained as the significant risk factors of *Entamoeba* complex infection by the multiple logistic regression model analysis. Male participants (OR = 1.63; 95% CI= 1.07, 2.48) and those aged between 18 and 40 years (OR = 7.82; 95% CI= 3.22, 14.34) had higher odds of having *Entamoeba* complex when compared with females and other age groups. Similarly, living in coastal/foothill areas increased individual's odds for *Entamoeba* complex infection by about three times (2.90; 95% CI = 1.98, 4.26). Moreover, the presence of another family member infected with *Entamoeba* complex increased the individual's odds of having an *Entamoeba* complex infection by four (OR = 3.80; 95% CI = 2.54, 5.69).

TABLE 4.6. Univariate analysis of factors associated with *Entamoeba histolytica*, *E. dispar* and *E. moshkovskii* infections among the participants from rural Yemen (n = 605)

Variables	*E. histolytica*			*E. dispar*			*E. moshkovskii*		
	%	OR (95% CI)	*P*	%	OR (95% CI)	*P*	%	OR (95% CI)	*P*
Age groups (years):									
≤ 10	17.9	1		12.8	1		17.2	1	
11 - 17	22.2	1.31 (0.80, 2.13)	0.280	13.9	1.11 (0.62, 1.96)	0.734	14.6	0.82 (0.48, 1.42)	0.481
18 - 40	25.7	1.59 (0.97, 2.61)	0.064	24.3	2.19 (1.29, 3.71)	0.003*	26.5	1.74 (1.06, 2.85)	0.027*
≥ 41	8.1	0.41 (0.12, 1.37)	0.135	13.5	1.07 (0.39, 2.92)	0.900	10.8	0.59 (0.20, 1.73)	0.328
Gender:									
Male	20.2	0.99 (0.66, 1.51)	0.993	16.5	1.20 (0.75, 1.90)	0.452	22.7	2.62 (1.59, 4.33)	< 0.001*
Female	20.2	1		14.2	1		10.1	1	
Location:									
Coastal/foothill areas	24.5	1.74 (1.16, 2.61)	0.007*	15.0	0.90 (0.58, 1.40)	0.647	27.8	4.22 (2.61, 6.81)	< 0.001*
Highlands	15.7	1		16.4	1		8.4	1	
Education level:									
Non-educated	21.9	1.71 (1.01, 2.92)	0.045*	16.2	1.18 (0.68, 2.03)	0.555	17.9	0.91 (0.56, 1.45)	0.713
Educated	14.1	1		14.1	1		19.3	1	
Occupational status:									
Not working	20.0	0.97 (0.60, 1.55)	0.889	14.3	0.64 (0.39, 1.05)	0.075	16.8	0.69 (0.43, 1.10)	0.113
Working	20.6	1		20.6	1		22.8	1	
Family size:									
≥ 8	19.6	0.92 (0.62, 1.38)	0.696	17.0	1.24 (0.80, 1.92)	0.345	19.9	1.27 (0.84, 1.93)	0.258
< 8	20.8	1		14.2	1		16.3	1	
Household monthly income:									
< YER 20,000	20.8	1.13 (0.73, 1.74)	0.583	16.9	1.35 (0.83, 2.21)	0.230	17.4	0.85 (0.55, 1.31)	0.458
≥ YER 20,000	18.8	1		13.1	1		19.9	1	

