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Intestinal Protozoa and Cryptosporidium genotypes in North of West Bank/ Palestine

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Dedication

This thesis is dedicated to:
The sake of Allah, my Creator and my Master, My great teacher and messenger, Mohammed (May Allah bless And grant him), who taught us the purpose of life, My homeland Palestine, the warmest womb; The great martyrs and prisoners, the symbol of Sacrifice; My great parents, who never stop giving of themselves in countless Ways, My beloved brothers and sister I dedicate this research.

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MMMY

الإقرار

أنا الموقعة أدناه مقدّمة الرسالة التي تحمل العنوان :

Intestinal Protozoa and *Cryptosporidium* genotypes in Children with Diarrhea in Northern Palestine

أقر بأن ما اشتملت عليه الرسالة إنما هو نتاج جهدي الخاص ، باستثناء ما تمت الإشارة إليه حيثما ورد ، وأن هذه الرسالة ككل ، أو أي جزء منها لم يقدّم من قبل لنيل أي درجة علمية أو بحث علمي لدى أي مؤسسة تعليمية أو بحثية أخرى.

Declaration

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student`s name: اسم الطالب:

Signature: التوقيع:

Date: التاريخ:

Abbreviations

PCR	Polymerase chain reaction
C.	Cryptosporidium
E.	Entameoba
IFAT	Indirect fluorescent antibody test
CIEP	Counter immune electrophoresis
CDC	Center of Disease Control
WHO	World Health Organization
AIDS	Acquired Immune Deficiency Disease
DNA	Deoxy nucleic acid
SSu	Small sub unit
RBC	Red blood cell
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction fragment length polymorphism

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Intestinal Protozoa and cryptosporidium genotypes in Children with Diarrhea in Northern Palestine

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Abstract

Objective: The aim of this project was to determine the predominant intestinal parasites infecting children with diarrhea residing in Northern Palestine. In addition, cryptosporidium isolates were genotyped.

Materials and methods: A total of 300 stool samples were collected from several areas in Northern Palestine and aliquoted into two vials; one was preserved in 5% formaldehyde for concentration, staining with malachite green and microscopic identification. The other was emulsified in 95% ethanol and stored at -20^{o} C for molecular manipulations and genotyping by PCR, nested PCR and restriction.

Results: There were 19.8% (58/60) of the samples tested microscopically positive for intestinal parasites. The percentage of *E. histolytica*, *G. lamblia* and *Cryptosporidium* was 69% (40/58), 17.2% (10/58) and 13.8% (8/58) respectively. Two additional cryptosporidium spp. were further identified by the highly sensitive and specific PCR procedure. There were 6 *C. hominis* and 4 *C. parvum*. Further Genotyping of cryptosporidium by PCR revealed that 5 *C. hominis* isolates belong to

family Ib and one isolate belong to Id. Furthermore, 2 isolates of *C. parvum* belonged to family IIa and 2 isolates belonged to IId.

Conclusion: the results of this study provide significant information about the predominant intestinal parasites among Palestinian children residing in Northern Palestine. In addition, the genotypes of cryptosporidium have also been determined. This can provide important epidemiological information on one hand regarding the status of parasitic infections among children. On the other hand, it can guide physicians and healthcare specialists to take the necessary measures to prevent the acquisition and spread of these parasites among young children.

Chapter One

Introduction

1.1 Introduction:

Protozoan organisms are unicellular animal like organisms, belonging to protista kingdom. The human body contains many types of protozoa inhabiting organs mainly gastro-intestinal tract. Gastrointestinal protozoa include both non-pathogenic species and pathogenic species which can cause serious problems especially in immune compromised people, and in children [1].

The World Health Organization (WHO) ranks diarrheal diseases as the second highest cause of morbidity and mortality in children in the developing countries. Enteric protozoa are one case of diarrheal disease in children [2]. Intestinal protozoa are transmitted by the fecal-oral route and their life cycles consisting mostly of a cyst stage and a trophozoite stage. Fecal-oral route involves the ingestion of food or water contaminated with protozoa cysts.

The cysts consist of a resistant wall and are excreted in the feces. The cyst wall functions to protect the organism from harsh in the external conditions. Cyst transforms into trophozoite after entering the gastrointestinal tract of the host and become active metabolism and usually motile. Unhygienic conditions promote transmission of most of protozoa

[1]. Gastrointestinal parasitic protozoa are worldwide distributed, but hazardous out-breaks mostly are in developing countries through fecal contamination as a result of poor sewage disposal and poor quality of water.

The main serious symptoms of these parasitic infections are diarrhea resulted from direct cytotoxic effects of the parasites, and the ability of invade and / or effects of the immune response on the intestinal epithelium [3].

1.2. Infectious intestinal protozoa taxonomy:

Gastrointestinal parasitic protozoa belong to various taxonomic groups; flagellates, amoebae, apicomplexa, ciliates, and others [4].

1.2.1 Dientamoeba fragilis:

This species was considered as an amoeba for a long time before it was reclassified as an aberrant trichomonad flagellate based on electron microscopic features. This type of protozoa has no cyst stage. The trophozoite's size ranges between 7-12 µm in diameter and has 1 to 4 nuclei, but the most common form is the binucleated form. It lives in colonic mucosal crypts, feeding on bacteria and rarely ingests RBCs. This species can't invade tissues but it may cause diarrhea and other symptoms such as abdominal pain, flatulence and fatigue. In the absence of cyst stage,

it has been proposed that trophozoites may survive in nematode eggs mostly *Enterobius vermicularis* and transmitted through them [3].

1.2.2 Entamoeba polecki:

They are mainly pathogen in animals such as pigs and monkeys; it was detected in human in Papua – New Guinea. It is not appearing to be significant human pathogen but symptomatic cases may be difficult to treat.

The cysts of this organism mainly have one nucleus with large central karyosomes and evenly distributed peripheral chromatin or peripheral chromatin massed at one or both poles, and the trophozoites have one nucleus with minute central karyosome with peripheral chromatin evenly distributed or massed at one or both poles, trophozoites ingest bacteria. A few cases were studied for this parasite and the main symptom was diarrhea [4].

1.2.3 Balantidium coli:

The largest protozoan parasite of human and it is the only ciliated parasite. *Balantidium coli* life cycle consist of two forms: trophozoite and cyst. Trophozoites are oblong, spheroid, or more slender of 30- 150 µm long by 25- 120 µm wide and the cysts are spheroid or ovoid of 40-60µm. Trophozoites have two nuclei , genes in the macronucleus control the everyday functions of the cell, such as feeding, waste removal, and

maintaining water balance and micronucleus is responsible for sexual reproduction [1].

Cysts enter via contaminated food and water into the human body and live in cecum and colon where they transform to trophozoites. Trophozoites multiply by transverse fission and feed on food particles and sometimes secrete proteolytic enzymes which digest host's epithelial lining causing ulcer. This type of parasite has low prevalence in Islamic societies because they prohibit consuming of pigs which are the reservoirs for the parasite [4].

1.2.4 Cyclospora cayetanensis:

This species was recently recognized as an agent for diarrhea in human, in fresh fecal samples, oocysts are 8 μm to 10 μm in diameter and contain membrane-bound refractile globules. Sporulation requires 5 to 11 days; mature oocysts contain two sporocysts about 4 μm in diameter [5]. Cyclosporosis is characterized by diarrhea, especially relapsing or cyclical, sometimes alternating with constipation. Patients may also exhibit fatigue, cramps, weight loss, and vomiting. Infection is typically concentrated in the jejunum, although in people with AIDS the bile duct may also be involved. The diarrhea is usually self-limiting in immunocompetent hosts but prolonged in AIDS patients [3].

1.3 Most common intestinal protozoa:

According to epidemiological studies of intestinal protozoa and the protozoa outbreaks around the world it was found that *Giardia*, *Cryptosporidium* and *Amoeba* species are the most commonly reported protozoa associated with enteric infections and those mainly associated with food and water transmission [6].

1.3.1 Giardia lamblia:

Giardia lamblia was the first described pathogenic protozoan in human [4]. *G. lamblia* is a flagellated protozoan that cause diarrhea. This parasite has a flagellate for whip- like movement. It has seven genetically distinct genotypes, designated A to G, and H newly identified in marine vertebrates. Only A and B infect humans [7].

1.3.1.1 Morphology:

1) Cyst - Measures 9 x 12 micrometers and contains 2 to 4 nuclei; the karyosome is centrally located, with little or no peripheral chromatin; and the parabasal bodies are present.

