MOLECULAR EPIDEMIOLOGY OF *GIARDIA* AND *CRYPTOSPORIDIUM* AMONG OUTPATIENTS IN SANA'A CITY, YEMEN

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Throughout my four years pursuing my PhD, many changes have happened in my country and onto me. I cry when I see all the painful moments as my country journeys towards a better tomorrow. When I see the blood that watered the land, I cried. Yemen, our country, a land of wisdom, carrying the heaviest pain with a smile. In the faces of suffering children, its people are still optimistic. I believe there is still much hope to reach great future.

I dedicate this work first to my country and all the people in Yemen.

I also dedicate this work to my beloved parents who deserve all the love and respect.

ABSTRACT

Giardia and *Cryptosporidium* are two common causative agents of protozoan infections in developed and developing worlds. Currently, there is insufficient information on the molecular epidemiology of these protozoa in Yemen. This study was conducted to identify the molecular epidemiology of these two protozoan parasites by evaluating the risk factors that contribute towards understanding the possible role of transmission of these protozoa among Yemeni patients. Samples were collected from September 2008 to March 2009. All information were gathered using a standard pre-tested questionnaire; permissions were obtained from the heads of every hospital and patients were randomly and voluntarily selected. In case of children, permissions were obtained from parents. On the same day of collection, parasitic identification was done directly by microscopy after formal ether concentration technique. The modified Ziehl-Neelsen stain was used to identify oocyst of *Cryptosporidium*. A total of 503 samples was collected, all samples were preserved for molecular genotype using potassium dichromate. The microscopy work was carried out in Yemen, while the molecular genotyping was done at Department of Parasitology, Faculty of Medicine, University of Malaya, Malaysia.

The overall prevalence of intestinal protozoan infections was 30.9%. Infection rates of *Giardia duodenalis*, *Entamoeba histolytica/dispar* and *Cryptosporidium* were 17.7%, 17.1% and 1%, respectively. Other parasites detected included *Ascaris lumbricoides* (2.4%), *Schistosoma mansoni* (0.3%), *Hymenolepis nana* (1.4%) and *Enterobius vermicularis* (0.4%). Multivariate analysis using forward stepwise logistic regression based on intestinal protozoan infections showed that contact with animals (OR = 1.748, 95% CI = 1.168–2.617) and taking bath less than twice a week (OR = 1.820, 95% CI = 1.192–2.779) were significant risk factors of protozoan infections. Molecular analysis of

Giardia targeted the genotyping of 16S rRNA gene and subgenotyping of *b*-giardin genes. For *Cryptosporidium,* the 18S rRNA and *gp60* genes were used for genotyping and subtyping respectively. Of the 89 microscopic positive *Giardia* samples, 65 were successfully sequenced, of which 43 (66%) were identified as *Giardia duodenalis* assemblage A and 22 (34%) as *G. duodenalis* assemblage B. The subtyping analysis based on *b*-giardin gene identified the presence of *G. duodenalis* assemblages A2, A3 and B3. Infections with assemblage A were significantly associated with animal contact ($P < 0.05$) and grass collection activity for animal feed ($P < 0.05$). For *Cryptosporidium* species, out of 335 samples that were randomly chosen for DNA extraction, 33 (9.9 %) were positive. Of these, 33 (96%) were identified as *C. parvum* whilst one case was caused by *C. hominis*. All seven *C. parvum* isolates subtype belonged to the IIaA15G2R1 subtype. The predominance of the zoonotic IIa subtype family of *C. parvum* highlights the likely occurrence of zoonotic transmission of cryptosporidiosis in Yemen. In conclusion, the present study on protozoan diseases indicated that low personal hygiene and contact with animals were important predictors for intestinal protozoan infections. Data of the study based on molecular work suggest that both anthroponotic and zoonotic transmissions play potential roles in the transmission of giardiasis in the community. However, this postulation needs confirmation with future molecular epidemiological studies in both humans and animals. The findings on risk factors combined with molecular genotyping will assist in developing effective control strategies in Yemen based on a better understanding of epidemiology of this parasite. The findings also warrant further genotyping studies among animals. In order to effectively reduce these infections, a multi-sectoral effort is needed including preventive measures such as good hygienic and animal husbandry practices, heightened provision of educational health programs and health services in all

governorates including rural areas. Furthermore, it is also essential to find radical solutions to the recent water crises in Yemen.