TABLE 4.6: Continued

Variables	*E.histolytica*			*E. dispar*			*E.moshkovskii*		
	%	OR (95% CI)	*P*	%	OR (95% CI)	*P*	%	OR (95% CI)	*P*
Presence of other family members infected with *Entamoeba* spp.									
Yes	25.9	1.82 (1.22, 2.72)	0.003*	15.1	0.93 (0.60, 1.45)	0.749	25.5	2.29 (1.51, 3.49)	< 0.001*
No	16.1	1		16.1	1		13.0	1	
Source of drinking water									
Unsafe (well, streams, rain, dams)	20.3	1.04 (0.66, 1.63)	0.879	19.0	3.44 (1.74, 6.81)	< 0.001*	19.0	1.24 (0.76, 2.02)	0.394
Safe (piped water)	19.7	1		6.4	1		15.6	1	
Presence of toilet in house									
No	22.4	1.32 (0.88, 1.97)	0.175	19.7	1.87 (1.19, 2.94)	0.006*	17.1	0.87 (0.57, 1.31)	0.490
Yes	17.9	1		11.6	1		19.3	1	
Washing hands before eating									
No	22.1	1.23 (0.82, 1.83)	0.322	18.4	1.41 (0.91, 2.19)	0.128	20.1	1.24 (0.81, 1.88)	0.319
Yes	18.8	1		13.9	1		16.9	1	
Washing hands after playing with soil or gardening									
No	18.1	0.80 (0.53, 1.20)	0.284	16.9	1.17 (0.75, 1.82)	0.481	19.7	1.19 (0.78, 1.80)	0.415
Yes	21.7	1		14.8	1		17.1	1	
Washing hands after defecation									
No	20.1	0.99 (0.66, 1.50)	0.970	17.9	1.30 (0.83, 2.03)	0.245	18.8	1.07 (0.70, 1.63)	0.767
Yes	20.2	1		14.4	1		17.8	1	
Cutting nails periodically									
No	18.8	0.81 (0.54, 1.21)	0.306	13.9	0.73 (0.47, 1.13)	0.155	19.6	1.26 (0.82, 1.93)	0.285
Yes	22.1	1		18.2	1		16.2	1	

TABLE 4.6: Continued

Variables		*E.histolytica*			*E. dispar*			*E.moshkovskii*	
	%	OR (95% CI)	*P*	%	OR (95% CI)	*P*	%	OR (95% CI)	*P*
Close contact with domestic animals									
Yes	19.9	0.91 (0.55, 1.50)	0.712	16.4	1.18 (0.73, 1.89)	0.496	19.1	1.41 (0.80, 2.51)	0.236
No	21.4	1		14.3	1		14.3	1	
Indiscriminate defecation									
Yes	19.6	0.92 (0.62, 1.37)	0.692	19.6	1.97 (1.24, 3.14)	0.004*	19.8	1.24 (0.81, 1.88)	0.319
No	20.9	1		11.0	1		17.3	1	
Washing vegetables before consumption									
No	21.7	1.16 (0.77, 1.74)	0.490	22.2	2.05 (1.32, 3.19)	< 0.001*	22.2	1.49 (0.98, 2.27)	0.062
Yes	19.3	1		12.2	1		16.0	1	
Washing fruits before consumption									
No	22.5	1.23 (0.80, 1.88)	0.342	19.8	1.52 (0.96, 2.40)	0.071	17.6	0.94 (0.60, 1.49)	0.802
Yes	19.1	1		13.9	1		18.4	1	
Boiling drinking water									
No	19.9	0.81 (0.41, 1.59)	0.531	15.5	0.86 (0.40, 1.83)	0.690	18.1	0.90 (0.44, 1.86)	0.783
Yes	23.5	1		17.6	1		19.6	1	

YER = Yemen Rial; (US$1 = YER216)

OR = Odds ratio; CI = Confidence interval; Reference group marked as OR = 1

* Significant association ($P < 0.05$).

Likewise, participants who used unsafe drinking water had 2.20 (95% CI = 1.37, 3.40) times the odds of having an *Entamoeba* spp. infection compared to those who had a piped water supply in their house. Moreover, non-educated participants had a 3.51 odds of infection compared to their educated counterparts (95% CI = 2.03, 6.05). Similarly, not washing vegetables before consumption increased the participant's odds of having the disease by 1.59 times (95% CI = 1.05, 2.39) when compared with those who always washed vegetables.

In the species-based regression models (Table 4.7), three factors were found to increase the participant's odds of having *E. histolytica* infection by 1.7 times, including living in coastal/foothill areas (95%CI = 1.16, 2.66), illiteracy (95%CI = 1.02, 3.02), and the presence of other family members infected with *Entamoeba* (95%CI = 1.13, 2.56). Similarly, using unsafe drinking water (OR = 2.89; 95%CI = 1.44, 5.80) and not washing vegetables before consumption (OR = 1.68; 95%CI = 1.06, 2.67) were identified as the significant risk factors of *E. dispar* infection.

Moreover, four variables were retained as the significant risk factors of *E. moshkovskii* infection. Participants aged between 18 - 40 years (95%CI = 1.18, 3.54) and those who live with other family members infected with *Entamoeba* (95%CI = 1.37, 3.42) were at about twice as likely to be infected with *E. moshkovskii*. Similarly, male gender (95%CI = 1.63, 4.84) and living in coastal/foothill areas (95%CI = 2.21, 6.01) increased the participant's odds of having the infection by 2.81 and 3.65 times when compared with females and those who live in highlands, respectively.