2) Trophozoite: It has four pairs of flagella – one anterior pair, two ventral pairs located, and one posterior pair. An axostyle and parabasal bodies are present. It uses “sucking discs” to adhere to intestinal wall; and it cripple

the intestine's ability to secrete enzymes and absorb food leading to appearance of disease symptoms 1-2 weeks after ingestion [8]. Fig (1.1)

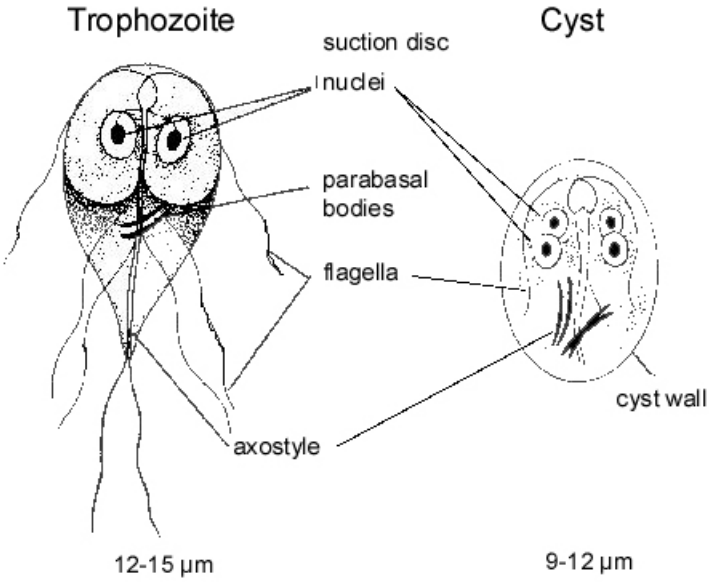


Figure (1.1): *G. lamblia* morphology: trophozoite and cyst stages [8].

1.3.1.2 Life cycle:

Humans ingest cysts from fecally contaminated environment (usually contaminated water); the organism excysts in the upper intestine; trophozoites divide by binary fission about every 12 hours and attach to the intestinal mucosa, sometimes entering secretory tubes, even the gall bladder. Trophozoites and cysts are passed in the feces.

Cyst excretion (shedding) may persist for months. Once outside the body, cysts can be ingested by another animal and hatch due to stomach acid and digestive enzymes and then the life cycle repeats [4].

1.3.1.3 Diagnosis:

1.3.1.3.1 Microscopy:

Stool samples can be directly examined for diagnosis of *Giardia* by wet mount preparation with sensitivity around 90%. Stool samples could be fresh or saved in formalin or ethanol and could be stained by Iodine, trichrome or iron hematoxylin staining [9].

Duodenal aspirate may be helpful in trophozoite recovery when stools are negative. Endoscopic brush cytology may be helpful and duodenal biopsy sometimes used for detection of *G. lamblia* trophozoite in limited cases [3].

1.3.1.3.2 Serology:

Antigen detection is highly sensitive and specific. Antibody detection may be useful in distinguishing acute or chronic infection, if available. Serology for *Giardia* IgG is useful for epidemiologic studies, since after infection, IgG antibodies level remain high for prolonged periods of time. It is not clear if testing for IgM antibodies is useful in the clinical management of giardiasis [10].

1.3.1.3.3 Molecular diagnosis:

PCR usefulness is being tested in surveillance of water supplies; PCR is more sensitive than other tests and have the ability to discriminate

between species and strains. Primers specific for *Giardia* targeted a 183-bp product from the small-subunit (SSU) rRNA gene, 218- and 171-bp amplicons from a giardin gene, and a 163-bp product from a heat shock protein (HSP) gene [3].

1.3.2 Entamoeba histolytica:

1.3.2.1 Morphology:

About 40–50 million people develop clinical amoebiasis each year, resulting on up to 100,000 deaths [11].

The trophozoite is amoeboid and 15-30 μm (sometime larger) in size and contains a single nucleus with a distinctive small central karyosome. The endoplasm is fine and granular, and may contain erythrocytes.

The cyst is spherical and measures 10-15 μm. It has a refractive wall and contains dark staining chromatoidal bodies and 1-4 nuclei with a central karyosome and an evenly distributed peripheral chromatin [4]. (Fig 1.2)

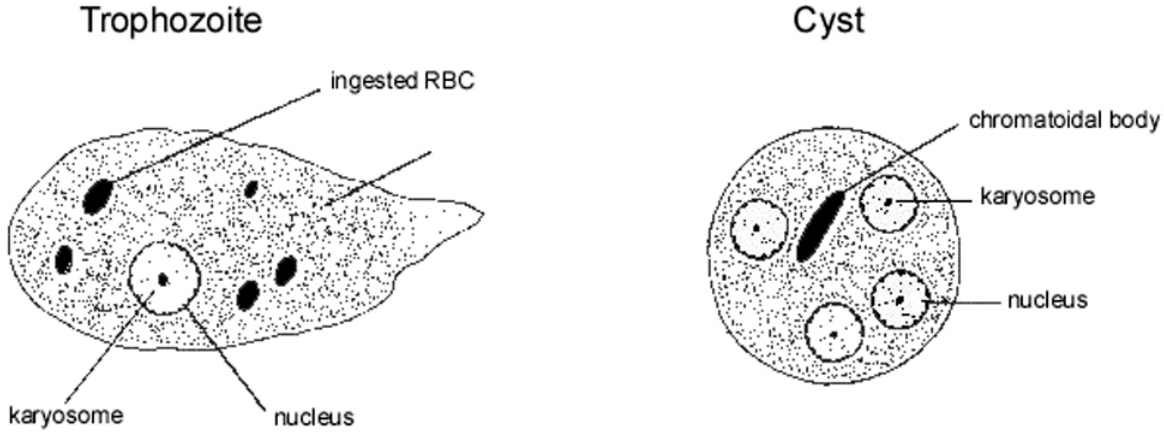


Figure (1.2): *Entamoeba histolytica* morphology: trophozoite and cyst stages [8].

1.3.2.2 Life Cycle:

The infection is acquired when cysts are ingested. The factors contributing to infection are similar to other organisms transmitted by the fecal oral-route. Excystation takes place in the intestine after passing through the stomach. A trophozoite emerges through the disrupted cyst wall and begins to replicate by binary fission. This trophic period occurs on the mucosa of the large intestine. Some of the trophozoites will not replicate and undergo encystations leading to the production of cysts. Up to 45 million cysts can be passed per day in the feces of an infected person [5].

The parasite can also penetrate the intestinal mucosa and epithelial cells and cause severe disease. The initial stage of invasive disease is an ulceration of the colon. Trophozoites are able to kill host epithelial cells in a contact dependent manner and gain access to the lamina propria. In addition, the trophozoites begin to ingest host cells instead of bacteria. The ingestion of host cells is indicated by the presence of trophozoites containing erythrocytes, or hematophagous amoeba [12].

During this phase the patient may exhibit dysentery and the stool may contain hematophagous trophozoites. The trophozoites destroy and ingest host cells leading to ulcer enlargement below the epithelial layer producing a characteristic 'flask-shaped' ulcer. The dysentery will worsen as the lesions continue to expand both laterally and downward into the lamina propria. Ulcers can coalesce leading to sloughing off of large sections of the intestinal epithelium. Peritonitis will result if the ulcer spans

the colon wall. Occasionally a tumor-like mass, known as an ameboma, will form in the intestinal wall. This severe pathogenesis is not advantageous for the parasite, since cysts are no longer produced after the ameba becomes invasive [5].

The amoeba can also become extra-intestinal and invade other tissues with the liver being the most commonly affect organ. This invasion of the liver is likely due to hematogenous spread via the portal vein. The lesions in the intestines and liver can also expand by a direct extension to the skin or lungs. Extra intestinal amebiasis is a progressive disease which will result in death if untreated [3].

1.3.2.3 Diagnoses:

1.3.2.3.1 Microscopy:

Direct microscopy should be done by mixing a small amount of the specimen in 0.9% sodium chloride solution (wet mount) or Lugol's iodine solution. This allows the detection of motile trophozoites of *Entamoeba histolytica and* can also provide information on the contents of the stool, that is, the presence of leucocytes and red blood cells. The second portion of the stool sample is then stained with trichrome and/or iodine to identify trophozoites and cysts [13].

1.3.2.3.2 Serology:

The combination of serology and stool antigen assays is more sensitive and specific than microscopy for the diagnosis of *Entamoeba histolytica* infection. The tests of choice for serology are indirect fluorescent antibody test (IFAT), counter immunoelectrophoresis (CIEP), or enzyme linked immunosorbent assay (ELISA). Detection for antigen can be used to distinguish between different types of amoeba in stool sample [14].

1.3.2.3.3 Molecular diagnosis:

PCR methods were found to be highly sensitive and specific for detecting parasitic DNA from microscopy positive samples using both manual and automated methods. DNA samples extracted from stool samples then analyzed by conventional PCR and real-time PCR which is more sensitive and specific than conventional PCR [15].

***1.3.3 Cryptosporidium*:**

1.3.3.1 *Cryptosporidium* taxonomy:

Cryptosporidium is among the most common parasitic enteric pathogens in humans. The organisms infect and reproduce in the epithelial cells of digestive and respiratory tracts [16]. There are more than ten named species of cryptosporidium including species that infect mammals, birds, reptiles and fish [17]. *C.parvum* (4 µm diameters) is the main species

responsible for disease in human. *C. parvum* has been divided into two separates species: *C. homonis* (previously *C. parvum* genotype 1), and (*C.parvum* genotype 2). *C. hominis* and *C. parvum* are found in humans and a number of other animals [18, 19].

