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Molecular Identification and Characterization of *Amoeba* Species from Different Geographical Regions in The West-Bank, Palestine

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DEDICATION

I’d like to dedicate this work to my wife, Shurouq, who has always believed in my capabilities and has supported me throughout my study journey. To my son, Zaid, who taught me to love without conditions. I also dedicate the work to my mom, Mariam and to my father soul Mohammad. I finally dedicate the work to my sincere brothers and sisters who have believed in my abilities to succeed and strive.

Malekshareef

DECLARATION

I certify that this thesis submitted for the degree of Master, is the result of my own research, except there otherwise acknowledged, and that this study (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signature: 

Malek Mohammad Awad Shareef

Date: 27.11.2022

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ABSTRACT

Amoebiasis is one of the most ten common intestinal parasitic diseases worldwide, with *Entamoeba histolytica* infecting around 500 million people causing 100,000 deaths each year. In Palestine, amoebiasis is reported to be a major health problem, it is routinely diagnosed using microscopy by identification of cysts and trophozoites in fresh stool samples. However, this diagnostic method may result in overestimating the patient numbers infected with *E. histolytica*, and leads to mistreatment of the nonpathogenic species of *Amoeba* (*E. dispar* and *E. moshkovskii*) that are morphologically indistinguishable from the pathogenic species. In this study we investigated the molecular epidemiology of *Amoeba* species among Palestinian population in different regions of the West Bank. In addition, we determined the sociodemographic and socioeconomic factors associated with *Amoeba* infection among patients. A total of 100 stool samples were collected from patients who have been presented to Palestinian Ministry of Health (PMOH) clinics and private labs, patients came with symptoms of intestinal infections (abdominal pain, diarrhea and / or dysentery). Sociodemographic data was collected using questionnaire for patients who were diagnosed with *Amoeba* infection. The samples were initially analyzed by direct wet mount microscopy and then by PCR with specific primers for detection of *E. histolytica*, *E. dispar*, and *E. moshkovskii*. The PCR results confirmed the diagnosis of *E. histolytica* in 74 samples, and *E. dispar* in 29 samples. Mixed infection of both *E. histolytica* and *E. dispar* was identified in 7 samples. In a comparison between microscopy and PCR methods for the identification of *E. histolytica* and *E. dispar*, 96 positive fecal samples were yielded by PCR while 100 positive samples diagnosed microscopically. Furthermore, PCR confirmed of 74% positive samples diagnosed microscopically are also positive for *E. histolytica*. The demographic data showed a significant correlation between *E. histolytica* infection and patient's age and educational

level; where the highest infection rates were found among school and preschool children. Our study highlights the need for additional representative large population-based molecular studies on the distribution and epidemiology of the diseases in Palestine. Further studies on the environmental and behavioral factors of patients should be performed on larger scale to determine the risk factors associated with amoebiasis infection in Palestine.

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LIST of ABBREVIATIONS

ddH2O: Double-distilled water

DNA: Deoxyribonucleic acid

E. Bangladeshi: Entamoeba Bangladeshi

E. coli: Entamoeba coli

E. dispar: Entamoeba dispar

E. hartmanni: Entamoeba hartmanni

E. histolytica: Entamoeba histolytica

E. moshkovskii: Entamoeba moshkovskii

E. poleki: Entamoeba poleki

EIA: Enzyme immunoassay

ELISA: Enzyme-linked immunosorbent assay

GEMCS: Global Enteric Multi-Center Study

IR: Incidence Rate

IRB: Institutional review board

MSM: Men who have Sex with Men

PMOH: Palestinian Ministry of Health

N: Negative control

NO: Nitric oxide

NaCl: Physiological saline 0.9%

OR: odd ratios

PMNs: Polymorphonuclear leukocytes

PCR: Polymerase Chain Reaction

SPSS: Statistical Package for the Social Sciences

18S rDNA: Small subunit rRNA genes

sIgA: specific secretory IgA

WHO: World Health Organizatio

Chapter One

Introduction

Amoebiasis is one of the most common intestinal parasitic diseases worldwide. In Palestine, it is considered to be endemic. Amoebiasis is routinely diagnosed by microscopic identification of cysts and trophozoites in fresh stool samples. This chapter demonstrates the background of this study, the primary research problem. Furthermore, the study's justification, aims and objectives and the hypotheses are all given here.

1.1 Background

Infections caused by intestinal parasites are a serious public health problem across the world, particularly amoebiasis, which is among the most ten common intestinal parasitic diseases (WHO 1987). Amoebiasis is caused by *Entamoeba histolytica*, and according to the WHO reports, it infects around 500 million people, causes amoebiasis in 50 million and results in 100,000 deaths yearly (WHO 1997).

In Palestine, intestinal parasite infections are reported to be endemic (Hussein 2011). Amoebiasis is one of the most common parasitic infection found to be occurred among Palestinians (al-Agha and Teodorescu 2000, Hussein 2011, Hamarsheh and Amro 2020, Hamarsheh 2021).

1.2 Problem Statement

In Palestine, amoebiasis is routinely diagnosed by microscopic identification of cysts and trophozoites in fresh stool samples by preparing wet or permanent stained preparations. Although, this method is a gold standard for diagnosis of amoebiasis, it is not species-specific it is prone to

errors and needs well-trained staff. An overestimating of *E. histolytica* may occur and lead to mistreating the other nonpathogenic species (*E. dispar* and *E. moshkovskii*) that are morphologically indistinguishable. This study aims to investigate the molecular epidemiology of *Amoeba* species among Palestinian population in different regions of the West Bank.

1.3 Study Justification

The current gold standard diagnostic methods of amoebiasis is based on microscopic examination of stool samples this method has low sensitivity and specificity. It is not possible to differentiate between different pathogenic and non-pathogenic species, which is important for treatment. Advanced approaches based on molecular biology methods proved to have higher accuracy in identification of *E. histolytica.*

Therefore, we aimed to use molecular methods based on polymerase chain reaction (PCR) to identify species of *Amoeba* in clinical samples and to investigate the epidemiology of amoebiasis principally caused by *E. histolytica* among Palestinian population.

1.4 Study Hypothesis

Conventional microscopic methods that are currently routinely used for diagnosis of amoebiasis overestimate and probably lead to misdiagnosis of amoebiasis caused by *E. histolytica* among Palestinians in West Bank.

1.5 Study Objectives

In the light of the above justifications and hypothesis mentioned, the current study has the following objectives:

1. To collect and identify clinical stool samples for Amoeba parasite using wet mount preparation methods from different directorates in the West Bank, Palestine.
2. To estimate the molecular prevalence of *E. histolytica*, *E. dispar*, and *E. moshkovskii* infections among Palestinians in the West Bank
3. To determine the sociodemographic factors that are associated with *Amoeba* infections through distribution of questionnaires and collection of epidemiological data

1.6 Summary

This thesis explains the research problem and its significance. Then provides a review of the available literature on this topic, further describes the study framework and variables. Furthermore, describing the disease and demographic characteristics and then moved to the molecular prevalence of the *Amoeba* species, which is the focus of this study. Finally, the major findings of the study are discussed along with the conclusions and limitations, then establishing the recommendations based on our findings.

Chapter Two

Literature Review

This chapter provides a literature review on amoebiasis disease epidemiology and etiology in Palestinian directorates, diagnosis and treatment strategies. A detailed information on previous studies of the diseases both in Palestine, neighboring countries and the World.

2.1 Global Epidemiology of amoebiasis

Amoebiasis is defined by The World Health Organization (WHO) as the infection with *Entamoeba histolytica* regardless of the symptomatology (WHO/PAHO/UNESCO,1997). Invasive amoebiasis is ranked as the third cause of death from human parasitic infections globally (Calle-Pacheco, Jiménez-Chunga, and Vivas-Ruiz 2022) following malaria and schistosomiasis (Rawat et al. 2020). It is believed that 500 million individuals are infected with amoebiasis worldwide (Guevara et al. 2019) or approximately 10% of the world's population (Pritt and Graham Clark 2008). *E. histolytica* is a potent parasite that infects around 50 million individuals annually, leading in 40,000-100,000 deaths yearly (van Hal et al. 2007). Children are at especial high risk of *E. histolytica* as they can suffer malnourishment and stunting due to repeated infection (Mondal et al. 2006). According to the Global Enteric Multi-Center Study (GEMCS) in Africa and South Asia, *E. histolytica* was one of the top ten pathogens causing moderate to severe diarrhea in children under the age of five. Furthermore, data from the GEMCS study showed that the *E. histolytica* is the enteric pathogen with the highest risk of death in the second year of life (Kotloff et al. 2013). Amoebiasis has a high worldwide burden due to its correlation with severe and life-threatening

symptoms, low infectious dosage, environmental stability, and chlorine resistance (Al-Dalabeeh et al. 2020).

In developing countries, amoebiasis was found with the highest burden (Ankri and Nagaraja 2019) and its high prevalence in these countries is due to the crowded and low hygienic living levels, in addition to poor sanitation (Parija and Khairnar 2005). In the developing areas of Asia, Africa, and central and south Africa amoebiasis is endemic (Shirley et al. 2018). The burden of amoebiasis in developing countries is difficult to quantify due to a variety of reasons including insufficient diagnostic capabilities, limited surveillance in the endemic areas, epidemiological complexities, and the low sensitivity of diagnostic modality that can be used (Shirley et al. 2018). However, some recent studies can give a prevalence estimation of *E. histolytica* infection in some regions of developing countries. For instance, by cross-sectional survey, *E. histolytica* was detected in 20% of fecal samples in Yemen using Polymerase Chain Reaction (PCR) (Al-Areeqi et al. 2017), and using fecal antigen detection in cross-sectional study of children hospitalized with acute diarrhea in Jeddah, Saudi Arabia, *E. histolytica* was detected in 20% of the children (Hegazi, Patel, and El-Deek 2013). Using the same detection method, 38% of patients were positive for *E. histolytica* in a case–control study of patients presenting with acute diarrhea in Cairo, Egypt (Abd-Alla and Ravdin 2002).