TABLE 4.7: Multivariate analysis of risk factors associated with *Entamoeba histolytica*/*E. dispar*/*E. moshkovskii*infections among the participants from rural Yemen (n = 605).

	Entamoeba histolytica		***Entamoeba dispar***		***Entamoeba moshkovskii***	
Variables	**AOR (95% CI)**	***P***	**AOR (95% CI)**	***P***	**AOR (95% CI)**	***P***
Age group (18 - 40 years)	-	-	1.64 (0.95, 2.85)	0.078	2.04 (1.18, 3.54)	0.011*
Gender (male)	-	-	-	-	2.81 (1.63, 4.84)	< 0.001*
Location (coastal/foothill areas)	1.75 (1.16, 2.66)	0.008*	-	-	3.65 (2.21, 6.01)	< 0.001*
Education level (non-educated)	1.75 (1.02, 3.02)	0.044*	-	-	-	-
Occupational status (working)	-	-	-	-	-	-
Presence of other family members infected with *Entamoeba spp.*	1.70 (1.13, 2.56)	0.011*	-	-	2.17 (1.37, 3.42)	0.001*
Source of drinking water (unsafe)	-	-	2.89 (1.44, 5.80)	0.003*	-	-
Presence of toilet in house (no)	-	-	1.29 (0.75,2.23)	0.355	-	-
Washing hands after playing with soil or gardening (no)	-	-	-	-	-	-
Washing vegetables before consumption (no)	-	-	1.68 (1.06, 2.67)	0.027*	-	-
Indiscriminate defecation (yes)	-	-	1.35 (0.77, 2.38)	0.296	-	-

AOR, adjusted odds ratio; CI, Confidence interval

- Significant key risk factors ($p < 0.05$)

CHAPTER 5: DISCUSSION

Results of the current study revealed that the prevalence of *Entamoeba* complex was high with more than half (53.6%) of the participants being infected. This is consistent with previous studies that investigated the prevalence of infection among schoolchildren from urban areas in Sana'a and Al-Mahweet provinces and restaurants workers in Sana'a City (the capital of Yemen) in which the prevalence rates of *Entamoeba* complex were 52% and 48.9% (Azazy *et al.,* 2002; Al-Shibani *et al.*, 2009b).

However, generally, the present study findings are far higher than those reported in other studies on Yemen and ranges from 6.4% to 33.7% (Azazy and Raja'a, 2003; Raja'a and Mubarak 2006; Al-Shibani *et al*., 2009a; Al-Haddad and Baswaid, 2010; Alyousefi *et al.*, 2011; Bin Mohanna *et al.*, 2014; Alsubaie *et al.*, 2016). Given that the current study covered a wider area consisting of four diverse provinces as well as a wider range of participants in terms of age, the variation in the prevalence rates reported could be attributed to demographic, socioeconomic and environmental differences.

The disparity in results could also reflect differences in hygiene and sanitation conditions and other unidentified factors among the targeted communities. Moreover, the variation in the prevalence could also be due to the high sensitivity and accuracy of the laboratory techniques used in the diagnosis in the current study.

A comparison of the findings of the present study with those of studies on neighbouring countries revealed that Yemen had the highest prevalence rate in the region. Previous studies in other Middle Eastern countries have reported a varying prevalence of *Entamoeba* complex:4.9% in Jordan (Youssef *et al.*, 2000), 5.5% in Iran (Sharif *et al*., 2015), 7.1% in Oman (Prakash, 2008), 8.2%-16.2% in Saudi Arabia (Al-Mohammed *et al*., 2010*;* Amer *et al.,* 2016), 16.8% in Egypt (Hegazy *et al*., 2014), 19.2% in the United Arab Emirates (ElBakri *et al*., 2013), 23.4% in Iraq (Al-Kubaisy *et*

al., 2014), and 33.1% in Sudan (Abdel-aziz *et al*., 2010).