Several *Cryptosporidium* species infect mainly animals such as *C. andersoni*, *C. wrairi*, *C. baileyi*, *C. galli*, *C. serpentis*, *C. saurophilum*, and *C. molnari*. [17] *C. felis*, *C. muris*, *C. canis*, and *C. meleagridis* are also found mainly in animals but have also been identified in some human individuals. Additional heterogeneity within species may lead to variations in infectivity and clinical expression in different hosts [20, 21].

Cryptosporidiosis is most common in children aged between 1 and 5 years. It is also more frequent in children less than two years old, although outbreaks occur worldwide in all age groups [22]. People with weak immune systems (those with AIDS, or persons who have undergone transplantation or are receiving chemotherapy) are likely to be most seriously affected [23].

1.3.3.2 Characteristics of Cryptosporidium

Cryptosporidium oocysts are highly resistant to many common disinfectants such as aldehyde-, ammonia-, alcohol-, chlorine-, and alkaline based commercial disinfectants. Oocysts are heat sensitive, a temperature of 65 °C inactive oocysts within 5-10 minutes. Over a period of 2 hours or more, desiccation is lethal to oocysts. Oocysts can remain viable for about

18 months in a cool, damp or wet environment [24], so they are common in rivers and lakes especially where there has been sewage or animal contamination. Oocysts are generally susceptible to freezing, but this varies by onset of freezing; snap freezing will destroy oocysts reliably, but with slow freezing, such as that found in natural environment, oocysts can be survive. Oocysts have been reported to survive temperatures as low as -22 °C [25].

1.3.3.3 Mode of transmission

Cryptosporidium is shed in the feces of infected humans and animals. People become infected by ingesting the organism [26]. Infected individuals can shed the organism in their stool for several weeks after they recover from the illness [27]. Because cryptosporidiosis is transmitted by the fecal-oral route, the greatest potential to transmit the organism comes from infected people who have diarrhea, people with poor personal hygiene and diapered children [28, 29].

Cryptosporidium can be spread by person-to-person or animal-to-person contact, contaminated food such as vegetables, fruits, raw meat and unpasteurized milk and by drinking contaminated water [30]. Cryptosporidial oocysts may be found in all types of water, including untreated surface water, filtered swimming-pool water, and even chlorine-treated or filtered drinking water [31]. Contamination of untreated surface water and filtered public water supplies is a growing concern, since water-

borne outbreaks have been reported worldwide [31]. Person-to-person spread of *C. parvum* is one of the most common modes of transmission.

1.3.3.4 Life cycle

Cryptosporidium is capable of completing all stages of its development (asexual and sexual) within a single host as shown in fig.1.3 [16]. The thick-walled oocyst is the resistant stage found in the environment. When mature oocysts (5 µm) are ingested, they undergo excystation in the small bowel, releasing four banana-shaped motile sporozoites that attach to the epithelial cell wall of the gastrointestinal tract [20, 32].

The sporozoites differentiate into a spherical trophozoite and mature asexually into two types of meronts, type I meronts contain 6-8 nuclei which release 6-8 merozoites intraluminally [33]. When the meront is mature, each merozoite is able to infect a new host cell and then develops either into type I meront or type II meront, which contains 4 merozoites when mature [20]. Merozoites from type II meronts also invade new host but undergo sexual maturation by differentiating into either microgamont (male) or macrogamont (female) stages [33]. After fertilization of the macrogamont by microgamonts, the fertilized macrogamont (zygote) then develops into an oocyst that sporulates within the infected host by undergoing mitosis [16].

There are two types of oocyst produced during the cycle, the thick-walled oocysts (80%) which are commonly excreted from the body in the

feces, and the thin-walled oocysts (20%) that are involved in autoinfection because they excyst within the gut, they release merozoites and infect new host cells[32,33]. Oocysts are infectious and can remain viable for many months at a wide range of temperatures. The new generation of the parasite can develop and mature within 12-14 hours helping it to produce huge numbers of parasitic cells in the gut and to secondary infection sites in the duodenum and large intestine [23].

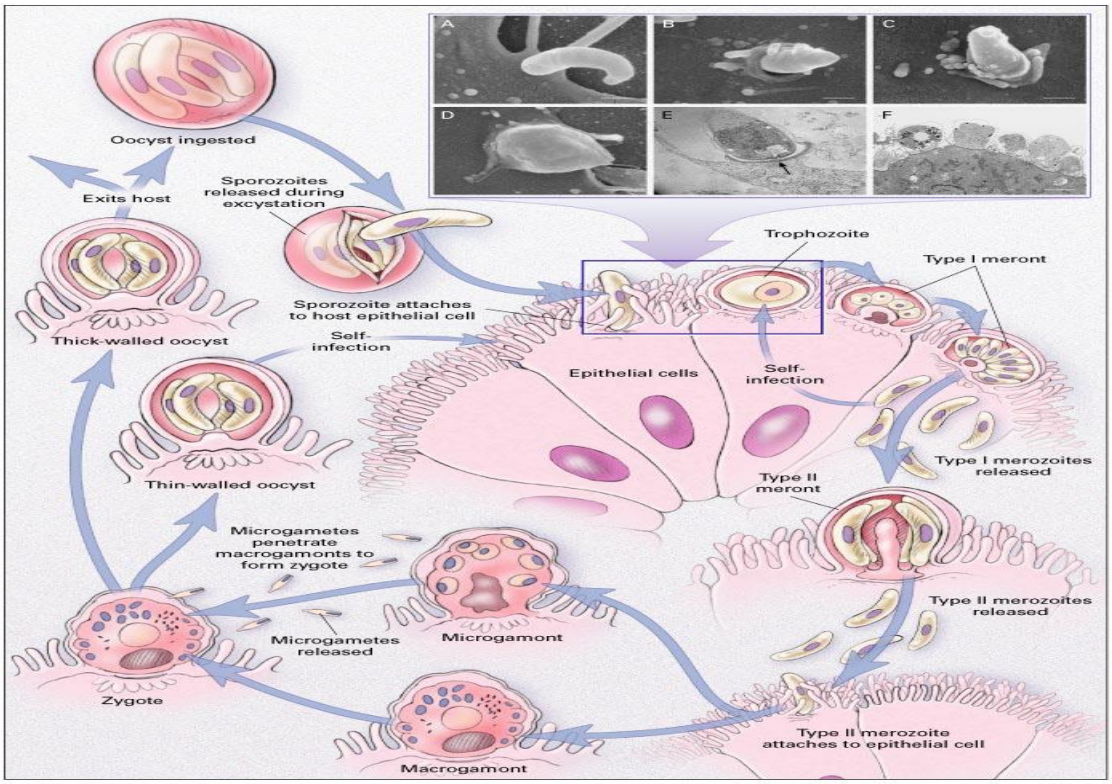


Figure (1.3): Life cycle of *Cryptosporidium* [16].

1.4 Literature review:

1.4.1 Prevalence of intestinal protozoa; *E. histolytica* and *G. lamblia*.

Hussein (2011) studied the prevalence of intestinal parasite infections in northern districts of West Bank, Palestine and to determine associated sociodemographic factors. The prevalence of parasitic infection was 22.2% and the rate of infections with Amoeba was 9.7%, *G. intestinalis* 4.1%, *Entrobius vermicularis* 1.6% and *Ascaris lumbricoides* 3.8%. Real-time PCR was performed to differentiate between *E. histolytica* and *E. dispar*, results of PCR showed that 14% of samples positive with microscopy for amoeba were positive for *E. histolytica*. There was significant association between parasite infections and parent's education, place of residence, and washing hands habits [34].

To evaluate the geographic distribution of *G. intestinalis* genotypes in Nablus, West Bank, Palestine, a genotyping study was performed using clinical fecal samples. Microscopic examination confirmed that 8 of 69 (11.6%) samples were *G. intestinalis* positive, and subsequent genotyping analyses targeting the small subunit ribosomal RNA (18S rRNA) and glutamate dehydrogenase (GDH) genes revealed the *G. intestinalis* genotypes in the 8 samples [35].

A study was carried between January 2000 and December 2009 to assess the prevalence of intestinal parasites among Jenin governorate. Stool samples were collected from Jenin governmental hospital and diagnosed.

The most common protozoa was *E. histolytica* (8.2-18.2%), *G. lamblia* was also present (0.18-0.66%) [36].

In another study aimed to assess the occurrence of gastrointestinal parasites among pre-school children, 679 stool specimens were collected from children aged <10 months to 60 months attending Ard El-Insan Association in Gaza. Stool specimens were inspected by a direct smear microscopy and sedimentation techniques. It found that 16.6% of the studied children were infected with intestinal parasites. Infection with *G. lamblia* showed the highest prevalence (10.3%) among other parasites detected. Intestinal parasite prevalence was higher among male children than females. All age groups were susceptible for parasitic infection and no clear trend due to age was noted [37].