In developed countries, amoebiasis is more common among returning travelers or immigrants from endemic areas (Al-Dalabeeh et al. 2020). In a study conducted by the GeoSentinel Surveillance Network on the international travelers, amoebiasis was reported to account for 12.5% of all microbiologically proven cases, with an incidence of 14/1000 among the returning travelers (Swaminathan et al. 2009). Although the frequency of amoebiasis in the United States is low, it nonetheless accounts for at least 5 deaths yearly (Gunther et al. 2011). In certain areas of the

industrialized countries of Europe, North America, and Asia, invasive amoebiasis was common to occur and transmit among men who have sex with men (MSM) (Hung et al. 2008). In a recent study conducted in Japan to detect anti *E. histolytica* antibodies titer, antibodies were detected in 21% of 1303 HIV positive patients (Watanabe et al. 2011).

2.2 Epidemiology of amoebiasis in Palestine

Amoebiasis is considered to be one of the most parasitic infections that occurs endemically among Palestinians (al-Agha and Teodorescu 2000, Hussein 2011, Hamarsheh and Amro 2020, Hamarsheh 2021). Data of the Palestinian Ministry of Health (PMOH) on amoebiasis among Palestinians in the last ten years shows that incidence rate ((IR) (cases per 100, 000 per year) of amoebiasis was the highest in 2013 (33.3) and then gradually decreased in the next years to reach (0.7) in 2021 (Figure 2.1). However, there has been no comprehensive epidemiological survey on amoebiasis in the West Bank.

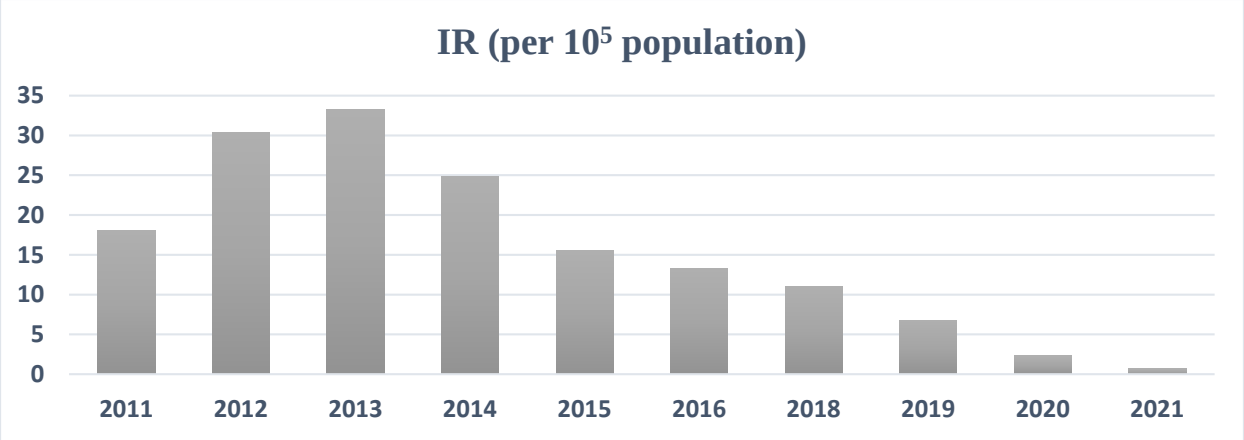

Figure 2.1: Incidence Rate (IR) of Amoebiasis among Palestinians Living in the West Bank, Palestine, in the Last Ten Years (2011-2021) (Source: MOH, 2022).

2.3 Etiology of amoebiasis

Amoebiasis is caused by the extracellular enteric protozoan, *E. histolytica* (Julio C. Carrero et al. 2020). Among the six *Entamoeba* species *(E. histolytica, E. dispar. E. moshkovskii, E. bangladeshi, E. coli, E. poleki*, and *E. hartmanni*) that colonize the human large intestines, *E. histolytica* is considered to be the only pathogenic species that invades the intestinal tract (S. Parija, Ponnambath, and Mandal 2014) and causes both intestinal and extra-intestinal infections (López-López et al. 2017).

2.3.1 *E. histolytica* sources and transmission:

E. histolytica can be found in sewage and contaminated water (López-López et al. 2017). The *E. histolytica* cysts can be transmitted through the ingestion of faecally contaminated food or drink (Guevara et al. 2019) and can affect every gender and age (Al-Dalabeeh et al. 2020).

2.3.2 *E. histolytica* forms and life cycle:

E. histolytica can exist in two forms: a resistant infective cyst and an invasive trophozoite (Huston, Haque, and Petri 1999). The trophozoites (with diameter of 10–50 µm) have a single nucleus with a central karyosome whereas the cysts (with diameter of 10–15 µm) typically have four nuclei (Figure 2.2) (Huston, Haque, and Petri 1999). Cysts are resistant to chlorine and gastric acidity.

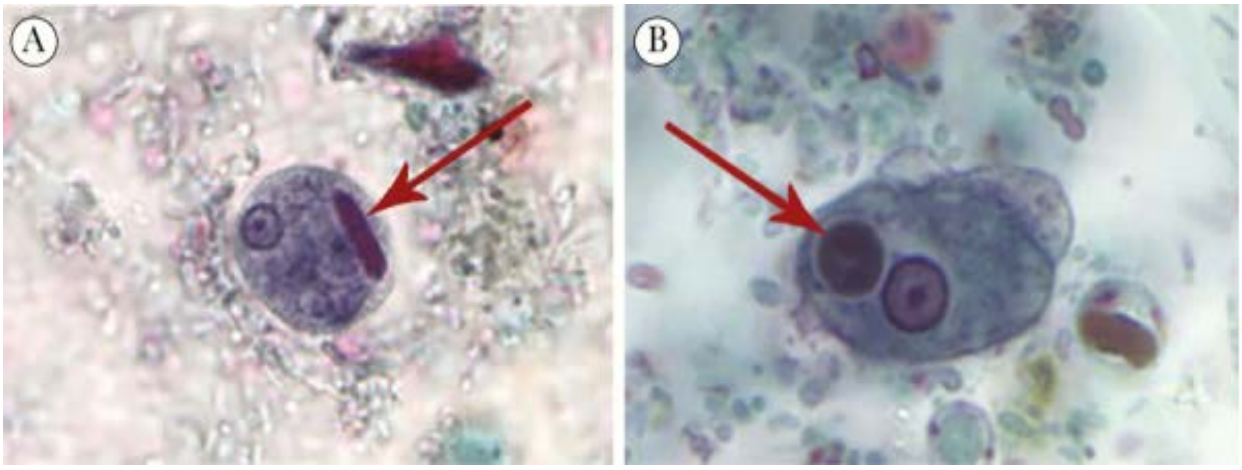

Figure 2.2. *Entamoeba histolytica* forms in stool stained with trichrome stain (A) *E. histolytica* cyst with the chromatoid body assigned with red arrow. (B) *E. histolytica* trophozoite with ingested erythrocyte indicated by the red arrow (Adopted from Shirley et al. 2018).

Once a human host ingest the cysts, excystation occurs within the lumen of the small intestine (López-López et al. 2017). In the excystation, nuclear division is followed by cytoplasmic division to produce eight trophozoites. Then, the trophozoites may take the most common path, the commensal colonization, where trophozoites remain in the caecum and large intestine lumens and adhere to colonic mucus and epithelial layers (Huston, Haque, and Petri 1999) and feed on enteric bacteria by phagocytosis (Wilson, Weedall, and Hall 2012). This adhesion is considered to be determinant for the human tissues invasion, severity of the amoebiasis, and cytotoxic activity (García, Gutiérrez-Kobeh, and Vancell 2015). Following that, trophozoite re-encystation occurs inside the lumen of the colon, leading to cyst excretion in the feces and the continuation of the life cycle (Huston, Haque, and Petri 1999) (Figure 2.3). Furthermore, some trophozoites may be excreted in feces outside of the human host, but they are unable to survive (Flaih et al. 2021).

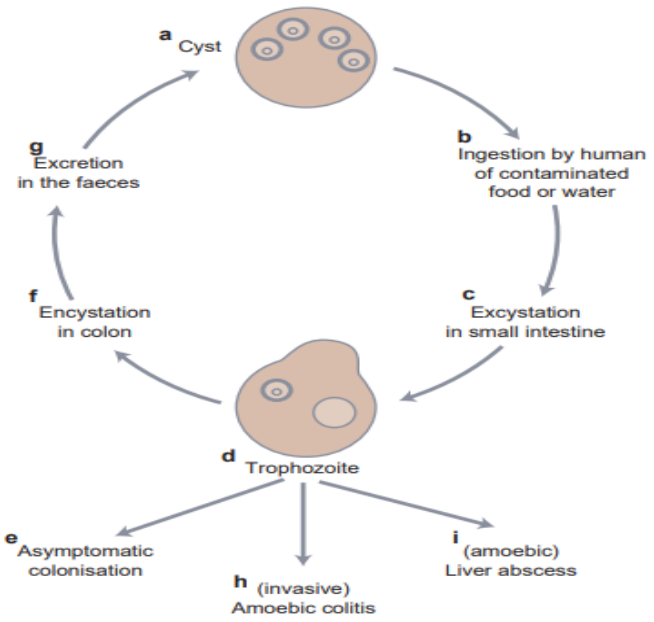

Figure 2.3. *Entamoeba histolytica* life cycle. This figure shows the life cycle of *E. histolytica* in two stages: cyst (a) and trophozoites (d), starting from ingestion of the cyst (b) and ending by excretion of cyst in the feces (g).