This study differentiates, for the first time, *E. histolytica* from *E. dispar* and *E. moshkovskii* in Yemen using the nested multiplex PCR assay. It was found that 122 (44.2%) and 95 (34.4%) of the 276 PCR-positive products were identified as *E. histolytica* and *E. dispar* which accounted for 20.2% and 15.7% of the total number of samples, respectively. Moreover, the present findings demonstrated that *E. moshkovskii* was identified in 110 (39.9%) isolates which accounted for 18.2% from the study participants (110/605). *Entamoeba histolytica*, *E. dispar* and *E. moshkovskii* were identified in 5.6%, 70.8% and 61.8%, respectively of 89 PCR-positive samples from Australia (Fotedar *et al.*, 2007a). Likewise, *E. moshkovskii* and *E. dispar* were reported in 25.4% and 23.2% of 181 asymptomatic children under 16 years old from Colombia, with *E. histolytica* was reported in one sample only (0.55%) (López *et al.*, 2015).

On the other hand, a much lower prevalence was reported among 120 faecal samples from the United Arab Emirates, with *E. histolytica*, *E. dispar* and *E. moshkovskii* were detected in 13.3%, 6.7%, and 3.3%, respectively (ElBakri *et al.*, 2013). Similarly, a previous study among 500 participants from Peninsular Malaysia revealed that *E. dispar* was the highest prevalent species (13.4%), followed by *E. histolytica* (3.2%) and *E. moshkovskii* (1.0%) (Anuar *et al.*, 2012c). However, when seeking to draw conclusions from a cross-country comparison, the differences in the socioeconomic status, environmental conditions, and other risk factors should be borne in mind as well as whether travellers and expatriates and institutionalized members of the population were included in the samples of the studies undertaken in these countries.

The present study showed that *Entamoeba* complex was significantly associated with age, as demonstrated by the convex pattern of distribution. The prevalence of infection was 45.3% among children aged ≤ 10 years and increased to reach its highest (83.1%) among participants aged 21–30 years then decreased to the lowest (32.4%)

among those aged more than 40 years. It was observed a similar pattern of distribution with *E. histolytica*, *E. dispar* and *E. moshkovskii* infections. In Brazil, Benetton *et al.* (2005) noticed that the prevalence of *E. histolytica* infection among outpatient clinics increases with age. However, the present finding contrasts that in previous studies on Saudi Arabia, Turkey, Cameroon and Malaysia which reported the highest prevalence among those aged below 12-15 years (Al-Shammari *et al.*, 2001; Ilikkan *et al.*, 2005; Anuar *et al*., 2012a; Mbuh *et al*., 2012). This could be attributed to the fact that most of adult in Yemen are chewing qat (A type of narcotic leaves or plant & people addict it as cocaine) every day (mostly after lunch) & usually without washing it; which can cause infection with a lot of microorganisms including parasites, while the children don't chew qat. Other explanation is that most of adult men are farmers and more exposure to the source of infection. Moreover, this finding needs more confirmation with future study including more areas of study.

In the present study, 55 microscopy-positive specimens were found to be negative by PCR. These results can possibly be explained by the fact that most of those samples were of low intensity which fell below the PCR detection limit (only 1-2 cysts were detected by either trichrome stain or formalin-ether sedimentation method), in addition to the presence of faecal inhibitor substances which were not completely removed former to PCR reaction. Another reason for that may be related to the presence of other *Entamoeba* species. However, this must be confirmed by further studies using more sensitive PCR techniques (khairnar and Parija, 2007) .This in agreement with a study conducted in Malaysia among orang asli reported that 30 faecal samples that were positive in microscopy, gave negative results by using PCR (Anuar *et al.*, 2013b).

The present study showed that *E. dispar* and *E. moshkovskii* infections were significantly associated with age, with the prevalence increased to reach its highest rate among participants aged 21-30 years then decreased to the lowest among those aged

more than 40 years. Moreover, *E. moshkovskii* prevalence was significantly higher among male participants while neither *E. histolytica* nor *E. dispar* showed similar findings. These differences could be explained by a higher exposure of adult males to the sources of infections as a result of their daily activities such as the higher access to contaminated outdoor food, contaminated water sources, and contact with infected

individuals.

Moreover, due to religious and cultural practices in rural Yemen, females spend most of their time at home for family domestic activities and they are not always allowed to work or to go for outdoor activities. In consistency with the present findings, a researcher reported that the prevalence of *E. dispar* infection among residents subjected to water shortage in the northeast region of Brazil increases with age (Calegar*et al.*, 2016). By contrast, a previous study among Malaysian rural communities which reported a significantly higher prevalence of infection with *E. dispar* among participants who their age below 15 years than those aged 15 years or more (Anuar *et al.*, 2012c). On the other hand, previous studies in Colombia and Malaysia found no significant association between gender and the three species (Anuar *et al.*, 2012c; López *et al.*, 2015). However, the possible different epidemiological characteristics of the studied Yemeni communities should be taken into consideration when comparing the results with other studies conducted abroad.