Data from the Epidemiology Department- Ministry of Health / Gaza were collected and analyzed statistically. The prevalence of intestinal parasites was studied for the period 1998–2007. The study shows that out of 471,688 patients (all ages) who had provided 1 stool specimen to the laboratories of primary health care centers in one of the 5 governorates of the Gaza Strip, 116,261 specimens were positive for intestinal parasites; representing an overall prevalence of 24.6%. *E. histolytica* and *G. lamblia* were the most frequently detected intestinal parasites. [38].

Intestinal parasitic infections were studied in Amman, Jordan, from 2003 until 2005. A total number of 1280 specimens were studied.

E.histolytica was found to be the most prevalent parasite with an infection rate of 27.81%. The highest incidence of this infection was at an early age (1-10 years) [39].

In Iraq a study was done in Kadhmiyah Hospital to determine the prevalence of *E. histolytica* and *G.lamblia* for 1520 stool samples from children of ages between 1 month-12years, the total infection of *E. histolytica* was 9.80%, and *G. lamblia* was 1.77%. Also it showed that there were significant relation between age group and infectivity rate of *E. histolytica* and *G. lamblia*[40].

Another study was held in south of Tehran, Iran to describe epidemiologic characteristics of intestinal parasites in the population there. 466 out of 4,371 patients (239 males and 227 females) were laboratory diagnosed for intestinal parasites at Zakaria Razi Laboratory from April 21, 2004 to October 20, 2005. *B. hominis* and *G. lamblia* were the most frequent intestinal parasites recorded [41].

In Sharjah, United Arab Emirates a survey of prevalence of intestinal parasites among people attending Ministry of Health hospitals was performed during the year 2008 and 2009. Stool examination from 10,514 patients was performed. 814 of the 10,514 examined specimens were found to be positive for intestinal parasites. The rate of infection in males (58%) was higher than in females (42%). Overall, *E. histolytica* (71.8%) and *G. lamblia* (17.5%) were the commonest intestinal parasites identified. [42].

A study was done in Saudi Arabia to determine the prevalence of *E. histolytica* and *E. dispar* by microscopy and two stool antigen detection kits. Stool specimens were collected from diarrheic patients in Makkah. And the authors reported that 76.9% out of the 156 samples were infected. Microscopic examination showed that, 64.8% were positive for *E. histolytica/E. dispar*, 1.9% were positive for *G. lamblia*, 1.9% were positive for *Cryptosporidium* spp. and 8.3% were positive for other parasites. 112 samples were found to be infected using Triage. 59.6% were infected with *E. histolytica/ E. dispar*, 1.9% was infected with *G. lamblia* and 1.9% with *Cryptosporidium* spp. [43].

To determine the prevalence of different types of parasitic infections among patients attending primary health care centers, and to find if infection differs with socio-demographic factors, a cross- sectional sampling survey was conducted in five health regions of Kuwait. One thousand questionnaires were distributed, and 912 completed questionnaires were received from the patients, who presented with gastroenteritis symptoms. A total of 912 participated in the study, including 607 (66.6%) males and 305 (33.4%) females based on stool examination, 255 (28%) subjects were found to be positive for different types of parasitic infections. There was no significant difference in the prevalence of parasitic infection among gender and nationality, but was significantly higher among children. Infection was significantly higher among people with low level education, as well as, those with low or middle class income

and also among the unmarried patients. The most common type of parasite found was *Enterobius vermicularis*, 27.1% and was significantly higher (74.6%) among children. The *E. histolytica* and *E. coli* was significantly higher among adults [44].

Stool specimens of 1282 schoolchildren from Abha- Saudi Arabia were collected in the period of October 1987 through March 1988, these samples were examined for the presence of intestinal parasites, and 313 specimens (24.4%) were found infected with one or more species of 11 intestinal protozoa and helminthes. The most common protozoa were *G. lamblia* (10.9%) followed by *E. histolytica* (4.1%), *Hymenolepis nana* was the most prevalence helminthes. The distribution of intestinal protozoa was analyzed according to age, and other variables and it was found that prevalence of E. *histolytica* increased with age, whereas *G. lamblia* infections were less common among older children [45].

An epidemiological study investigated the prevalence of *E. histolytica* and its relation with residency, sex, age, economical status, and maternal education, among 200 children, including 117 boys and 83 girls, aging less than 1-12 years, attending the pediatric hospital in Erbil/Kurdistan region-Iraq, between the beginnings of November 2010 to the end of March 2011. The rate of infection was 30% (34.69% in urban and 25.49% in rural regions). The higher rates of infection were among

girls (33.73%), aged 4-6 years (52.38%), with moderate economical status (34.54%), illiterate mothers (39.24%) [46].

Out of 1261 stool specimens collected from children in Dohuk city, northern Iraq, the prevalence of *G. lamblia* infection was 38.5%. The highest rate of infection was in orphan care centers (48.1%) and the lowest in the pediatric hospital (31.3%). The age group 10–12 years had the highest rate (81.2%) and 7–9 years the lowest (22.9%); boys had a higher rate than girls. Some infected samples (70/486) showed double or triple infections and *G. lamblia* was combined with *Hymenolepis nana*, *Blastocystis hominis*, *E. histolytica* and *Iodamoeba buetschlii* [47].

Giardia parasite is prevalent endemically in Taif city. Infection is more prevalent in children under 5 year's old and elderly people. The sickness is more intense in immune compromised people. The disease is usually diagnosed by stool examination by the microscope, for the identification of the both trophozoite and cyst stages. Molecular characterization or diagnosis was used as an alternative method for the diagnosis of infection based on polymerase chain reaction (PCR). The prevalence of *G. duodenalis* was 15% in stool samples collected from different hospital in Taif. By means of RAPD technique, most *G. duodenalis* isolates were genetically similar, forming two main groups [48].

A systematic review and meta-analysis aimed to estimate the epidemiology of *G. lamblia* in the Republic Islamic of Iran found that the prevalence of *G. lamblia* was 14.7%. By age classification, the prevalence was 15.1% amongst fewer than 10 years children, 19.2% amongst adolescents and youngest of fewer than 20 years, and 6.7% amongst adults of between 20-30 years old ($p<0.001$) [49].

Another study determined the prevalence of a protozoan flagellates *G. lamblia* parasite in South Libya Sebha Province, and showed that out of 501 stool specimens the overall prevalence of *G. lamblia* infection was 3.19% and there were no significant differences between incidence in males and females. There was significant differences between age groups, with the age from 11-20 had the highest rate of infection (3.92%). The age groups less than or equal 10 years had the rate of infection 3.64% and older than 60 years were healthy. The age groups 21-30, 31-40, 41-50, the infected percentage were, 3.26%, 2.86%, 2.63%, respectively [50].

1.4.2 Genotyping and prevalence of *Cryptosporidium*:

There are a number of species of the *cryptosporidium* parasite reported from different countries [17]. Taxonomy of parasite species was initially based on oocyst morphology but molecular typing was reported to be the best technique in differentiation of cryptosporidium species [17].

In Palestine, a number of investigations regarding the burden of cryptosporidiosis were reported. [51,52,53]. Abu-Alrub *et al*. and Da'as studied the prevalence of cryptosporidiosis in children aged 1 month to 13 years and 1-5 years, respectively [51,52]. Abu-Alrub studied the prevalence of Cryptosporidium in sample consisting of 760 children aged 1month-13years old in different regions of west bank , microscopy was used for detection of cryptosporidium in diarrheal stool samples and the results showed 11.6% (88/760) Cryptosporidium positive samples. Da'as also studied the prevalence of Cryptosporidium in North of west bank for children aged 0- 5 years old which found to be 13.6%. Hussein has proved based on molecular technique that the causative agent of diarrhea outbreak in Nablus city was *C. parvum* [53].

In stool surveys of patients with gastroenteritis, the reported prevalence of Cryptosporidium is 1–4% in Europe and North America and 3–20% in Africa, Asia, Australia, and south and Central America [54].

A study from Jordan in 2004, reported that the causative agent of cryptosporidiosis in children admitted to north Jordanian pediatric hospital was *C. parvum.* Phylogenetic analysis of PCR product sequenced showed that most *C. parvum* isolates belonged to subtypes families IIa and IId, two of the samples were of the subtype family IIc, and one sample belongs to IIf family. One of the *C. hominis* containing samples belongs to Id family, one belongs to Ie family and two belongs to Ib family [55]. In a recent

study on Jordanian children, 4 different species based on PCR techniques has been reported. These parasites types are *C. parvum*, *C. hominis*, *C. meleagridis* and *C. canis* [56].

Stool samples were obtained from 1275 children with diarrhea over a 2 years period in Egypt, 214 (17%) were found to be infected with *Cryptosporidium* [57].

In another study in Kuwait stool samples were collected from 2548 children with diarrhea aged between 6 months and 16 years during the period September 2005–March 2008. DNA preparations of 83 samples positive by microscopy identified *C. parvum* in 61 of the children followed by *C. hominis* in 22 children and four children had both *C. parvum* and *C. hominis* [58].