ABSTRAK

Giardia dan *Cryptosporidium* adalah dua agen lazim penyebab jangkitan protozoa di dunia maju dan membangun. Pada masa ini, terdapat maklumat yang tidak mencukupi mengenai epidemiologi molekul kedua-dua protozoa ini di Yemen. Kajian ini dijalankan untuk mengenalpasti epidemiologi molekul kedua-dua penyakit protozoa dengan menilai faktor risiko yang menyumbang terhadap pemahaman mengenai penularan protozoa ini di kalangan pesakit di Yemen. Sampel telah dikumpulkan dari September 2008 hingga Mac 2009. Dalam kajian ini, semua maklumat dikumpulkan menggunakan soal selidik pra-uji berpiawai. Kebenaran telah diperolehi daripada ketua setiap hospital dan semua pesakit yang telah dipilih secara rawak dan secara sukarela. Dalam kes kanak-kanak, kebenaran telah diperolehi daripada ibubapa. Pada hari yang sama pengumpulan sampel, pengenalpastian parasit telah dilakukan secara langsung dengan mikroskopi selepas teknik kepekatan formol eter. Teknik Ziehl Neelsen yang dimodifikasikan telah digunakan untuk mengenalpasti oosista *Cryptosporidium*. Sebanyak 503 sampel telah dikumpulkan, dan semua sampel diawetkan bagi kerja molekul menggunakan kalium dikromat. Kerja mikroskopi telah dilakukan di Yemen, manakala kerja-kerja molekul telah dilakukan di Jabatan Parasitologi, Fakulti Perubatan, Universiti Malaya, Malaysia. Prevalens jangkitan protozoa usus secara keseluruhan adalah 30.9%. Kadar jangkitan *Giardia duodenalis*, *Entamoeba histolytica/dispar* dan *Cryptosporidium* adalah masing-masing 17.7%, 17.1% dan 1%. Parasit lain yang dikesan termasuk *Ascaris lumbricoides* (2.4%), *Schistosoma mansoni* (0.3%), *Hymenolepis nana* (1.4%) dan *Enterobius vermicularis* (0.4%). Analisis multivariat menggunakan regresi ke hadapan langkah demi langkah logistik berdasarkan jangkitan protozoa usus menunjukkan bahawa hubungan dengan haiwan (OR = 1,748, 95% CI = 1,168-2,617) dan mandi kurang daripada dua kali seminggu (OR = 1,820,

95% CI =1,192-2,779) adalah faktor risiko yang ketara bagi jangkitan protozoa. Analisis molekul *Giardia* telah mensasarkan genotiping gen rRNA 16S dan subgenotiping gen *b*-giardin. Bagi *Cryptosporidium,* 18S rRNA dan gp60 masing-masing telah digunakan untuk genotiping dan subgenotiping. Daripada 89 sampel mikroskopik yang positif bagi *Giardia,* 65 sampel berjaya dijujukkan, yang mana 43 (66%) telah dikenalpasti sebagai *Giardia duodenalis* perkumpulan A dan 22 (34%) sebagai *Giardia duodenalis* perkumpulan B. Analisis subtip berdasarkan gen *b*-giardin mengenalpastikan kehadiran *G. duodenalis* perkumpulan A2, A3 dan B3. Jangkitan dengan perkumpulan A adalah ketara dan dikaitkan dengan sentuhan haiwan (P <0.05) dan aktiviti pengumpulan rumput untuk makanan haiwan (P <0.05). Bagi spesies *Cryptosporidium,* daripada 335 sampel yang dipilih secara rawak untuk pengekstrakan DNA, 33 (9.9%) adalah positif bagi *Cryptosporidium*. Daripada jumlah tersebut, 33 (96%) telah dikenalpasti sebagai *Cryptosporidium parvum* manakala satu kes disebabkan oleh *Cryptosporidium hominis*. Kesemua tujuh pencilan subtip *C. parvum* adalah milik subtip IIaA15G2R1. Predominasi famili subtip zoonotik IIa *C. parvum* membuktikan kemungkinan berlakunya jangkitan kriptosporidiosis zoonotik di Yemen. Kesimpulannya, kajian kehadiran penyakit protozoa menunjukkan bahawa tahap kebersihan diri yang rendah dan hubungan dengan haiwan merupakan ramalan penting bagi jangkitan protozoa usus. Data kajian berdasarkan kerja molekul mencadangkan bahawa kedua-dua penularan antroponotik dan zoonotik memainkan peranan yang berpotensi dalam penularan giardiasis dalam masyarakat. Bagi *Cryptosporidium,* predominasi famili subtip zoonotik IIa *C. parvum* membuktikan kemungkinan berlakunya jangkitan kriptosporidiosis zoonotik di Yemen. Walau bagaimanapun, penganggapan ini memerlukan pengesahan lanjutan dengan kajian epidemiologi molekul kriptosporidiosis dan giardiasis dalam kedua-dua golongan manusia dan