2.3.3 Pathogenicity of *E. histolytica* and Disease Outcome:

The other and the less common path of trophozoites is the invasion causing pathogenic amoebiasis (Wilson, Weedall, and Hall 2012). This path is characterized by three events: death of the host cell, inflammation, and invasion. Trophozoites can kill host cells by a variety of ways, including triggering programmed cell death, phagocytosis, and trogocytosis. (Ralston et al. 2014). As a result of cascading secretory proinflammatory cytokines, trophozoite-bound epithelial cells undergo apoptosis (Wilson, Weedall, and Hall 2012).The parasite causes pathogenic amoebiasis through different mechanisms, including those that allow it to resist and disrupt the host's innate and adaptive immune responses. One of these mechanisms is secretion of amoebapore-A, upon the direct contact between the trophozoite and the host cell, leading to forming of pores in the target cell membrane with no need for a specific receptor (Wilson, Weedall, and Hall 2012).

2.3.4 Human Immune Response to *E. histolytica*:

E. histolytica faces plenty of innate defenses in human such as the intestinal mucosa and epithelial barrier, lytic serum components, granulocytes, and phagocytes (Guo, Houpt, and Petri 2007). In the human body, there are many immune responses mechanisms to protect against the intestinal infection. Stimulation of intestinal secretory response by mucosal delivery of amoebic antigens where specific secretory IgA (sIgA) antibodies were detected in many compartments associated to mucosa. In addition, different anti-amoebic sIgA antibodies have been found in feces, bile, breast milk, and saliva of amoebiasis patients (J. C. Carrero et al. 2007).

Other immune response associated with releasing of nitric oxide (NO) and reactive oxygen species by immune effector cells involve in in the destruction of *E. histolytica* trophozoites. Nitric oxide was found to be effective in inhibiting *E. histolytica* alcohol dehydrogenase 2 and cysteine proteinases which are considered as virulence factors (Siman-Tov and Ankri 2003).

Although host cells develop various mechanisms for pathogen elimination, *Amebae* continuously devise diverse strategies for evading host defense and enhancing their own survival. For instance, immunoglobulins that bind to surface proteins can inhibit adhesion and then activating the complement pathway. Trophozoites are able to escape this line of immunity by a process known as 'capping and shedding,' in which bound antibodies are transferred to the back of the trophozoite, producing a 'uroid,' and then shed. After that the host immune system becomes briefly 'blind' to the parasite till more surface receptors bind, at which time the process starts again (Espinosa-Cantellano and Martínez-Palomo 1994).

2.4 *Entamoeba dispar*

In 1993, *E. dispar* was described as a distinct species from *E. histolytica*. *E. dispar* is morphologically similar to *E. histolytica* by microscopic examination (Wilson, Weedall, and Hall 2012). It has been reported to infect about 12% of the world's population (da Silva et al. 2021). For a long time, *E. dispar* has been considered as a noninvasive and avirulent species and mostly associated with local inflammatory response (Espinosa Cantellano, Castanon Gutierrez, and Martinez-Palomo 1997) and asymptomatic cases (da Silva et al. 2021) as it is unable to break down the mucus barrier or cause damage in the intestinal epithelial cell (Bansal et al. 2009).

In comparison to *E. histolytica*, a high number of *E. dispar* genes related to pathogenicity are found to be down-regulated. Among them, Gal/GalNAc lectin, cysteine proteases, peroxiredoxin, and others. Furthermore, in a comparative study on the erythrophagocytosis between *E. histolytica* and *E. dispar*, the latter was found to have lower capacity of phagocytosis (Talamás-Lara et al. 2014). In addition, *E. histolytica* was evidenced to develop larger lamellipodia, indicating a stronger adherence to fibronectin whereas *E. dispar* developed filopodia that covered a smaller region, and that is partially why *E. histolytica* is more pathogenic and has a behavior similar to tumor cells in invasion and migration (Talamás-Lara et al. 2020).

Not until a few years ago some strains of *E. dispar* were isolated from patients with symptomatic non-dysenteric colitis (Graffeo et al. 2014) and patients with amoebic liver abscesses (da Silva et al. 2021).

2.5 *Entamoeba moshkovskii*

E. moshkovskii was described for the first time in 1941 from samples taken from a wastewater treatment system in Moscow. It was morphologically identical to *E. histolytica*. However, the in

vitro growth conditions that fit the growth of *E. moshkovskii*, including temperature tolerance range of 4 ◦C and 41 ◦C, low amount of nutrients, and hypotonicity, completely deviated from the conditions that suit *E. histolytica* growth (Tshalaia 1941). Since 1941, *E. moshkovskii* has been isolated in several countries from various sources such as fresh and wastewaters, rivers, lakes, streams, and human feces (Heredia, Fonseca, and López 2012).

E. moshkovskii was often found as coinfection with either *E. histolytica* or *E. dispar* in areas where amoebiasis is endemic like Bangladesh, and particularly among children (Ali et al. 2003). In a study conducted in India on patients with gastrointestinal symptoms, coinfection of *E. moshkovskii* with *E. histolytica* was found to be less frequent to occur (Khairnar and Parija 2007). Another study conducted in Pakistan, to assess *Entamoeba* species prevalence in individuals with chronic diarrhea, reported the presence of *E. moshkovskii* mono-infection in patients who were suffering gastrointestinal symptoms like chronic diarrhea (Yakoob et al. 2012). The great variability in the frequencies of *E. moshkovskii* infection can be attributed to different factors that need to be considered in each study including the sanitary conditions of the studied area, lifestyles, socioeconomic conditions, nutritional status, and the population (Heredia, Fonseca, and López 2012). In general, *E. moshkovskii* was concluded to be a common infection particularly in patients who have risk factors for amoebiasis (Heredia, Fonseca, and López 2012).

2.6 Laboratory Diagnostic Techniques for *Entamoeba* Species

As the most common presentation of amoebiasis is asymptomatic infection, amoebiasis poses a diagnostic challenge because humans can be infected with the other morphologically identical *Entamoeba species*, i.e. *E. dispar* and *E. moshkovskii*, resulting in the use of unnecessary antiamoebic treatment in many cases (Pritt and Graham Clark 2008). Microscopy, antigen detection, antibodies detection, molecular-based assays, and serology are among the diagnostic

approaches available to aid in diagnosis of amoebiasis, each with its advantages and disadvantages. The traditional approach for diagnosis of *E. histolytica / E. dispar / E. moshkovskii* infection is by direct microscopic examination of stool specimens. The main disadvantage of this technique its low sensitivity (Heredia, Fonseca, and López 2012). In particular, most cases of extra-intestinal abscess occur without concomitant intestinal infection, therefore microscopic examination of stool is less sensitive for the identification of amebic liver abscess (Shirley et al. 2018). This study focused on the microscopic examination as well as the use of molecular techniques for diagnosis of amoebiasis.

2.6.1 Microscopic examination of stool samples:

In general, microscopic examination for clinical diagnosis of *Entamoeba* species in stool can be performed using different methods, including wet mount preparation (direct saline), concentration, and permanently stained smears. The sensitivity of microscopy methods for diagnosis does not exceed 60% (Haque et al. 1998). Using permanent stained smears, stained with trichrome or iron hematoxylin, outperforms the wet and concentration methods in recovery and identification of *Entamoeba* species (Fotedar et al. 2007a). The use of a light microscope to diagnose *E. histolytica* can easily result in false positives due to the misidentification of polymorphonuclear leukocytes (PMNs) as cysts, macrophages as trophozoites, and misidentification with other *Entamoeba* species. (Fotedar et al. 2007, Saidin, Othman, and Noordin 2019). The specificity of this approach is 9.5% compared with the ProSpecT enzyme immunoassay (EIA) antigen detection tests and *Entamoeba* test (Pillai et al. 1999).

2.6.2 Fecal Molecular Assays:

In areas where amoebiasis is prevalent and causes considerable morbidity and mortality, molecular-based assays are not frequently employed. Instead and due to a shortage in facilities in Palestinian health services for diagnosis of amoebiasis using molecular approaches, the diagnosis is still made via microscopic examination (Fotedar et al. 2007a).

2.6.2.1 Fecal Sample Complexity:

Using fecal sample in the molecular-based assays is considered to be complex and problematic due to several reasons, principally, fecal sample contains many PCR inhibitors such as heme, bile salts, complex carbohydrates, and bilirubin that may give false-negative results. In addition, fecal samples may contain other organisms as normal flora, pathogenic or nonpathogenic which will be co-extracted along with the parasite DNA (Holland et al. 2000). Furthermore, storing and fecal samples at ambient temperature may cause fast degradation of the parasites particularly trophozoites, which affects the amount and quality of the extracted DNA (Lebbad and Svärd 2005). As a result, the sensitivity of DNA assays utilizing unpreserved fecal specimens varies with storage time (Lebbad and Svärd 2005). Therefore, the preferable preservation strategy is to freeze the fecal sample and store it at -20°C until processing (Ramos et al. 1999).

In regards to the aforementioned issues about the complexity of using fecal samples in the molecular-assays, QIAGEN company developed a special DNA extraction kit for stool samples (The QIAamp DNA stool kit) that proved to be reliable and reproducible. The QIAamp extraction kit was modified to improve reproducibility and sensitivity by increasing the time and temperature of proteinase K digestion step and through adding an extra washing step before the DNA elution step (Roy et al. 2005).

2.6.2.2 Conventional Polymerase Chain Reaction (PCR):

PCR-based approaches are the gold standard in the diagnosis of amoebiasis and are the choice of the developed countries to perform studies in both clinical and epidemiological streams (Calderaro et al. 2006, Visser et al. 2006, Lebbad and Svärd 2005,Rivera, Tachibana, and Kanbara 1998). PCR has been proven to be sensitive enough to detect as few as five cysts in a stool sample, as well as to be rapid and selective in distinguishing *E. histolytica* from *E. dispar* (Rivera, Tachibana, and Kanbara 1998a).