Stool specimens were collected from children aged <5 years in six pre-school crèches and clinics in the Jeddah area of Saudi Arabia. (25%) of the children had diarrheal disease but the other were asymptomatic. Stool samples were stained and examined for the oocysts of *Cryptosporidium* species and other enteric protozoa, (32%) of the symptomatic children but only (4.7%) of the asymptomatic were found positive for Cryptosporidium [59].

A total of 391 fecal samples were collected from Egyptian patients in ten hospitals of the Great Cairo area between May 2008 and March 2009.Twenty three out of 391were immune reactive to *Cryptosporidium*.

The cryptosporidium oocyst wall protein (COWP) PCR was successful in 18 samples; fifteen samples out of 18 positive COWP genes PCR were typed by RFLP analysis. Nine isolates were *C. hominis* genotype, three *C. parvum* and three mixed infections [60].

To detect cryptosporidium infection in clinical samples, 177 children aged 1 to 5 years old, suffering from diarrhea, selected from outpatient clinic, children Hospital, Cairo University with 35 healthy children in the same age as control group were selected. Samples were diagnosed using RIDA Quick Cryptosporidium Copro antigen detection kit. In addition, stool specimens were examined following the kinyoun acid-fast staining method, 27 children in the study group were positive for cryptosporidium infection using antigen detection method (15.3%), while 20 were positive using the acid-fast technique (11.3%). All children in the control group were negative for cryptosporidium infection [61].

To identify the genotype of Cryptosporidium in diarrheic stool, stool samples from diarrheic patients admitted to Dokuz Eylül University Medical Faculty-Turkey and samples of patients affected by a diarrhea epidemic in Izmir were collected and sent to laboratory. A total of 162 stool samples were examined by microscopic and molecular methods, by microscopy, 18 stool samples were positive for *Cryptosporidium*. Using PCR found Cryptosporidium in 15 of these cases. Six out of 144 Microscopy negative samples were showed positive result for Cryptosporidium using molecular diagnosis. The restriction fragment

length polymorphism (RFLP) method was applied in PCR positive samples. *C. meleagridis* was found in 1 case, and *C. parvum*-specific bands were seen in 20 cases. It was found that 88.9% of those diagnosed with Cryptosporidium spp. drank artesian and well water, and 11.1% drank bottled water. [62].

Another study aimed to search for *C. parvum* and to determine the prevalence of this parasite among children in Kut city, Iraq. Six hundred stool samples were collected from children less than 12 years old from October 2011 to May 2012. Stool samples were diagnosed by microscopy and ELISA. Results indicated that 203 cases gave positive results (33.83 %) and 397 cases gave negative results (66.17%) with microscopy. The higher infection, 115 (19.17%) appeared in age (<1).[63].

1.5 Significance of the study:

The study which has been performed in the genetic laboratory (at An-Najah National University) showed the role of cryptosporidium in the diarrhea outbreak which took place in 2008 in Nablus region. [53], although *C. parvum* was found to be the causative agent of the outbreak, little data is available about the role of other species in Palestine. Identification of species of the parasite has implications for taxonomy as well as for understanding the mode of transmission of the parasite especially if the transmission is zoonotic.

1.6 Objectives

The main objective of this study is to identify genotypes of *Cryptosporidium* among Palestinian population.

Other objectives include:

1. Exploring the prevalence of cryptosporidium among children aged between 0-10 years old who attended health care centers and have diarrhea.
2. Exploring the prevalence of other intestinal parasites among study participants more specific, *E. histolytica* and *G. lamblia*.

CHAPTER TWO
MATERIALS AND METHODS

2.1 Study area and study population

This study was conducted on newborn to 10 years old Palestinian children living in the northern region of the West Bank, Palestine. It included Nablus, Tulkarm and Salfit Governorates.

2.2 Study population and sampling procedure:

In 2012, the Palestinian Central Bureau reported that the total population of Northern West Bank districts was 707,817. The total number of children ≤10 years old was 141,155 [64], the distribution of children population in the study area is shown in Table 2.1.

Table 2.1: Total number of children in different regions ≤ 10 years old [64].

	Nablus	**Tulkarm**	**Salfit**	**Total**
City	31511	12612	2179	46302
Villages	44757	22582	14320	81659
Refugee camps	8605	4589	None*	13194
Total	84873	39783	16499	141155

* No camps in Salfit governorate.

Stool samples were collected from 300 children with diarrhea selected from health clinics, UNRWA clinics, and hospitals in the

designated study areas. Self-designed questionnaire was used to collect relevant demographic information and clinical data of the children participants. The study period was from October 2012 until March 2013. The sample size was calculated according to the following equation:

$$\text{Sample Size (SS)} = \frac{(1.96)^2\,(1-P)}{\varepsilon^2\,P}$$

Where:

E is known as the relative precision = 0.2

P is the estimated prevalence rate according to neighboring countries = 0.24

The number of children enrolled in the study has been determined for each governorate by the following equation:

$$\frac{\text{Number of children per area X300}}{\text{Total number}}$$

The number of children selected from each study area was calculated in a similar fashion and the results are shown in Table 2.2.

Table 2.2: Number of samples collected from children living in the designated study areas.

	Nablus	**Tulkarm**	**Salfit**	**Total**
City	67	27	5	99
Villages	95	48	30	173
Refugee camps	18	10	0*	28
Total	180	85	35	300

2.3 Stool samples collection:

The stool samples were collected from children less than 10 years of age suffering from gastrointestinal illness. Sample collection took place from October 2012 to March 2013. The samples were obtained from medical health centers, UNRWA clinics, and hospitals. An ethical consideration and consent by the parents or guardians of the children was signed before getting the samples. The samples were collected in a special tightly capped leak proof containers. Each sample was divided into two portions, one preserved and stored in 10% formalin and the other in 96% ethanol. Grouping of samples was done in the place of collection before the samples were stored in fridge at 4°C temperature in the Genetics Laboratory at An- Najah National University .

2.4 Identification of intestinal protozoa by direct microscopic examination and staining:

Microscopic examination was performed on all stool samples using Malachite green negative stain for detection of *Cryptosporidium* species and Iodine stain for other intestinal parasites and stages. Stool samples preserved in 10% formalin and stored were used for this purpose.

2.4.1 Malachite green staining procedure:

Malachite green negative staining has been used as described by Elliot et al. [65] and performed as follows:

Stool smears were prepared, air dried and then fixed in methanol for 1 min. The smears were then stained with malachite green for 5 min. After brief washing with 50% ethanol, followed by washing in water, the smears were destained for 2 to 3 minutes in 0.5% aqueous Acetic acid depending on the thickness of smears. Finally the smears were dried on warm surface and examined microscopically.

2.4.2 Iodine staining procedure:

Direct iodine mounts were prepared and used for exploring the presence of protozoan trophozoites and cysts. The use of iodine in the mounts determines the internal structures of the parasitic forms and facilitates their identification. Iodine mounts were prepared in the same way as saline mounts except that a drop of Lugol's iodine is added to the mounts instead of saline. The addition of iodine kills the organisms but permits better identification of cysts. The working solution of iodine should not be too dark or morphological detail will be obscured.

The stock solution of Lugol's iodine was prepared by dissolving 10 grams potassium iodine in 100ml of distilled water then 5 grams iodine were added, and mixed until dissolved. The solution was filtered and placed in a brown tightly stopper bottle. Lugol's iodine was added to the stool smear and covered with a cover slip and examined within five- to 15 minutes using the l00 X oil objective [66].

2.5 DNA extraction:

All 300 stool samples stored in 96% ethanol were subjected to DNA extraction. DNA was extracted from stool samples using QIAamp DNA stool kit (Qiagen, Germany), according to manufacturer's instructions. DNA was extracted from approximately 150 mg stool samples which were incubated at 100°C for 60 minutes, digested with proteinase K (3 mg/ml) in lysis buffer at 56°C for 30 minutes, and extracted by spin-column filtration. Extracted DNA was stored at -20°C before use.

Quantization for DNA was done by photometer at 280nm and qualification of DNA was at 260/280 nm ratio, then all DNA were run in 1.5% agarose gel to check for DNA.

2.6 PCR:

For genotyping of *Cryptosporidium*, a PCR protocol was adopted to amplify a 18s rDNA locus using a previously described method by Xiao et al. [67]. This PCR technique was used for identifying *Cryptosporidium* spp. among other species in the total samples. The entire SSU rRNA gene was amplified from samples by conventional polymerase chain reaction by using the forwarding primer 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and reverse primer 5'-GAGTAAGGAACAACCTCCA-3'. Each PCR consisted of 35 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 60 s, with an initial denaturation at 94°C for 5 min and a final

extension at 72°C for 10 min. Amplification using above primers gave positive samples with around 835 bp. To confirm the presence of *Cryptosporidium* spp., the PCR product was further digested with V*sPI* restriction enzyme that resulted in 550-bp fragment (positive sample) on 2% gel.