haiwan di Yemen. Penemuan sekarang yang mengabungkan faktor risiko dengan pencirian molekul akan membantu dalam membangunkan strategi kawalan yang berkesan di Yemen berdasarkan pemahaman yang lebih baik tentang epidemiologi parasit ini. Hasil kajian juga membuktikan perlunya pengajian genotip selanjutnya di kalangan haiwan seperti yang ditonjolkan dalam kajian ini selain kajian genotip antara manusia dan haiwan yang ada hubungan rapat. Untuk mengurangkan jangkitan ini secara efektif, usaha pelbagai sektor diperlukan termasuk langkah-langkah pencegahan seperti amalan kebersihan yang baik, amalan penternakan yang baik, peruntukan meningkatkan program pendidikan kesihatan dan perkhidmatan kesihatan di semua wilayah termasuk kawasan luar bandar. Selain itu, adalah juga penting untuk mencari penyelesaian radikal kepada krisis air di Yemen yang berlaku kebelakangan ini.

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LIST OF ABBREVIATIONS

%	Percentage
µl	Microliter
uM	Micromolar
AIDS	Acquired Immunodeficiency Syndrome
Bp	Base pair
G	Gram
HIV	Human Immunodeficiency Virus
Ml	Milliliter
mM	Millimolar
NJ	Neighbor-Joining
^{o}C	Degree Celcius
P value	Level of significance
RPM	Revolution per minute
UV	Ultraviolet
pp	Posterior probabilities

CHAPTER I

INTRODUCTION

1.1 GENERAL INTRODUCTION

Giardia and *Cryptosporidium* are significant causes of diseases and morbidity in humans and mammals. These parasites are significant causes of diarrhea and nutritional disorders in institutional and community settings, affecting growth and cognitive functions of infected individuals and may cause production losses in livestock. The main impact of these diseases is found in developing countries, including Yemen. Transmission of giardiasis and cryptosporidiosis is typically associated with poor faecal-oral hygiene. Both parasites are included in the WHO Neglected Diseases Initiative because they impair the ability to achieve full potential, development and socio-economic improvements, and they have a common link with poverty (Savioli *et al*., 2006).

The real importance of these protozoan infections comes from the broad global deployment and the morbidity that affect the health of the community in addition to at least 325 water-associated outbreaks of parasitic protozoan diseases that have been reported. North American and European outbreaks accounted for 93% of all reports and nearly two-thirds of outbreaks occurred in North America. Over 30% of all outbreaks were documented from Europe, with the UK accounting for 24% of outbreaks, worldwide. *Giardia duodenalis* and *Cryptosporidium parvum* account for the majority

of outbreaks (132; 40.6% and 165; 50.8%, respectively), *Entamoeba histolytica* has been the aetiological agents in nine (2.8%) outbreaks (Karanis *et al*., 2007).

Giardia duodenalis is a flagellated protozoa infecting humans and a wide range of animals worldwide with higher prevalence in developing countries (Islam and Meyer, 1990). Although 200 million individuals acquire symptomatic giardiasis every year, the majority of human giardiasis are asymptomatic (WHO, 1996a). Chronic giardiasis may cause malnutrition, growth retardation and impairment in cognitive functions in early childhood (Ortega and Adam, 1997).