In a comparison study in using of enzyme-linked immunosorbent assay (ELISA) based kits and PCR amplification of the small subunit rRNA genes (18S rDNA) for detection of *E. histolytica* and *E. dispar*, the rRNA PCR has been evidenced to be almost 100 times more sensitive than ELISA kits (Mirelman, Nuchamowitz, and Stolarsky 1997). Many studies adopted the constant genetic diversity identified between the 18S rDNAs of *E. histolytica* and *E. dispar* as a target for species differentiation (Que and Reed 1991, Clark and Diamond 1992). In addition to the 18S rDNA, different genes were targeted to differentiate between *Amoeba* species including, M17 gene (Gomes et al. 1999, Tannich and Burchard 1991), 30 - kDa antigen gene (Rivera, Santos, and Kanbara 2006, Rivera, Tachibana, and Kanbara 1998b), and cysteine proteinase genes (Freitas et al. 2004). Despite PCR-based approaches effectiveness in detection all three *Entamoeba* species, their applicability in routine diagnosis is currently limited due to difficulties in DNA extraction from fecal samples, generation of nonspecific DNA fragments, and the high cost and time required for DNA amplification and detection (Fotedar et al. 2007a).

2.7 Treatment of Amoebiasis

Basically, amoebiasis is treated with amebicides depending on the location and severity of infection (Li *et al*. 2021). Symptomatic amoebiasis in tissues is primarily treated by hydration and the use of metronidazole, nitazoxanide, dehydroemetine, chloroquine, and/or tinidazole. Metronidazole is dosed for adults as 750 mg/day orally every 6 to 8 hours for 7 to 10 days. Whereas Tinidazole is dosed as 800 mg/ day orally, 3 times per day, over 7 days for adults. In case of luminal infection, diloxanide furoate or iodoquinoline are usually used for treatment (Li *et al*. 2021). Among these medications, metronidazole (MTZ) is the most commonly prescribed and used to treat invasive amoebiasis (Gonzales, Dans, and Sio-Aguilar 2019). Metronidazole is a prodrug that reduced by the thioredoxin reductase of the parasite and, most likely, ferredoxin to produce a nitroradical anion or, if further reduced, a reactive nitroimidazole, both of which are toxic to the parasite (Leitsch *et al*. 2007). Treatment with metronidazole is found to be associated with different side effects including nausea, headaches, ataxia, anorexia, and skin rashes (Li *et al*. 2021). However, partial resistance to metronidazole has been described in an in vitro experiment among some clinical strains of *E. histolytica* which suggests that metronidazole-resistant strains are emerging and other treatment choices should be investigated (Bansal et al. 2004). These treating drugs of amoebiasis are considered by some authors as unnecessary treatment in case of *E. dispar* infection even in patients who are suffering symptomatic nondyesntric colitis (Pestehchian *et al*. 2011, Araujo *et al.* 2008). However, in a case report study for Italian patient diagnosed with enteritis due to *E. dispar,* amoebiasis therapy was decisive for the complete recovery (Graffeo et al. 2014).

Chapter Three

Study Framework

This chapter depicts the conceptual framework for our study, as well as the dependent and independent variables, in addition to their definitions.

3.1 Conceptual Framework

The diagnosis of amoebiasis by microscopy was predicted to increase the misdiagnosis of the disease especially in the endemic areas. As time goes by, the molecular diagnosis of amoebiasis and molecular epidemiological data collection confirmed its highly importance to distinguish between pathogenic from the nonpathogenic *Amoeba* species. This will help the parasitologists to diagnose the disease and prescribe the appropriate anti-amoebic drug.

3.2 Study Variables

In this study, the outcome variable was amoebiasis. Whereas, independent variables include the demographic data, infection risk factors, and disease outcomes. The demographic data include; gender, age, region, educational level, marital status, and living-conditions. The disease outcomes consist of age at diagnosis, individual and family infection history, presence of disease symptoms, and sample characteristics. Furthermore, different environmental and behavioral factors were studied and include toilet facility, source of drinking water, using history of public bathrooms, contact with domestic animals, and hands washing habits.

Chapter Four

Methodology

4.1 Study Design

This molecular-epidemiology study is a cross-sectional study conducted in Palestinian population living in the West Bank from September 2019 until March 2021. Stool samples were collected from different regions in West Bank. Samples were collected from individuals diagnosed with abdominal pain and had typical clinical picture of *Amoeba* infection by Palestinian Health Services clinics, Directorate of Health in each district, Palestinian Ministry of Health and private medical centers and laboratories. Participants were randomly selected from urban and rural areas of all Palestinian districts in the West Bank. The sample size of this study is 100 stool specimens. A questionnaire was used to collect sociodemographic data from the patients (Appendix 1).

4.2 Study Tools

For the purpose of the study, sociodemographic data were collected using a face-to-face interview, where the study objectives were explained for patients and each patient signed on a written consent to participate in this study (Appendix 3). Data was collected using questionnaire for patients who were diagnosed with *Amoeba* infection, stool sample was obtained from each participant in sterilized screw-capped containers to avoid any external contamination, labeled with specific coding system, then microscopically examined and kept at -20 C° . All samples were transported to the Department of Life Sciences Laboratories at Al-Quds University for further molecular

analysis. Questionnaires were kept in special lockers and the patient's information were kept confidential.

4.3 Study Population and Areas

A total of 100 stool samples were collected by specialized lab technicians at both Palestinian Ministry of Health and private medical clinical centers and laboratories. Stool samples were collected in 9 different Palestinian districts in the West Bank; Hebron, Nablus, Ramallah, Bethlehem, Jenin, Tulkarm, Salfit, Jericho, and East Jerusalem.

4.4 Microscopic Examination of Stool Samples

All stool samples were first examined microscopically by direct wet-mount method in which one drop of physiological saline (0.9% NaCl) was placed on a clean glass slide and about 2mg of fresh stool was added and mixed using small wooden stick applicator, a cover slip was placed on the top of the sample and examining under the microscope using high dry power (40X magnification).

4.5 DNA Extraction from Stool Samples

Prior to DNA extraction, an approximately of 20 grams of tool samples were mixed with 1ml of phosphate buffered saline (pH 7.2), washed three times and the fecal materials and debris precipitated by centrifugation for 5 minutes at 14,000x. DNA extraction was carried out directly on washed and cleaned stool samples using a QIAamp ® DNA stool mini kit (QIAGEN, Hilden, Germany) according to the manufacturer instructions. Briefly, a suspension of 0.5 ml of stool material were added to sterile 2 ml micro-centrifuge tube and then the tube was placed in ice. Then, 1 ml of InhibitEX Buffer was added to each stool sample and vortexed very well for 1 minute or until the suspension is homogenized, the suspension was heated for 5 minutes at 70°C and vortexed for 15 seconds. The sample then centrifuged for 1 minute to pellet stool particles. Then, 200 µL of

the supernatant were pipetted into a new 1.5 ml micro-centrifuge tube containing 15 µL of *Proteinase K*. A total of 200 µL of buffer AL were then added and vortexed for 15 second to form a homogeneous solution. The mixture incubated for 10 minutes at 70 °C. A volume of 200 µL of ethanol (96–100%) were added to the lysate, and mixed by vortex. Then 600 µL of the lysate were carefully added to QIAamp spin column and centrifuged for 1 min. Then, the QIAamp spin column was placed in a new 2 ml collection tube, and the tube containing the filtrate was discarded. After that, 500 µL of buffer AW1 were added to QIAamp spin column and centrifuged for 1 minute. And for the second time, the QIAamp spin column was added in a new 2ml collection tube, and the collection tube containing the filtrate was discarded. Then, QIAamp spin column was opened and 500 µL of buffer AW2 were added and centrifuged for 3 minutes. The collection tube containing the filtrate was discarded. Later, the QIAamp spin column was placed in a new 2ml collection tube and centrifuged for 3 minutes, and the old collection tube with the filtrate was discarded. The QIAamp spin column was transferred into a new labeled 1.5ml micro-centrifuge tube and 200 µL of Buffer ATE were pipetted directly onto the QIAamp membrane, incubated for 1 minute at room temperature, then the DNA was eluted after centrifugation at maximum speed for 1 minute.

4.6 DNA Concentration Measurement

DNA concentration and its purity was measured and evaluated for each sample using NanoDrop spectrophotometers (Thermo Scientific™ NanoDrop™ 2000/2000c Spectrophotometers, Thermo Fisher Scientific, USA).

4.7 PCR Amplification Conditions

PCR amplifications were carried out for targeted fragments on the *M17* and 18S rRNA genes based on documented previous studies (Fotedar et al. 2007a) . The PCR experiments were carried out using GoTaq® Green Master Mix (Promega, USA), according to the manufacturer's recommendations. Briefly, 25 µl reaction mixture was prepared by adding 1.5 µl of the extracted DNA, 2 µl of the forward and the reverse primers, 12.5 µl of Master Mix, and 7 µl of ddH2O. Forward and reverse oligonucleotide primers targeting the signature sequence of each *Amoeba* species were used for PCR assay listed in Table 4.1.

To ensure that the PCR mixture was not contaminated, a PCR negative control was used (by preparing an extra reaction mix for each amplification and substituting the DNA with ddH2O). The PCR tubes were amplified, with a thermocycler machine (FlexCycler2 PCR Thermal Cycler, Analytik Jena, Germany), using the following conditions for amplification of the targeted genes: Initial DNA denaturation, at 94.0°C for 3 minutes; 35 cycles of: DNA denaturation, at 94.0°C for 60 seconds; primer-annealing, at 58.0°C for 60 seconds; and primer-extension, at 72.0°C for 60 seconds; the last cycle of primer-extension, at 72.0°C for 7 minutes).