For genotyping of the *cryptosporidium spp*., the 835-bp fragments were used to subtype parasites at the GP60 gene locus using a two step nested PCR that amplified around 830 bp fragment [68, 69, and 70].

For subtyping of *C. parvum* based on GP60 locus, a PCR amplification used sense primer gp15ATG (5'-CGGGATCCATATGAGAT TGTCGCTCATTATC) and antisense primer gp15STOP (5'-GGAATTCT TACAACACGAATAAGGCTG), which amplified a ca. 1-kb fragment extending from the translational start codon to the termination codon of the gp15/45/60 gene [68]. Amplifications were performed for 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 45 s, and extension at 72°C for 60 s. All gp15/45/60 PCR fragment products were purified using a QiaQuick PCR Prep kit (Qiagen), and the sequences were determined by cycle sequencing using the same primers (sequencing was done in Bethlehem university).

For subtyping of *C. hominis* based on GP60 locus, PCR amplification at the 18S rDNA locus was used. A PCR product of ~830 bp was amplified by a nested PCR using the forward primer actin AII F1 (5′-ATGCCVGGW RTWATGGTDGGTATG-3′) and the reverse primer actin

Act6R (5′-GGDGCAACRAC YTTRATCTTC-3′) using the conditions: 50 PCR cycles of 94°C for 30 s, 58°C for 20 s, and 72°C for 40 s, with a final extension of 72°C for 7 min. The DNA fragment products were further sequenced using al3535 primer (5’-GGA AGG AAC GAT GTA TTT-3’) [69, 70].

Nucleotide sequences were analyzed using ChromasPro version 2.3 (http://www.technelysium .com.au) and aligned with reference genotypes from GeneBank using Clustal W (http://clustalw.genome.jp).

2.7 Data statistical analysis:

Chi-square test was conducted using of the SPSS statistical package version 15. SPSS was used to analyze the data of questionnaire and determine if there a relationship between the variables and the infection with intestinal protozoa. Values were considered to be statistically significant when the p-value obtained was less than 0.05.

The study was approved by the institutional Review Board of the An-Najah National University.

CHAPTER THREE

RESULTS

In this study the mean age of participants was 5 ± SD years old. The percentage of males was 56.6% (170/300) showing a slightly higher percentage than female participants.

3.1 Demographic data:

Table (3.1) shows the age distribution of children at the time of the study. The mean age of children participants is 5 ± SD. The highest rate of participants was 29.6% (89/300) of the newborn to one year group. The results for the presence of parasitic infection in the samples tested by microscopic examination are shown in Table 3.1.

Table 3.1 Microscopic examination showing positive results for the children age groups tested.

	Intestinal Parasites			
Age Group	***E.histolytica***	***G. lamblia***	***Cryptosporidium spp.***	**Total**
0 -5	34	8	9	51
>5-10	6	2	1	9
Total	40	10	10	60

There were 20% (60/300) of the samples tested showed positive infection for one or more intestinal parasite. It is interesting to note that 67% (40/60) of the parasites was *E. histolytica. G. lamblia and*

cryptosporidium spp., were present in 16.6% (10/60) each in the tested samples. Furthermore, children newborn to five years of age had the highest rate for all the parasites 56.6% (34/60) for *E. histolytica,* 13.3% (8/60) for *G. lamblia* and 15% (9/60) for *Cryptosporidium spp.* Children with ages equal or greater than 5 years old had lower prevalence for intestinal parasites 15% (9/60) , whereas *E. histolytica* prevalence was 10% (6/60), *G. lamblia* was 3.3% (2/60) and *Cryptosporidium spp.* Was 1.6% (1/60) , as shown in Table 3.1.

3.2 Prevalence of intestinal parasites among Palestinian children

The results of our study showed that 20% of participants are infected with one of the parasites identified in this report (Table 3.2). Distribution of infection among male children was slightly higher than that of females (11.3% in males compared to 9.7%). However, the difference between the rate of infection between males and females was not statistically significant (P-value >0.05) (Table 3.2).

Table 3.2: Prevalence of intestinal parasites among gender.

Gender	positive	negative	total	p-value
male	34	136	170	1.00
female	26	104	130	
total	60	240	300	

The effect of various demographic variables was analyzed. Among the various variables studied, only living in a house with animals in or around it and hand washing had significant association with parasitic infection. Table 3.3.

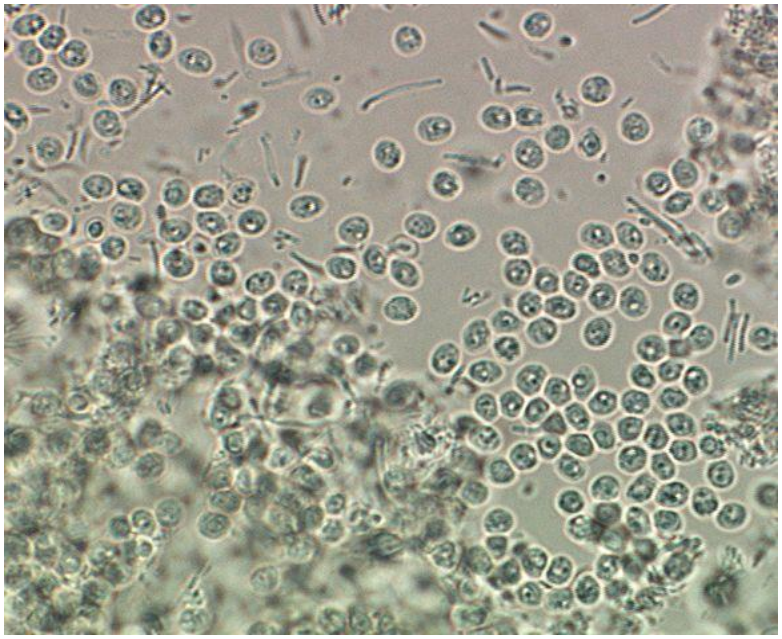
Table (3.3): Relationship of parasitic infection with various environmental risk factors there is a significance difference if p ≤0.05.

Characteristic		Number		Percent		P - value
		+ve	-ve	+ve	-ve	
Place of residence	City	19	80	6.3%	26.6%	0.374
	Village	38	135	12.6 %	45%	
	Camps	3	25	1%	8.3%	
Father's education	Less than tawjihi	28	88	9.3 %	29.3 %	0.549
	Tawjihi	14	78	4.6%	26%	
	Diploma	5	21	1.6 %	7 %	
	Bachelor	12	45	4%	15%	
	Post graduate	1	8	0.3 %	2.6 %	
Mother's education	Less than tawjihi	21	79	7 %	26.3 %	0.986
	Tawjihi	17	73	5.6%	24.3%	
	Diploma	4	17	1.3 %	5.6%	
	Bachelor	18	71	6%	23.6%	
	Post graduate	0	0	0	0	
Family size	Less than 5	27	101	9%	33.6%	0.683
	5 and more	33	139	11 %	46.3 %	

Family income	Less than 2000 NIS	14	54	4.6%	18%	0.890
	2000 NIS and more	46	186	15.3 %	62%	
Animals near home	Yes	29	69	9.6 %	23 %	**0.004**
	No	31	171	10.3%	57%	
Drinking water source	Tap water	51	216	17 %	72 %	0.076
	Well	2	15	0.66 %	5%	
	Spring	4	4	1.3 %	1.3 %	
	Filtered water	3	5	1%	1.6 %	
Washing hands before eating	Yes	55	219	18.3 %	73%	0.918
	No	5	21	1.6%	7 %	
Washing hands after go to W.C	Yes	54	23	18 %	7.6 %	**0.001**
	No	6	4	2%	1.3 %	
Diarrhea infection before	Yes	40	166	13.3 %	55.3 %	0.709
	No	20	74	6.6 %	24.6%	
Parasitic infection before	Yes	16	51	5.3 %	17 %	0.368
	No	44	189	14.6 %	63%	
Family member infected before	Yes	20	59	6.6%	19.6 %	0.169
	No	40	181	13.3 %	60.3%	
Fruit and vegetable washing	Yes	60	240	20%	80%	-
	No	0	0	0	0	
Playing with pets	Yes	13	70	4.3 %	23.3%	0.245
	No	47	170	15.6 %	56.6%	

3.3 Microscopic Diagnosis:

Microscopic diagnosis for stool samples was done using Iodine mounts and Malachite green staining which showed the presence of several species of intestinal parasites which included protozoa and helminthes. Figure (3.1) shows the Cryptosporidium spp. oocysts stained with malachite green.



Figure(3.1): Microscopic detection of Cryptosporidium . Oocysts collected from the stool of children with gastroenteritis were stained with malachite green (100X magnification).