In addition, Acuna-Soto and his colleagues have reviewed 66 hospital-based reports of invasive amoebiasis (including dysentery, colonic perforation, , peritonitis, appendicitis, liver abscess and amoeboma) and 20 community–based parasitological surveys conducted between 1929 and 1997 and concluded that the percentage of men affected by all forms of invasive amoebiasis was three times higher than women (with a higher male frequency was found in all age groups) while almost identical rate of asymptomatic infection with *E. histolytica* was observed in both sexes in all age groups (Acuna-Soto *et al.*, 2000).

The present study also investigated the risk factors of *E. histolytica*, *E. dispar* and *E. moshkovskii* infections. The age and gender were kept as significant risk factors of *E. moshkovskii* infection only. Interestingly, the current study showed a significantly higher prevalence of *E. histolytica* and *E. moshkovskii* infections in the coastal/foothill

areas than the highlands of Yemen. Similarly, the present study identified the use of unsafe drinking water as a strong significant risk factor of *E. dispar* infection. A previous study on Sana'a City (Alyousefi *et al.*, 2011) and other reports elsewhere found that using unsafe drinking water was a risk factor for *Entamoeba* complex and other intestinal parasitic infections (Benetton *et al*., 2005; Anuar *et al*., 2012a; Al-Delaimy *et al*., 2014).

In fact, there is an obvious difference in the sources of drinking water with villages in the highlands of Yemen still rely on rainwater (dams, tanks and troughs) and ground water (drilled artesian pump-based wells).While streams and traditional dug wells are the main drinking water sources in the coastal/foothill areas. Duringthe rainy season, the floods flow through the valleys spreading human and animal excreta into fields, dams, pools, wells and other water sources. People collect water from wells, pools and dams, and store that contaminated water in tanks and troughs, and the water is also handled by bare hands. Hence, contamination of drinking water with *Entamoeba* cysts during the collection, transport and storage of water is also possible (Wright *et al.*, 2004). A recent study among 200 children in Al-Mahweet province found a significantly higher prevalence of intestinal parasitic infections particularly *Entamoeba* spp. among children who use stream water (60%) as a source of drinking water compared to those who use water from dams (8.5%), ponds (5%) and wells (16.5%) (Alwabr and Al-Moayed, 2016). However, in contrast, a recent study on rural and urban children in Ibb province found a higher prevalence of *Entamoeba* spp. infection among children using a piped water supply for drinking water compared to those using water from other sources (Alsubaie *et al.*, 2016). It could be postulated that contamination of drinking water sources is more likely to be higher in the coastal/foothill areas; however, it is not possible to infer causality using this cross-sectional study. Hence, further studies to explore the presence of parasites in drinking water in the study areas are

required to better understand these contradictory findings.

Indeed, within the family, the present findings showed that the presence of other infected family members significantly increased the odds of an individual who are infected with *E. histolytica* and *E. moshkovskii*. A previous study from Malaysia found a similar finding with *E. dispar* (Anuar *et al*., 2012c). The infection could easily be passed on through contaminated food and drinks prepared by infected family members who have inadequate personal hygiene. The trophozoite of *Entamoeba* spp. survives for a short time but the cyst lives longer and withstands unfavourable conditions; it can be found on the hands and clothes of children as well as in containers used for washing clothes and in shower trays, which implies that contact with an infected person's belongings and water they have used also transmits the infection (Roberts, 2009). In general, several previous studies have revealed that the presence of other family members infected with soil-transmitted helminthiasis, giardiasis, and schistosomiasis is significantly associated with a higher prevalence of these infections (Spencer, 1981; Gathiram and Jackson, 1987; Ruiz-Palacios *et al*., 1992; Braga *et al.*, 2001*;* Sady *et al*., 2013; Al-Delaimy *et al.*, 2014).