Parasitic infection was confirmed by the expected PCR band sizes of 482 bp, 101 bp, and 580 bp for *E. histolytica*, *E. dispar* and *E. moshkovskii* respectively through gel electrophoresis on 2% (weight/volume) agarose gels. The gel was prepared by mixing two grams of agarose powder (SigmaAldrich, St.Louis, USA) in 100 ml 1X TAE buffer (Thermo Scientific™, Lithuania). Gel allowed to solidify on room temperature on gel casting tray, 5 µl of PCR product was mixed with

2.5 µl loading dye and loaded into the gel in the electrophoresis champer along with 100bp DNA size marker, electrophoresis was carried out at 90V for 45 minutes. The DNA bands were visualized using ChemiDoc imaging system (ChemiDoc™, BioRad, USA).

Table 4.1: Primer's information which have been used in this study to detect *Amoeba* species by PCR assays

Species	Target	Primer Sequence (5' - 3')	Expected Product Size	Reference
E. histolytica	M17	F: 5' GCAACTAGTGTTAGTTA3' R: 5'CCTCCAAGATATGTTTTAAC 3'	482 bp	(Fotedar et al. 2007a)
E. dispar	18S rRNA	F: AGGAGGAGTAGGAAAATTAGG 3' R: 5 ' TTCTTGAAACTCCTGTTTCTAC 3'	101 bp	(Fotedar et al. 2007a)
E. moshkovskii	18S rRNA	F: 5'ATG CAC GAG AGC GAA AGC AT3' R: 5' TGA CCG GAG CCA GAG ACAT 3'	580 bp	(Hamzah et al. 2006)

4.8 Statistical Analysis

Data were coded, inputted and statistically analyzed with using SPSS statistical analysis software version 25.0 (SPSS, Chicago, IL, USA). Numbers and percentages were used to describe categorical variables. Descriptive statistics were carried out to demonstrate disease relationship with age, gender, residence area, work, and social and behavioral factors. Using univariate statistical model, Pearson's Chi-square test was used at a threshold of significance of $P < 0.05$ to evaluate the relationships of infection frequencies among groups. The dependent variables are infection frequencies, whereas the independent variables are environmental, socio-demographic variables, and participant clinical conditions. To assess the strength of the association between

parasitic infection determinants and infection burden, odd ratios (OR) and 95 % confidence intervals were calculated.

4.9 Ethical Consideration

The study principles and methods were approved by the institutional review board (IRB) committee at Al-Quds University and the Palestinian Ministry of Health. In addition, the questionnaire and the patients' data were securely stored electronically on a safe drive accessed with username and password. Furthermore, informed written consent was obtained from all the patients prior to the study. In case of children, the informed written consent was taken from their parents (appendix 2).

Chapter Five

Results

5.1 Study Population Demographic Data

Fecal samples were collected from 100 patients from nine different governorates in the West Bank. Samples were collected over the course of two years, from September 2019 to March 2021, and during different seasons of the year. The distribution of collected samples was as the following; out of 100 samples, 15 were from Bethlehem, 9 from East Jerusalem, 28 from Hebron, 5 from Jenin, 2 from Jericho, 21 from Nablus, 12 from Ramallah, 3 from Salift, and 5 from Tulkarm. The median age of patients was14.0 (range 1 to 76 years). Of those patients, 44 were females and 56 were males. Regarding the patients' residence, 58% of them live in rural areas including refugee camps, while the others reside in urban areas of the West Bank.

5.2 Microscopic Diagnosis of Amoebiasis

The medical records of patients, who gave fecal samples (100 patients) and analyzed in this study, showed that patients complained with at least two of the following symptoms, abdominal pain, fever, nausea, vomiting, diarrhea and/ or dysentery. Most of the collected fecal samples were physically abnormal in case of smell and texture. Two bloody samples were reported. Up to 88% of the samples were liquid, mucoid, and yellowish in color. The microscopic examination and diagnosis of 75% of patients was made in private health clinics and laboratories, while the remaining samples (25%) were examined and diagnosed at PMOH clinics and laboratories. The microscopic examination was carried out by direct saline (wet) mount under high dry power (x40).

E. histolytica cysts or trophozoites were observed under microscope and *Amoeba* infection was reported in all samples, (Figure 5.1).

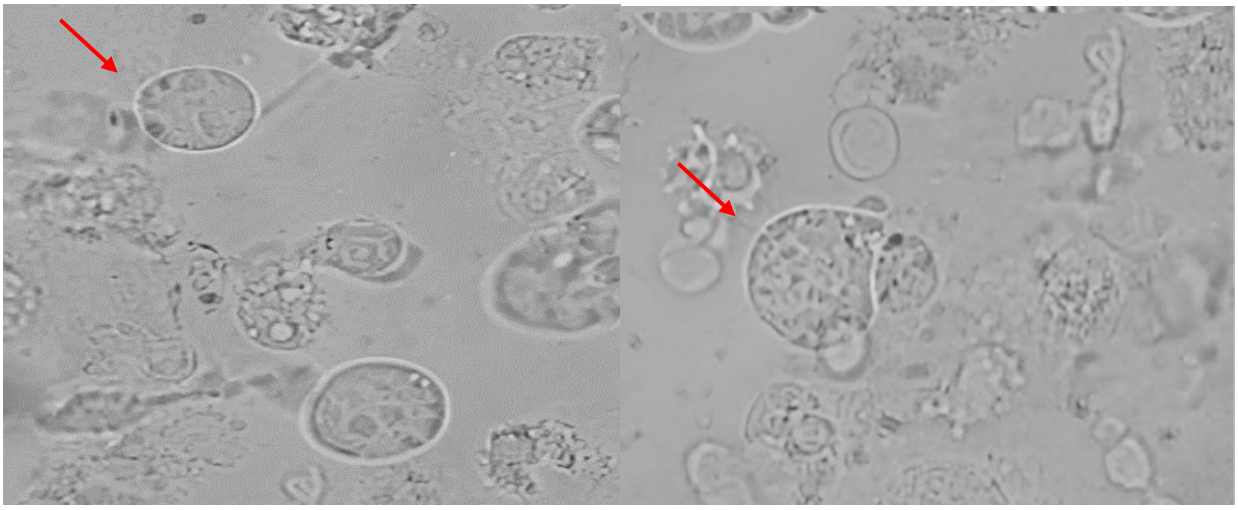

Figure 5.1: Microscopic examination of fecal samples confirm presence of *E*ntamoeba spp. Sample code HH14, arrow pointed to *E. histolytica*-cysts (left) and trophozoites (right).

5.3 Prevalence of *E. histolytica, E. dispar* and *E. moshkovskii*

For samples that were found positive by microscopic examination of wet mounts for *Amoeba*, conventional PCR was used to discriminate between *Amoeba* species. PCR amplification targeted the signature sequence of small ribosomal RNA gene in *E. dispar* and *E. moshkovskii*, and specific sequence of *M17* gene in *E. histolytica*. PCR amplifications confirmed that 67% of patients were positive for *E. histolytica* (mono-infection) (Figure 5.2), 22% were mono-infection but with *E. dispar* (Figure 5.3), none of the samples were positive with *E. moshkovskii*, 7% were positive for both *E. dispar* and *E. histolytic*a (mixed-infection) (Figure 5.4). On the other hand, 4% were negative for all the three PCR assays. *E. histolytica* and *E. dispar* were found to be spatially distributed among Palestinians in all governorates, except Jericho and Bethlehem (Table 5.1).

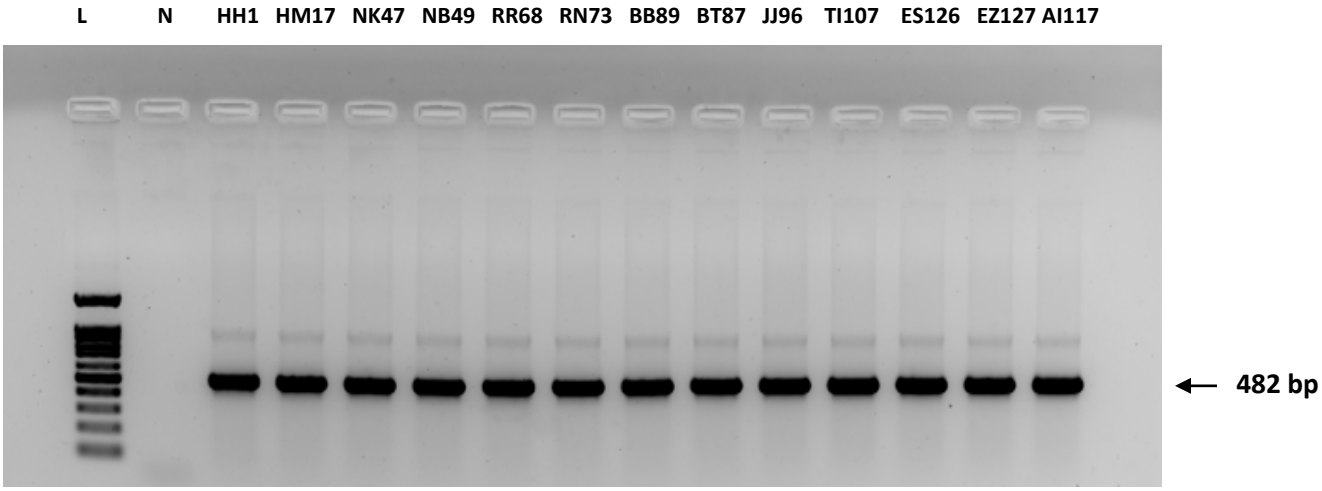

Figure 5.2: Gel electrophoresis of amplified targeted sequence on *M17* gene used to detect *E. histolytica*. Amplicons (with **482 bp**) is specific for *E. histolytica* in clinical samples from patients in different Palestinian directorates and regions including Hebron (coded H), Nablus (N), Ramallah (R), Bethlehem (B), Jenin (J), Tulkarm (T), Ezarieyeh (E), and Abudis (A). L and N indicates the DNA ladder (100 bp-ladder) and the negative control, respectively.