Prevalence of intestinal protozoan parasites among our group was using microscopic diagnosis was found to be 19.3% (58 samples out of 300). Prevalence of protozoan parasites *E.histolytica* cryptosporidium was determined to be 13.3%, 3.3% and 2.6%, respectively. Microscopic examination of stool samples of our participants without stain showed that

the prevalence of helminthes *Ascaris lumbricoides* and *Entrobius vermicularis* were found to be around 0.6% for each organism. Table 3.4.

Table 3.4: Prevalence of intestinal parasites diagnosed by microscopy.

Parasite	Positive		Negative	
	No.	%	No.	%
E. histolytica	40	13.3	238	79.3
G. lamblia	10	3.3		
Cryptosporidium	8	2.6		
A.lumbricoides	2	0.66		
E. vermicularis	2	0.66		
Total	62	20.6%		

3.4 Distribution of protozoa parasites among resident areas:

The protozoan parasites distribution among the selected areas was found to be highest rate in Nablus region with 9.6% (29/300). Tulkarm district was the second with parasitic infection among study sample 6.3% (19/ 300). Salfit region was found to have the lowest prevalence of 4% (12 out of 300 samples). Table (3.5)

Table (3.5): distribution of parasitic protozoa infection among selected regions

Result	Nablus		Tulkarm		Salfit	
	No.	%	No.	%	No.	%
Positive	29	9.6	19	6.3	12	4
Negative	151	50.3	66	22	23	7.6

3.5 DNA extraction and PCR Results:

Extracted DNA from the 300 samples were run on photometer to check for DNA integrity and resulted in bands as shown in (figure 3.2).

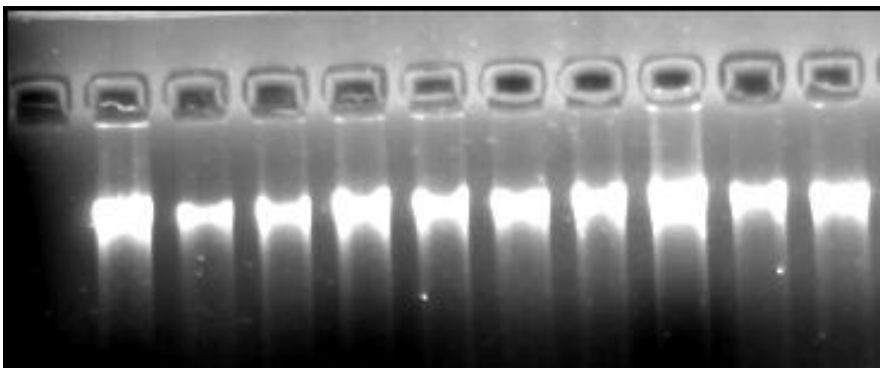


Figure (3.2): Genomic DNA extracted from 150 mg stool samples according to manufacture instructions (Qiagen, Germany).

DNA Samples were analyzed primarily with DNA probes specific for *Cryptosporidium spp*. Analysis of stool samples based on molecular technique (PCR) found 2 positive samples with cryptosporidium not detected by microscopic examination.

Using primers specific to *Cryptosporidium spp*. where an 835-bp fragment was amplified by PCR technique and by using *VsPI* enzyme 550 bp fragments was resulted, which confirm the presence of *Cryptosporidium* DNA. Figure 3.3.

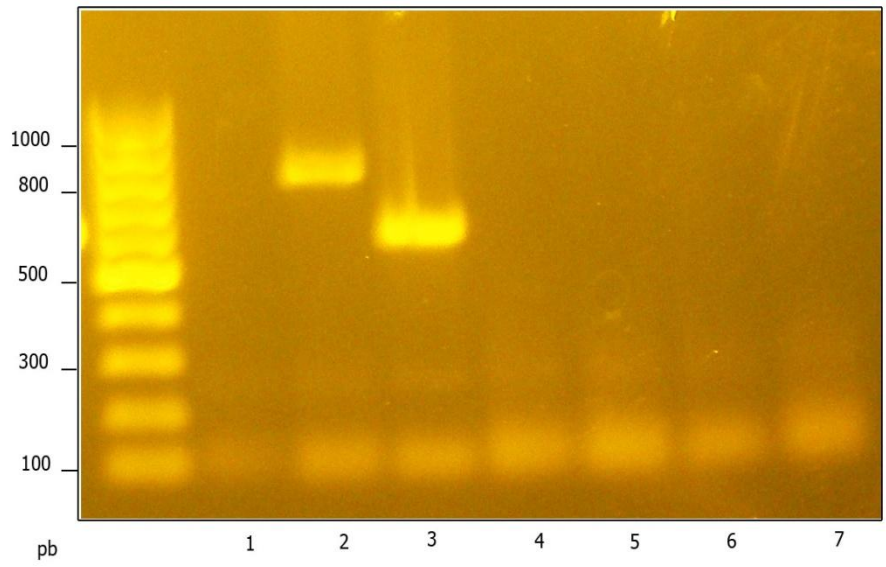


Figure (3.3) Detection of Cryptosporidium by PCR amplification. Lane 1, negative control (No DNA). Lane 2, DNA template from patient with cryptosporidium. Lane 3, DNA template from patient with cryptosporidium digested with V*sPI*. Lanes 4-7, DNA template from healthy individuals (No cryptosporidium with malachite green). To the left of the lanes is the 100-bp ladder.

Genotyping of *Cryptosporidium* spp. by PCR was based on the gp60 locus. Two sets of PCR were used to detect *C. hominis* and *C. parvum* spp. The results showed the presence of *C. hominis* spp. in 6 samples, and *C. parvum* in 4 samples. The most prevalent family of *C. hominis* spp. was I b in 5 samples and 1 sample of Id family. For *C. parvum*, 2 samples carried the genes for II a family and 2 samples for II d family. (Table 3.6)

Table (3.6): *Cryptosporidium* genotypes according to PCR and genotyping.

Species	GP60		No. of subtypes
	Family	Subtype	
C. hominis	**I b**	**I bA6G3**	**2**
	I b	**IbA9G3**	**2**
	I b	**I bA20G2**	**1**
	I d	**I dA21**	**1**
C. parvum	**II a**	**IIaA15G1R1**	**2**
	II d	**II da2OG1**	**2**

DNA sequencing showed a presence of 5 subtypes of Ib family and 1 subtype of Id family related to species *C. hominis*, also 2 samples belong to IIa family and 2 belong to IId family of C. parvum species were recognized.

PCR is the best diagnostic technique for Cryptosporidium since that microscopy is not efficient in all cases of Cryptosporidium infection especially if the oocysts are rare in stool sample but the parasite still infectious.

Chapter four

Discussion

4.1 Discussion:

The burden of parasitic diseases is still alarming world-wide particularly among children. Death caused by parasitic diseases is ranked as second among all cases of deaths especially in the developing countries. Interest in infection by cryptosporidiosis is relatively new. This is because the increasing number of immunocompromised patients.

This study showed high prevalence of *E. histolytica* among other protozoan parasites including *G. lamblia* and Cryptosporidium spp. This finding is similar to reports from previous studies from different regions.

Our finding is in agreement with results reported previously [34] which found a high prevalence of *E. histolytica* (71/735) where it studied the prevalence of protozoan parasites in North west Bank among healthy school children. Similar results were reported also from UAE where *E. histolytica* showed high prevalence (71.8%) compared with *G. lamblia* (17.5%)[42]. And it was similar to results from Iraq and Jordan where *E. histolytica* showed 9.80%, 94.17 % respectively [39, 40].

Washing hands after using bathroom and presence of animals near home had a significant difference with infection of protozoa another study in Ethiopia showed 91.9% of study population positive for intestinal

protozoa belongs to participants who had no washing facilities [71] and as other study in Palestine showed significant difference between infection and washing hands [34].

Prevalence of parasitic protozoa didn't affect with sex difference and this result is also showed in other parts of world, in Iran, Egypt, Ethiopia, Kuwait, Turkey, and Jordan [41, 61, 71, 44, and 39].

The most affected age group was (0-5 year) which shows highest prevalence in this study (51/300), this age group was also affected in other previous studies such as in Abu-Alrub study where highest prevalence of *Cryptosporidium* was in ages (1 day-5years) [51], and in Cairo where the highest rate of infection was 88.8% for the age group between (1-2 years) [57]. And 14.4% prevalence of *Cryptosporidium* in the age group less than 5 years in another study in Palestine [34].

WHO reported 1-4% of *Cryptosporidium* in Europe and North America, and 3-20% in Africa, Asia, Australia, and South America [72]. This study showed cryptosporidium prevalence equals 3.3% which resemble the world prevalence.

In other countries and cities such as India, Iraq, Pakistan, Irbid-Jordan, Gaza, and Bethlehem the prevalence of *Cryptosporidium* was (7.3%, 8.8%, 10.3%, 1.5%, 14.6%, and 13.5%) respectively [51].

The difference between prevalence in this study and the other studies listed is related to differences in hygienic conditions between these regions; countries with high population intensity have poor hygienic conditions these countries have higher prevalence for *Cryptosporidium* compared with countries with low population intensity such as region included in this study.