Moreover, the present findings revealed that the prevalence rate of *E. histolytica* infection was significantly associated with a low educational level, which is in agreement with previous reports on *Entamoeba* complex elsewhere (Al-Shammari *et al*., 2001; Al-Mohammed *et al*., 2010; Alemu *et al*., 2011; Dawet *et al*., 2012; Gelaw *et al*., 2013). Probably, illiterate participants would have less health-related knowledge and awareness about intestinal parasitic infections compared to educated individuals. Therefore, these individuals may also contribute to disseminating infection among other family members especially if they are responsible for food preparation.

In the same vein, the present study found a strong association between *E. dispar* infection and not washing vegetables before consumption, which is in agreement with a

preceding study on Malaysia (Anuar *et al*., 2012c). The vegetables could be contaminated by either an infected person or contaminated soil; by human or animal excreta. A similar finding has been reported in previous studies on enteric parasites from different countries including Brazil, Malaysia, and West African countries (Braga *et al.,* 2001; Amoah *et al*., 2007; Al-Delaimy *et al*., 2014; Elyana *et al.*, 2016).

Moreover, the present study showed that employed participants and those who do not have a toilet in their houses as well as those who do not wash their hands after playing with soil or gardening were more prone to infection by *Entamoeba* complex. However, these factors were not retained as significant risk factors by the multivariate logistic regression analysis. Previous studies on Malaysia conducted among the Orang Asli population conclude that working status and absence of toilet facilities in the house and are significant risk factors of intestinal parasitic infections (Nasr *et al*., 2013; Al-Delaimy *et al*., 2014; Elyana *et al*., 2016).

In general, the association between low socioeconomic status and the prevalence of *Entamoeba* spp. infection is well documented. It has been reported that the prevalence of *Entamoeba* spp. Among those who have a low-to-average socioeconomic status is higher than those who have an average or above socioeconomic status (Ravdin, 1995*;* Tellez *et al.,* 1997*;* Pham Duc *et al.,* 2011). Such an association was not found by the present study and this could be explained by the high prevalence of poverty in the studied communities. Lastly, previous studies on Yemen (in Sana'a City), Vietnam and Malaysia have reported a greater risk of *Entamoeba* infection among participants who have close contact with domestic animals (Pham Duc *et al.,* 2011*;* Alyousefi *et al.,* 2011*;* Anuar *et al.,* 2012b).

In the present study, there was no significant variance in the prevalence of *Entamoeba* between those who had animals at their households and their counterparts.

This could be explained by the fact *E. histolytica*–like group almost exclusively infects humans, with exception of *E. dispar* that may also infect chimpanzees, baboon and macaques (Hooshyar *et al*., 2015); animals that do not exist in the study area. However, recent studies suggested a role for the domestic animalsas dogs and cats in transmitting intestinal parasites as the infective cysts or eggs can be easily get picked up on the fur of these animals and then transferred into the houses or to the hands of their owners (Anuar *et al.,* 2012b*;* Choy *et al.,* 2014*;* Elyana *et al*., 2016).Until now, the only pathogenic species is *E. histolytica*; and many studies support the idea that *E. moshkovskii* is a non-pathogenic amoeba (Scaglia *et al*., 1983; Beck *et al*., 2008). In accordance, the findings of the current showed a significantly higher frequency of *E. moshkovskii* single infections among the asymptomatic individuals compared to those with GIT symptoms. Nonetheless, a previous study from Malaysia showed the presence of GIT symptoms such as weight loss, lack of appetite and nausea among *E. moshkovskii*-positive individuals (Anuar *et al.* 2012a). Similarly, a study in India described an association between *E. moshkovskii* infection and dysentery (Parija and Khairnar, 2005).

Moreover, in Australia, a case-control study noticed *E. moshkovskii* in 11 patients who presented with diarrhoea and other GIT clinical symptoms, and no other pathogens, such as bacteria and viruses, were isolated from their faecal specimens (Fotedar *et al*., 2008). These controversial findings call for further studies to identify potential pathogenicity and virulence of *E. moshkovskii* in causing GIT disorders.