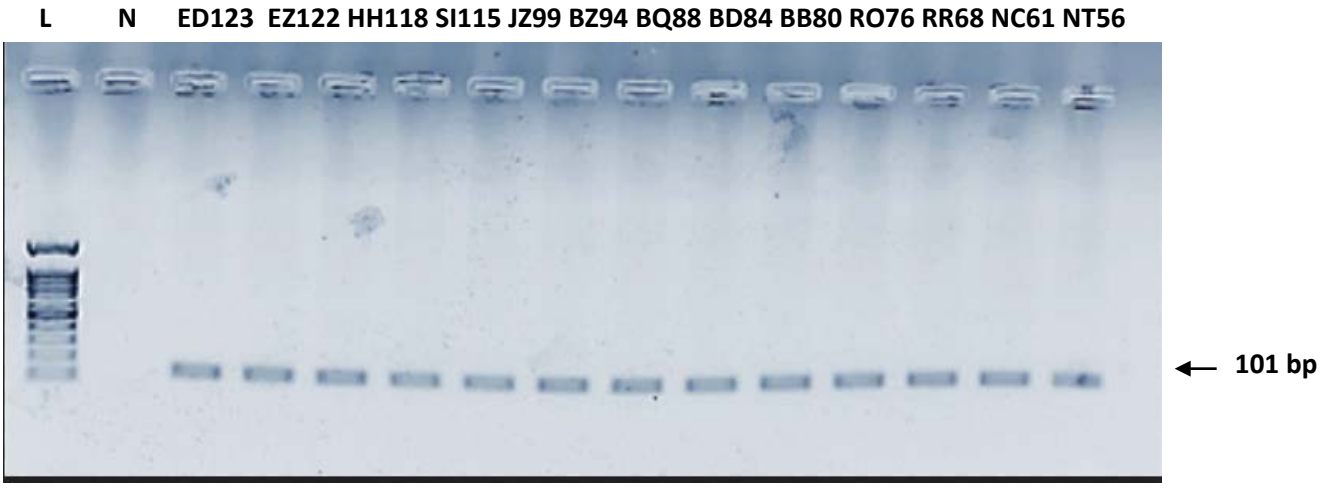

Figure 5.3: Gel electrophoresis of amplificated targeted sequence on 18S rRNA gene to detect *E. dispar*. Amplicons size of **101 bp** is specific for *E. dispar* in clinical samples from patients in different Palestinian directorates and rejoins including Hebron (coded H), Nablus (N), Ramallah (R), Bethlehem (B), Jenin (J), Tulkarm (T), Ezarieyeh (E), Salfit (S), and Jericho (J). L and N indicates the DNA ladder (100 bp-ladder) and the negative control, respectively.

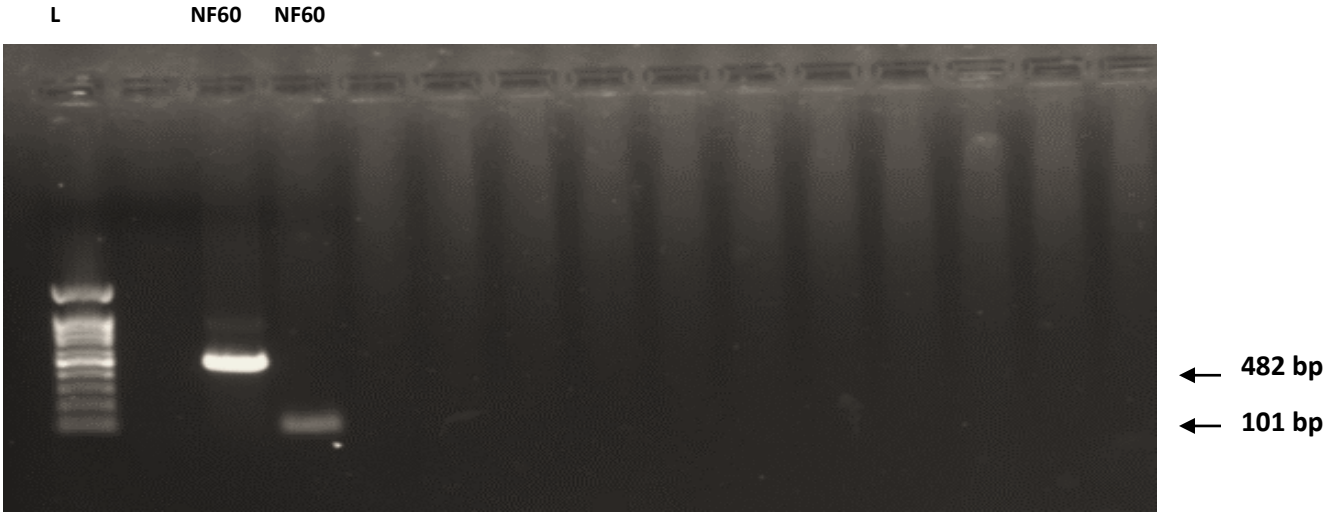

Figure 5.4: Gel electrophoresis for a patient with mixed *Amoeba* infection with both *E. histolytica* and *E. dispar*. NF60 sample shows a band size of **482** and **101 bp diagnostic** for *E. histolytica* and *E. dispar*, respectively. The first lane (L) represents DNA ladder (100 bp).

Table 5.1 Distribution of *E. histolytica* and *E. dispar* in the West Bank Governorates

Governorate	Positive microscopy samples	EH infection	ED infection	EH and ED coinfection	Negative PCR samples
Bethlehem	15	11	4	0	0
East-Jerusalem	9	7	2	0	0
Hebron	28	17	6	3	2
Jericho	2	2	0	0	0
Jenin	5	4	1	0	0
Nablus	21	12	6	2	1
Ramallah	12	8	2	1	1
Salfit	3	2	1	0	0
Tulkarm	5	4	0	1	0
Total	100	67	22	7	4

5.4 Socio-Demographic Characteristics and Prevalence of *E. histolytica* Infection

The overall prevalence of *E. histolytica* is 74%, there is a marked and significantly higher infection rate among patients aged less than 15 years (47 out of 56 were found positive), while patients aged between 15 and 30 years (15 out of 22 were found positive for *E. histolytica*) ($P < 0.001$). Forty-one (55%) of the total *E. histolytica* infected patients were originated from rural areas. Infection rate was found slightly higher in patients from rural areas when compared with others in urban areas. However, there is no significant correlation between the *E. histolytica* infection and residence areas ($P = 0.114$). Gender and marital status were not significantly ($P= 0.240$) associated with the *E. histolytica* infection, although males (59.4%) had slightly higher infection rate compared to females. It is surprising that 39% of patients infected with *E. histolytica* were living

in poor conditions. The univariate analysis showed that the amoebiasis infection was independent of living-condition (*P*= 0.623). Regarding the educational level, 73% of *E. histolytica* infections were among students and pre-school population (*P*= 0.064).

5.5 Socio-Demographic Characteristics and Prevalence of *E. dispar* Infections

The overall infection rate of *E. dispar* is 29%. Patients aged less than 15 years (51.7%) have highest infection rate, followed by patients aged between 15 and 35 years (20.6%). The majority of *E. dispar* infected patients (72.4%) were originated from rural areas. In addition, the highest infection rate was observed among students (62%). Living-conditions correleated with *E. dispar* infection, 51.7% of patients living in poor conditions (*P* = 0.236).

5.6 Association of Amoebiasis with Certain Environmental and Behavioral Factors and Infection History of Participants

Patients having unhygienic toilet facility were more likely to be infected with *E. histolytica* than those having hygienic toilet facilities (OR = 1.41; 95% CI = 1.104, 1.715; *P* = 0.028). In total, 27% of participants infected with *E. histolytica* were in contact with domestic animals such as cats and dogs. Furthermore, 37.8% of these patients were also with bad hands-washing habits. However, the univariate analysis showed no significant association between *E. histolytica* infection and hand washing (*P* = 0.956) or contact with domestic animals (*P* = 0.715). There is a 23% of the patients were positive for amoebiasis were previously infected with *E. histolytica* and 54% of them had previous family history of amoebiasis.

Chapter Six

This molecular-epidemiological study was conducted to investigate the molecular epidemiology of amoebiasis among Palestinians who are living in the West Bank, Palestine. The findings of this study showed the overall *E. histolytica* infection rate is lower than expected based on traditional diagnostic methods.

6.1 Discussion

In Palestine, infection of intestinal parasites is a serious public health problem especially among school and preschool children (Hussein 2011, Hamarsheh and Amro 2020). Our study focused on infection caused by *Entamoeba* species, the amoebiasis, among Palestinians who are living in the West Bank. In Gaza, the prevalence of *E. histolytica* and *E. dispar* was reported to be 15% among children with acute gastroenteritis (Abu Elamreen, Abed, and Sharif 2007, Hamarsheh and Amro 2020). In another study conducted among 735 schoolchildren in Northern Districts of West Bank, the prevalence of *E. histolytica and E. dispar* infection was highest among the other intestinal parasitic infections, with rate of 9.7% compared to *Giardia intestinalis* (4.1%), *Enterobius vermicularis* (1.6%), and *Ascaris lumbricoides* (3.8%) (Hussein 2011). These high prevalence rates of amoebiasis are similarly occurred in different developing countries, like; India (Nath *et al*. 2015), Turkey (Ustun *et al.* 2003), Yemen (Al-Areeqi *et al*. 2017), and others.

In our study, a total of 100 stool samples were collected form patients who have been presented to MOH clinics and different private clinics complained with symptoms of intestinal infections (complained abdominal pain, diarrhea and / or dysentery), the samples initially analyzed by direct wet mount microscopy and then by PCR with specific primers that previously used for detection

of *E. histolytica*, *E. dispar*, and *E. moshkovskii* (Fotedar et al. 2007a). Our study results confirm the diagnosis of *E. histolytica* in 74 samples, and *E. dispar* in 29 samples. Mixed infection of both *E. histolytica* and *E. dispar* was identified in 7 samples. Our results are in agreement with another study conducted in Gaza Strip using 92 stool samples, in which reported *E. histolytica*, *E. dispar*, and mixed infections (69.6% (64), 22.8% (21), and 7.6% (7) respectively (Al-Hindi *et al*. 2005).