Of *Cryptosporidium* spp., *C. hominis* was the most common followed by *C. parvum*, this result also found in other parts of the world. In Peru a study found 70% *C. hominis* prevalence compared with 13% for *C. parvum*[70], in England and Wales also *C. hominis* had highest prevalence (50.29%) compared with *C. parvum* (45.6%).

4.2 Conclusion:

Our study showed that *E. histolytica* is the most common intestinal parasitic protozoan (prevalence 13 %), followed by *G. lamblia* and *Cryptosporidium* (prevalence 3.3 %). The majority of infections were detected in children less than 2 years old. Two species of *Cryptosporidium* were found in this study; *C. hominis* and *C.parvum*. *C.homins* was more prevalent than *C.parvum*.

4.3 Recommendations:

The prevalence of intestinal parasitic protozoa can be reduced by different way depended on the factors studied in this study. To minimize rate of intestinal protozoan infection we can educate mothers and children to avoid direct contact to animals. Also washing child hands and helping mother to increase the hygiene conditions at home play an important role to decrease the infections.

Further work is still needed to identify risk factors. In particular sampling of domestic animals and subtyping of C. parvum from these animals is needed to determine the extent of zoonotic transmission of Cryptosporidium in children.

4.4 Limitation of the study:

A limitation of the study was the relatively large sample size whereas a lot of intestinal pain patients don't attend health centers, self healing infections may decrease the real prevalence of parasites calculated by this study.

Also this prevalence is only for patients with healthy immune system, the prevalence is higher in immunocompromised people.

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Appendix:

English questionnaire:

Serial number :			
Name:			
Sex:			
Age:			
Residence:	☐ City:	☐ Village:	☐ Camp:
Level of education of father :	☐ Less than Tawjihi. ☐ Tawjihi. ☐ Diploma. ☐ Bachelor's degree. ☐ Higher education.		
Level of education of mother :	☐ Less than Tawjihi. ☐ Tawjihi. ☐ Diploma. ☐ Bachelor's degree. ☐ Higher education.		
How many members in the family?			
Income of the family?			
Are there animals near home?	☐ Yes	☐ No	
Source of drinking water?	☐ Tap water. ☐ Well water. ☐ Filtered water. ☐ Spring water.		
Does your child clean his hands before eating?	☐ Yes.	☐ No.	
Does your child clean his hands after going to W.C?	☐ Yes.	☐ No.	
Has this child suffered diarrhea recently?	☐ Yes.	☐ No.	
Has this child suffered from parasites before?	☐ Yes.	☐ No.	

If yes, which type?				
Does any family member suffer of intestinal parasite?	☐	Yes.	☐	No.
If yes, which type?				
Do you clean vegetables and fruits before eating?	☐	Yes.	☐	No.
Does your child play with pets?	☐	Yes.	☐	No.

Arabic questionnaire:

الرقم المتسلسل:			
الإسم:			
الجنس:			
العمر:			
مكان الإقامة:	☐مدينة	☐ قرية	☐مخيّــم
المستوى التعليمي للأب:	☐أقل من توجيهي ☐دبلوم ☐دراسات عليا	☐توجيهي ☐بكالوريوس	
المستوى التعليمي للأم :	☐أقل من توجيهي ☐دبلوم ☐دراسات عليا	☐توجيهي ☐بكالوريوس	
عدد أفراد الأســرة :			
دخل الأســرة :			
هل يوجد حيوانات قريبة من مكان سكنكم؟	☐ نعم	☐ لا	
ما هو مصدرمياه الشرب؟	☐ماء حنفيّة ☐ ماء مفلتر	☐ ماء من البئر ☐ ماء ينبوع	
هل يغسل طفلك يديه قبل الاكل ؟	☐نعم	☐ لا	
هل يغسل طفلك يديه بعد الخروج من الحمّام ؟	☐ نعم	☐ لا	
هل أصيب طفلك بالإسهال مؤخراً ؟	☐نعم	☐لا	
هل سبق وان أصيب طفلك بأحد الطفيليات ؟	☐ نعم	☐ لا	

إذا كان الجواب نعم ، ما هي ؟	
هل سبق وأن أصيب أحد أفراد الأسرة بالطفيليات المعويّة ؟	☐ نعم ☐ لا
إذا كان الجواب نعم ، ما هي ؟	
هل تغسلين الخضروات والفواكه قبل الأكل؟	☐ نعم ☐ لا
هل يلعب طفلك مع الحيوانات الأليفه ؟	☐ نعم ☐ لا

62

CONSENT FORM FOR RESEARCH STUDY

Title of Project: Intestinal Protozoa and cryptosporidium genotypes in Children with Diarrhea in Northern Palestine

Name of Researcher: Maisaa Mohammad Mahmoud Yacoub

	Please tick To confirm
❖ I confirm that I understood understand the information for the above study.	☐
❖ I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
❖ I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	☐
❖ I agree to my GP being informed of my participation in the study.	☐
❖ I agree to take part in the above research study.	☐

Please Note:
Signing your name means you agree to be in this study and that you were given a copy of this consent form.

Name of Patient	**Date**	**Signature**
Name of Person taking consent (if different from researcher)	**Date**	**Signature**
Researcher	**Date**	**Signature**

When complete, 1 copy for patient, 1 copy for researcher site file

An-Najah
National University
Faculty of Medicine

بسم الله الرحمن الرحيم

جامعة
النجاح الوطنية
كلية الطب

IRB Approval letter

Study title:
Genotyping of Cryptosporidium Species in North of West Bank / Palestine

Submitted by:
Maisaa Mohammad Mahmoud Yacoub

Date Reviewed:
June 21, 2012

Date approved:
July 4, 2012

Your study titled " **Genotyping of Cryptosporidium Species in North of West Bank / Palestine**" Was reviewed by An-Najah National University IRB committee & approved on July 4, 2012

IRB

Samir Musmar,MD, FAAFP
IRB Committee Chairman,
An-Najah National University

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جامعة النجاح الوطنية
كلية الدراسات العليا

الأوليّات المعوية والتصنيف الجيني لطفيل الكريبتوسبوريديوم في شمال الضفة الغربية/ فلسطين

إعداد

ميساء محمد محمود يعقوب

إشراف

الدكتور أيمن حسين

قدمت هذه الأطروحة استكمالا لمتطلبات درجة الماجستير في العلوم الحياتية بكلية الدراسات العليا في جامعة النجاح الوطنية في نابلس، فلسطين.

2014

الأوليات المعوية والتصنيف الجيني لطفيل الكريبتوسبوريديوم في شمال الضفة الغربية/ فلسطين

إعداد

ميساء محمد محمود يعقوب

إشراف

الدكتور أيمن حسين

الملخّص

الهدف : هدفت هذه الدراسة إلى تحديد أكثر الأوليات المعوية إنتشاراً لدى الأطفال المصابين بالإسهال والقاطنين في مناطق شمال الضفة الغربية، بالإضافة إلى التصنيف الجيني لطفيل الكريبتوسبوريديوم.

إستراتيجيّات البحث وطرائقه : تم جمع 300 عينة براز من الأطفال الذين شملتهم الدراسة وقسمت كل عينة إلى نصفين تم حفظ أحدهما في مادة الفورمالين 10% من أجل التشخيص المجهري والصباغة ، وتم حفظ الجزء الآخر في كحول الإيثانول 95% من أجل عمليات إستخلاص المادة الوراثية (DNA) والتصنيف الجيني عن طريق عملية (PCR) .

النتائج: بعد إجراء الفحص المخبري بإستخدام المجهر والصبغات وجد أن 19.8% (60/58) من العينات كانت إيجابية للطفيليات المعوية ، وكانت نسب إنتشار الأميبا والجياردیا والكريبتوسبوريديوم بالترتيب كما الآتي : 69% (58/40) ، 17.2% (58/10) ، 13.8% (58/8) . كما تم الكشف عن عينتين إضافيتين من الكريبتوسبوريديوم بواسطة التشخيص الجيني.

تم إجراء عملية PCR لجميع عينات الكريبتوسبوريديوم ووجد أن ستة منها تنتمي للنوع *C.hominis* وأربعة منها تنتمي للنوع *C.parvum* ، كما تم إجراء عملية PCR أخرى لتحديد التصنيف الجيني للأنواع المذكورة كشفت عن 5 عينات من نوع *C.hominis* تنتمي للعائلة Ib

وعينة واحدة تنتمي للعائلة ld ، بينما كانت إثنتان من عينات النوع الآخر تنتمي للعائلة lld والأخرى لعائلة lla.

الإستنتاج: تدعم نتائج هذه الدراسة المعلومات المتوفرة مسبقاً عن الطفيليات المعوية الشائعة في مناطق شمال فلسطين ، كما أن تحديد أنواع طفيل الكريبتوسبوريديوم في هذه المنطقة يشكل مصدراً مهماً للمعلومات الطبية المتعلقة بإنتشار المرض مما يوفر فرصة للأطباء والأخصائيين لمنع إنتشار ها المرض والحد من العدوى بين الأطفال.