In Malaysia, a study carried out by Anuar *et al.* (2012a) found that subjects with single infection of *E. moshkovskii* presented with weight loss, lack of appetite and nausea. Likewise, a study done by Shimokawa *et al*. (2012) in Mirpur, Dhaka showed that cases with *E. moshkovskii* infection can develop gastrointestinal symptom as diarrhea among Bangladeshi children. Their study also reported that *E. moshkovskii* can

cause weight loss and colitis in mice. Interestingly, Yacoob *et al*. (2012) also demonstrated that *E. moshkovskii* significantly associated with diarrhoea in Pakistan. Several studies showed the association of *E. moshkovskii* infection with gastrointestinal symptoms and/or dysentery as in Bangladesh, India, Turkey and Australia (Ali *et al*. 2003; Parija and Khairnar, 2005; Tanyuksel *et al*. 2007; Fotedar *et al.* 2008). However, in the present study, it was not possible to conclude a causal association between *Entamoeba* infection and symptoms because there were two limitations in the methodology of the present study. First, the prevalence of multiple infections was high among the population studied with *Giardia duodenalis*, *Hymenolepis nana*, *Ascaris lumbricoides*, and hookworm being the most common intestinal parasites concurrently detected with the *Entamoeba* parasite. Second, bacterial and viral causative agents were not ruled out in the current study.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

Overall, the present study revealed that intestinal amoebiasis is highly prevalent among rural communities in Yemen with the occurrence of the three microscopically-identical species (*E. histolytica*, *E. dispar,* and *E. moshkovskii*). The study found an age-dependency pattern of distribution for both *Entamoeba* complex infection as well as the species-specific infections. Some variables were identified as the key risk factors of *Entamoeba* complex infection in these communities with a different species-specific grouping of risk factors.

It is expected that the findings of this study will be helpful to the policy makers and health authorities for implementing effective control and preventive measures to significantly decrease the prevalence and burden of amoebiasis as well as other intestinal parasitic infections in these rural communities in the studied provinces and in other provinces of Yemen that may share similar epidemiological characteristics. Throughout Yemen, rural communities share the same environmental, socioeconomic status and health care conditions. Therefore, the findings of the present study could be generalized to rural populations in other governorates in Yemen. However, further studies among other rural as well as urban populations are mandatory to confirm these assumptions.

The following are the main findings of the present study:

i. The overall prevalence of *Entamoeba* complex infection was 53.6% (324/605). Of these, single infections of *Entamoeba* complex were detected in 14.2% (46/324) of the samples and 85.8% (278/324) were mixed infections of *Entamoeba* spp. with other intestinal parasite species.

ii. The overall prevalence of *Entamoeba* complex infection was significantly higher among male participants in comparison with females (P = 0.008), and among those aged 21 – 30 years with an age-dependency distribution represented by a convex pattern of distribution (χ^2 = 39.373, P< 0.001).

iii. Six factors were identified by the multiple logistic regression model analysis as the significant risk factors of *Entamoeba* complex infection among the studied population. These factors are age of ≥ 18 years, the presence of another family member infected with *Entamoeba* spp. using unsafe drinking water, illiteracy, living in coastal/foothill areas, and not washing vegetables before consumption.

iv. Based on the nested multiplex PCR assay, *E. histolytica* was detected in 20.2% (122/605) of the samples while *E. dispar* and *E. moshkovskii* isolates were recovered in 15.7% (95/605) and 18.2% (110/605) of the samples. In other words, the occurrence of *Entamoeba* complex among the 276 *Entamoeba*-PCR-positive samples was 44.2%, 34.4%, and 39.9%, respectively.

v. The study revealed different sets of species-specific risk factors by the multiple logistic regression model analysis. Living in coastal/foothill areas, illiteracy, and the presence of other family members infected with *Entamoeba* was the significant risk factors of *E.histolytica*infection. Likewise, not washing vegetables before consumption and using unsafe drinking water were the risk factors of *E. dispar*. Moreover, age between 18 - 40 years, living with other family members infected with *Entamoeba*, male gender, and living in coastal/foothill areas were the significant risk factors of *E.moshkovskii* infection.

6.2 RECOMMENDATIONS

Further epidemiological and molecular studies on *Entamoeba* spp. using environmental samples such as soil and water samples are required for better understanding of the source

of *Entamoeba* spp.in these communities as well as to provide new insights on the transmission dynamics of these different *Entamoeba* spp.

Overall, the risk factors recognized by the present study should be considered by any prevention and control programme against intestinal parasitic infections. Proper attention should be given to the implement of using molecular methods or other species-specific diagnostic assays, diagnose and differentiate the pathogenic *E. histolytica* from *E. dispar*, and *E. moshkovskii*.