The three *Entamoeba* species; *E. histolytica*, *E. dispar*, and *E. moshkovskii*, look the same under the microscope, yet differ biochemically and genetically (Fotedar *et al.* 2007b). In a comparison between microscopy and PCR methods for the identification of *E. histolytica* and *E. dispar*, 96 positive fecal samples were yielded by PCR while 100 positive samples diagnosed microscopically. Furthermore, PCR confirmed of 74% positive samples diagnosed microscopically are also positive for *E. histolytica.* In the same context, Al-Hindi *et al.* in their study, reported that nearly 30% of suspected clinical amoebiasis cases were found to be negative for *E. histolytica* (Al-Hindi *et al.* 2005). In consistent with results from other studies (Helmy, Rashed, and Abdel-Fattah 2007, Dagci *et al.* 2007), these findings demonstrate the significance of the use of PCR technology for diagnosis of amoebiasis, especially to differentiate between pathogenic and non-pathogenic *Entamoeba* species. In addition, using PCR in diagnosis of amoebiasis will significantly reflects positively on the use of effective therapy. Furthermore, reducing *E. histolytica* overestimation in stool analysis performed by the routine methods without confirmation.

The demographic data of our patients showed no significant differences in patient's gender, marital status, residence areas (rural or urban), and living economic conditions (low-income, moderate-income, and high-income). On the contrary, in a study that was conducted in in Erbil City, northern Iraq, the highest infection rates were significantly higher in females than males and in low-income people than in good-income (Mahmood and Bakr 2020). In another study that was conducted in India, high rates of infection were also significantly associated with the low economic conditions, consumption of raw vegetables and habit of not washing hands before meals (Singh *et al. 2021)*. Moreover, in our study, there was a significant correlation between *E. histolytica* infection and patient's age, and educational level; where the highest infection rates were found among school and preschool children. This is may be due to the frequent contacts between children at nurseries and schools. In Malaysia, Shahrul Anuar *et al.* (2012) also found a significant association between prevalence of infection and age with higher rates observed among patients aged less than 15 years (Shahrul Anuar *et al.* 2012). The latter result was also documented in larger molecular epidemiology study carried out in India by Nath *et al.* (2015). In addition, Singh *et al.* (2021) documented a significant association between infection with *E. histolytica* and the uneducated patients. Our study documented that there is no statistically significant association between *E. histolytica* infection and habit of not washing hands before meals, consumption of raw vegetables, type of drinking water, and close contact with domestic animals. On the contrary, research from Yemen and Malaysia indicated increasing in the prevalence of *Entamoeba* infection among people who have a close contact with domestic animals (Anuar *et al.* 2012, Alyousefi *et al.* 2011). We believe that this may be to either the smaller sample size we used or problems in reporting this information by patients participated in the study, an increased sample size and more detailed questionnaire are needed for further investigation in this field.

6.2 Conclusions

The present study conducted among Palestinians from all over the West Bank showed the highest prevalence of *E. histolytica* among participants who were aged 15 years and less. In addition, we also documented the high efficiency of molecular based technique like PCR method diagnosis of *Entamoeba* species, this will significantly reduce misdiagnosis of the disease in Palestine where high infection rates of amoebiasis have been reported. Furthermore, adoption of molecular techniques in amoebiasis diagnosis will help in estimating the true epidemiology of this disease in different districts of the West Bank.

6.3 Strengths and Limitations

This cross-sectional study investigates the molecular epidemiology of *Entamoeba* species among Palestinians from different governments of the West Bank; an efficient and well-established PCR method have been used to detect the *Entamoeba*. Regardless of the efforts to strengthen the study, several limitations rose and were hard to overcome. First, the limited funds allocated to the study. There is a limited size of the samples collected from different districts and this might have limited our ability to find an association between the infection rate and many variables. Population-based molecular epidemiological prevalence estimations among Palestinians are rare; therefore, comparable molecular prevalence estimates among the Palestinian population were not available. Furthermore, the difficulty of transporting samples from governorates to the laboratory and keeping them in freezer is challenging and may resulted in the destruction of some of them. This study did not involve cooperation of many departments at PMOH in the collecting and transportation of samples in some governorates, which may have limited the chance for collecting more samples.

6.4 Recommendations

Our study highlights the need for additional representative large population-based molecular studies on the distribution and epidemiology of the diseases in Palestine. Further, more studies on the environmental and behavioral factors of patients should be performed on larger scale to determine the risk factors associated with amoebiasis infection in Palestine. Moreover, the history of infection should be extensively considered and studied to reduce the high burden of diagnosis and treatment of the same patients.

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Appendices

Appendix 1

Arabic Study Questionnaire

الالتهاب الأميبي

Amoeba Infection

الشخص المسؤول عن جمع العينة: .....

تاريخ جمع العينة: .....

مكان جمع العينة: .....

كود المريض: .....

الرجاء التكرم بالإجابة عن الأسئلة التالية فيما يتعلق بمعلوماتك الديموغرافية والتي تتضمن الحالة الاجتماعية، مستوى التعليم، مكان السكن، ومعلوماتٍ أخرى.

المعلومات السكانية				
الجنس	1. ذكر	2. أنثى		
تاريخ الميلاد	...../...../.....			
الحالة الاجتماعية	1.أعزب	2.متزوج	3.منفصل/مطلق	4.أرمل
مكان الولادة	.....			
ما هو عدد أفراد عائلتك؟	.....			
ما هو ترتيبك بين أفراد عائلتك؟	.....			

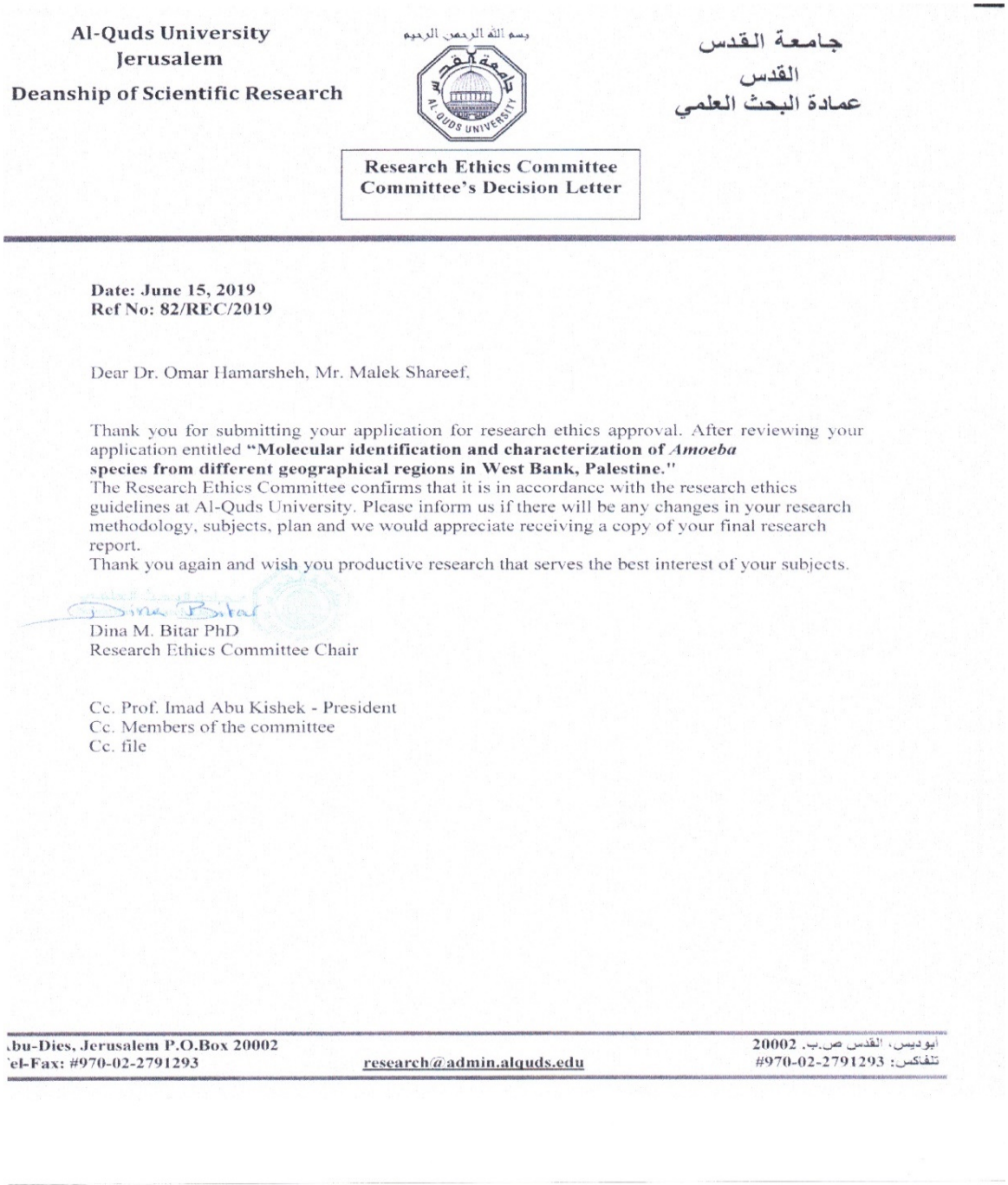
ما هي أعلى شهادة حصلت عليها؟	1. غير متعلم 2. أساسي 3. ثانوي 4.دبلوم 5 .بكالوريوس 6. دراسات عليا
في أي مدينة تسكن حالياً؟	1. نابلس 2. الخليل 3. رام الله 4. بيت لحم 5. جنين 6. القدس الشرقية 7. أريحا 8. سلفيت 9. غير ذلك .....
أين تسكن ؟	1. مدينة 2. بلدة 3. قرية 4. مخيم 5. غير ذلك .....
المعلومات الوظيفية	
هل لديك وظيفة حالياً؟	1.نعم 2. لا
ما هو عملك الحالي؟	.....
ما هو متوسط دخلك الشهري؟	1. 1500 فما دون 2. 1500-3500 3. 3500 فما فوق
المعلومات السكنية	
ما هو نوع السكن الذي تعيش فيه؟	1. منزل خاص 2. شقق سكنية 3. خيمة 4. سكن في مزرعة 5. غير ذلك .....