6.3 LIMITATIONS OF THE STUDY

Certain limitations should be considered when interpreting the findings of the current study infections such as using unsafe sources for drinking water, but it is not possible to confirm the causality between the variables using the present cross-sectional study. Hence, further studies to investigate the presence of *Entamoeba* spp. parasites in drinking water in the study areas are required. Similarly, some risk factors were significantly associated with one or two *Entamoeba* species but not with the other species although the three species are all transmitted in the same way, this could be explained by the findings of the present study that the prevalence of these *Entamoeba* species and their major risk factors in Yemen was high and their distribution varied considerably with geographic areas. Another explanation could be the intensity of infection with each species. It may be assumed that in a positive sample, most of the detected cysts are belong to one or two species while very few are belonging to other species. However, unknown parasite-related factors including potential parasite-environment interactions could not be ruled out.

Moreover, this study had to depend on a single stool specimen, hence the prevalence of *Entamoeba* spp. is likely to be underestimated due to the temporal variation in cyst shedding over hours and days. However, examining faecal samples by different techniques

including the gold standard (trichrome staining) and concentration methods (formalin-ether sedimentation) as well as the molecular PCR assay could assist to conquer this limitation. In the present study, 55 microscopy-positive specimens were found to be negative by PCR. This could be attributed to the fact that the majority of those samples were of low intensity which fell below the PCR detection limit (only 1-2 cysts were detected by either trichrome stain or formalin-ether sedimentation method) as well as the presence of faecal inhibitor substances which were not completely eliminated prior to PCR reaction. Using more sensitive methods such as real-time PCR should be considered in future studies. A previous study showed that real-time PCR assay exhibited superior sensitivity in detecting *Entamoeba* parasites in faecal samples when compared with microscopy, stool antigen detection and conventional PCR (Roy *et al*., 2005). Another limitation was that bacterial and viral causative agents were not ruled out in the current study, hence reporting a causal association between *Entamoeba* infection and symptoms was not possible.

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LIST OF PUBLICATIONS AND PRESENTATIONS

A. Publications during candidature, directly arising from this dissertation

Al - Areeqi, M. A., Sady, H., Al - Mekhlafi, H. M., Anuar, T. S., Al - Adhroey, A. H., Atroosh, W. M., Dawaki, S., Elyana, F. N., Nasr, N. A., & Ithoi, I. (2017). First molecular epidemiology of *Entamoebahistolytica*, *E. dispar* and *E. moshkovskii* infections in Yemen: Different species - specific associated risk factors. *Tropical Medicine and, International Health22*, 493-504.

B. Publication related, but not directly arising from this thesis

Atroosh, W. M., Al-Mekhlafi, H. M., Al-Jasari, A., Sady, H., Dawaki, S. S., Elyana, F. N., **Al-Areeqi, M. A.**, Nasr, N. A., Abdulsalam, A. M., & Subramaniam, L. R. (2016). Different patterns of pfcrt and pfmdr1 polymorphism in *Plasmodium falciparum* isolates from Tehama region, Yemen. *PeerJ, 4*, e2191.

Dawaki, S., Al-Mekhlafi, H. M., Ithoi, I., Ibrahim, J., Abdulsalam, A. M., Ahmed, A., Sady, H., Atroosh, W. M., **Al-Areeqi, M. A**., & Elyana, F. N. (2016). Prevalence and risk factors of Schistosomiasis among Hausa communities in Kano state, Nigeria. *Revista do Instituto de Medicina Tropical de São Paulo, 58*, 54.

Dawaki, S., Al-Mekhlafi, H. M., Ithoi, I., Ibrahim, J., Atroosh, W. M., Abdulsalam, A. M., Sady, H., Elyana, F. N., Adamu, A. U., Yelwa, S. I., &**Al-Areeqi, M. A.** (2016). Is Nigeria winning the battle against malaria? Prevalence, risk factors and KAP assessment among Hausa communities in Kano State. *Malaria journal, 15*, 351.

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C. Conference presentation from this dissertation:

Mona A. Al-Areeqi, HanySady, Hesham M. Al-Mekhlafi, JohariSurin. Epidemiology of *Entamoeba histolytica*/*Entamoeba dispar*/*Entamoeba moshkovskii* complex infection in rural Yemen. 52nd ANNUAL SCIENTIFIC CONFERENCE OF THE MALAYSIAN SOCIETY OF PARASITOLOGY AND TROPICAL MEDICINE (MSPTM) 2-3 March 2016 (Oral presentation).