ما هو مصدر ماء الشرب في مكان سكنك؟	1. أنابيب 2. بئر 3. صهاريج 4. مياه معدنية 5. غير ذلك		
أين يقع الحمام في منزلك؟	1. داخل المنزل 2. خارج المنزل 3. غير ذلك		
ما هو نوع الصرف الصحي في منزلك؟	1. شبكة صرف صحي 2. حفرة امتصاصية غير ذلك		
هل تعاني من مشكلات في تمديدات الصرف الصحي الخاصة بالمنزل؟	1. نعم 2. لا		
ما هو نوع مرحاض الصرف في منزلك؟	1. مرحاض أرضي (عربي) 2. مرحاض غربي مزود بنظام الشطف (إفرنجي) 3. غير ذلك		
العادات			
كم طولك بالسنتيمتر؟	.....		
كم وزنك بالكيلوغرام؟	.....		
هل لديك اختلاط مباشر بالحيوانات؟	.....		
هل تقوم بغسل يديك باستمرار؟	دائماً	أحياناً	أبداً
هل تقوم باستخدام الصابون أو المعقمات؟	دائماً	أحياناً	أبداً
هل تكتفي بمسح يديك عوضاً عن غسلها؟	دائماً	أحياناً	أبداً
هل تتناول الخضار أو الفواكه دون غسلها؟	دائماً	أحياناً	أبداً
هل تقوم بشرب الماء من أماكن مجهولة المصدر؟	دائماً	أحياناً	أبداً
هل تستخدم الحمامات العامة؟	دائماً	أحياناً	أبداً
الالتهاب الأميبي – الحالي			
هل تعاني من آلام في البطن؟	1. نعم 2. لا		
هل تعاني من الإسهال؟	1.نعم 2.لا		
كم عدد مرات الإخراج خلال اليوم الواحد ؟	.....		
هل كان الإخراج مصحوبا بالمخاط؟	1. نعم 2. لا		
كم استمر الإسهال بالأيام؟	.....		
هل عانيت أو تعاني من ارتفاع درجة حرارتك؟	1. نعم 2. لا		

هل تشكو من إرهاق أو تعب عام ؟	1. نعم 2. لا
الالتهاب الأميبي- السابق	
هل سبق وأن تم تشخيصك بالالتهاب الأميبي؟	1. نعم 2. لا
هل تلقيت العلاج اللازم ؟	1. نعم 2. لا
هل تم تشخيص أحد أفراد عائلتك بالالتهاب الأميبي؟	1. نعم 2. لا

Appendix 2

Research Ethics Committee's Approval



Al-Quds University
Jerusalem
Deanship of Scientific Research

بسم الله الرحمن الرحيم

جامعة القدس
القدس
عمادة البحث العلمي

Research Ethics Committee
Committee's Decision Letter

Date: June 15, 2019
Ref No: 82/REC/2019

Dear Dr. Omar Hamarsheh, Mr. Malek Shareef,

Thank you for submitting your application for research ethics approval. After reviewing your application entitled **"Molecular identification and characterization of *Amoeba* species from different geographical regions in West Bank, Palestine."**
The Research Ethics Committee confirms that it is in accordance with the research ethics guidelines at Al-Quds University. Please inform us if there will be any changes in your research methodology, subjects, plan and we would appreciate receiving a copy of your final research report.
Thank you again and wish you productive research that serves the best interest of your subjects.

Dina M. Bitar PhD
Research Ethics Committee Chair

Cc. Prof. Imad Abu Kishek - President
Cc. Members of the committee
Cc. file

Abu-Dies, Jerusalem P.O.Box 20002
Tel-Fax: #970-02-2791293
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ابوديس، القدس ص.ب. 20002
تلفاكس: #970-02-2791293

Appendix 3

Informed Consent Form

الموافقة عن علم على المشاركة في دراسة الالتهاب الأميبي

موافقة وتفويض

التاريخ

_____

الرقم

_____

أنا الموقع أدناه:_____وبعد أن شرح لي أخصائي المختبر _____ أوافق بمحض إرادتي على دخولي أو دخول (ابني/ابنتي) و المشاركة بالدراسة المتعلقة بالالتهاب الأميبي، وأوافق على دراسة عينات البراز بهدف تشخيص نوع الأميبا الجزيئي والمساهمة في الكشف عن مدى وعوامل انتشارها لدى السكان الفلسطينين في الضفة الغربية. كما أفوض فريق الدراسة بإعطاء ما يلزم من المعلومات عني للاستخدامات العلمية شريطة ألا يعطى اسمي الصريح في حالة النشر.

أعلم أني أستطيع الانسحاب من الدراسة في أي وقت وأن مشاركتي في الدراسة لن تكلفني أي تكلفة مالية وعلى ذلك أوقع.

توقيع المريض/ ولي أمر المريض:

_____

توقيع أخصائي المختبر المسؤول عن جمع العينة:

_____

التعريف والتوصيف الجزيئي لأنواع الأميبا في مناطق جغرافية مختلفة في الضفة الغربية

اعداد: مالك محمد عوض شريف

اشراف: د. عمر حمارشة

الملخص

يعدٌّ داء الأميبا أحد أكثر ألامراض الطفيلية والمعوية شيوعًا وانتشاراً في العالم، حيث تصيب الأميبا من نوع المتحولة الحالة للنُسج "*Entamoeba histolytica*" حوالي 500 مليون شخص حول العالم مسببةً ما يقارب 100,000 حالة وفاة كل عام. وفقاً لإحصائياتٍ أجرتها وزارة الصحة الفلسطينية فإن داء الأميبا يمثل مشكلة صحية كبيرة واسعة الانتشار بين السكان الفلسطينيين في الضفة الغربية وقطاع غزة. يتم تشخيص هذا المرض بشكلٍ روتيني باستخدام الفحص المجهري لفحص وجود الشكل الهاجع "الكيسات" والشكل النشيط "الأتاريف" في عينات البراز الحديثة الإخراج من المريض باستخدام المجهر الضوئي. في معظم الأحيان تؤدي طريقة التشخيص الروتيني باستخدام المجهر إلى زيادةٍ غير دقيقة في تشخيص المصابين بالأميبا المتحولة المسببة للمرض من نوع المتحولة الحالّة للنُسج "*E. histolytica*" في هذه الدراسة، قمنا بفحص عينات براز لمرضى يعتقد أنهم مصابون بداء الأميبا من السكان الفلسطينيين المقيمين في تسع مناطق مختلفة من الضفة الغربية وهي الخليل، بيت لحم، نابلس، رام الله، جنين، طولكرم، سلفيت، اريحا والعيزرية باستخدام تقنيات البيولوجيا الجزيئية "PCR". إضافةً إلى ذلك، قمنا بتحديد العوامل الاجتماعية والديموغرافية والاقتصادية المرتبطة بالإصابة بداء الأميبا. بالمجمل، تم جمع 100 عينة براز من المرضى الذين قاموا بمراجعة عيادات وزارة

الصحة والمختبرات الخاصة، والذين عانوا أعراض التهابات معوية تشمل الإسهال، ألم تشنجي أو مغص في البطن، الهزال، والحمى. علاوةً على ذلك، قمنا بجمع معلومات ديموغرافية خاصة بالمرضى لأهداف الدراسة باستخدام استبيان تم تعبئته من المعلومات التي قدمها المرضى المشمولين في الدراسة بشكل مباشر. بدايةً، تم فحص العينات باستخدام الفحص المجهري المباشر ثم بواسطة تقنية تفاعلات البلمرة المتسلسلة "PCR"لتحري المادة الوراثية للأميبا في البراز باستخدام مشرعات "Primers" محددة وموثقة للكشف عن الحمض النووي الخاص بالأميبا. أكدت نتائج تفاعلات البلمرة المتسلسلة تشخيص الأميبا من المتحولة الحالّة للنُسج. *E. histolytica* في 74 عينة والاميبا من نوع المتحولة المتغيرة *E. dispar* في 29 عينة. كذلك تم الكشف عن عينات تحتوي على نوعيّ الأميبا المذكورين سابقاً معاً في 7 عينات. بالمقارنة بين تقنية الفحص المجهري وتقنية تفاعلات البلمرة المتسلسلة لتشخيص الاميبا المسببة للمرض، أكدت تقنية PCRعلى أنّ 74٪ من العينات الإيجابية التي تم تشخيصها مجهريًا كانت إيجابية أيضًا باستخدام PCR. كما أظهرت البيانات الديموغرافية وجود ارتباط كبير بين العدوى بالأميبا من نوع *E. histolytica* والفئة العمرية للمريض والمستوى التعليمي له، حيث وجدت أعلى معدلات الإصابة بين أطفال المدارس ومرحلة ما قبل المدرسة. قامت دراستنا بتسليط الضوء على الحاجة الملحة لمزيدٍ من الدراسات باستخدام تقنيات البيولوجيا الجزيئية على عددٍ أكبر من العينات للحصول على صورة أكثر وضوحاُ حول انتشار وتوزيع داء الأميبا في فلسطين. كذلك توصي دراستنا بضرورة إجراء المزيد من الأبحاث حول العوامل البيئية والسلوكية للمصابين على نطاق أوسع لتحديد عوامل من المحتمل ان تكون مرتبطة بعوامل الخطر للإصابة بداء الأميبا في فلسطين .