Genotyping studies have shown that *G. duodenalis* is a complex species comprised of at least eight assemblages (i.e., A-H), with the latest addition of assemblage H, which was recently reported in marine vertebrates (Lasek-Nesselquist *et al*., 2010). Among them, assemblage A infects humans and animals and has been considered the most potential zoonotic assemblage (Thompson *et al*., 2000) with reported occurrence in farm and companion animals (Feng and Xiao, 2011). In endemic areas, where humans and animals live in close contact, transmission from humans to animals or vice versa has been suggested (Traub *et al*., 2004). One such group of animals are lambs which have been found to be frequently infected by zoonotic potential assemblages (Lebbad *et al*., 2010, Robertson, 2009). Although assemblage B tends to infect humans (Caccio *et al*., 2005), it has also been reported in animals to a lesser extent (Thompson, 2004). Thus far, assemblages C-H are by and large host specific (Monis *et al*., 2003a).

More recent utilization of typing tools based on *b*-giardin has sub-divided assemblage A into subtypes A1 to A8. Of these, A1, A3 and A4 have been implicated in zoonotic transmission (Lalle *et al*., 2005b, Trout *et al*., 2003). Within assemblage B,

subtypes B1, B3, B4, B5 and B6 have been identified, with only assemblage B3 being detected in both humans and animals (Lalle *et al*., 2005b). Several studies failed to find a correlation between *G. duodenalis* assemblages with clinical symptoms, but the issue remain unresolved (Mahdy *et al*., 2009).

Cryptosporidium is a protozoan parasite in humans and animals and has a worldwide distribution. *Cryptosporidium* infection has been implicated as a cause of diarrhea in multiple waterborne and foodborne outbreaks (Fayer *et al*., 2000, Slifko *et al*., 2000, Mac Kenzie *et al*., 1994). Humans can also acquire infection through direct contact with humans (anthroponotic transmission) or animals (zoonotic transmission). Although cryptosporidiosis causes self-limiting diarrhea in immunocompetent persons, it may lead to life-threatening diarrhea in immunocompromised patients (DuPont *et al*., 1995, Kjos *et al*., 2005). The disease has also been considered as a marker of malnutrition in early childhood (Mak, 2004, Huang and White, 2006).

The genus *Cryptosporidium* is a multispecies complex. To date, approximately 26 species and more than 60 genotypes have been identified (Fayer, 2010a, Traversa, 2010, Fayer and Santin, 2009). Most of the species or genotypes infect a particular host with a few notable exceptions. *Cryptosporidium parvum* infects human and several species of animals, and is the major species responsible for zoonotic cryptosporidiosis in many developed countries. Another species, *C. hominis,* commonly infects humans, thus is responsible for anthroponotic transmission, with a high prevalence in developing countries compared to industrial nations (Raccurt, 2007, Xiao, 2009).

Subtyping of *C. parvum* based on the 60 kDa glycoprotein (*gp60*) gene identified at least 12 subtype families (IIa – IIl). The zoonotic IIa subtype is predominant in developed nations and is rarely isolated from humans in developing

nations where the anthroponotic IIc subtype family is the most common cause of human *C. parvum* infections. The *C. parvum* IIa subtype family has a high genetic diversity with IIaA15G2R1 being predominant in human and calves. Other subtypes are less common (Xiao, 2009, Xiao, 2010).

The epidemiology of *Cryptosporidium* in Middle Eastern countries is different from other developing countries as earlier studies reported that *C. parvum* had higher prevalence than *C. hominis* in Saudi Arabia (Al-Brikan *et al*., 2008), Kuwait (Sulaiman *et al*., 2005), Jordan (Hijjawi *et al*., 2010), Turkey (Tamer *et al*., 2007), and Iran (Nazemalhosseini-Mojarad *et al*., 2011). However, in Egypt, *C. hominis* was more prevalent than *C. parvum* (Abd El Kader *et al*., 2011, Eida *et al*., 2009). Subtype analysis based on *gp60* has shown that the IId subtype family is the predominant subtype in Middle Eastern countries whereas the IIc subtype family is the dominant *C. parvum* in developing countries (Xiao, 2010).

The focus of the present study is in Yemen, a developing Middle Eastern country located at the southern part of the Arabian Peninsula with a total population of 23 million. The country depends totally on ground and rain water as a source of water. Recently, the country has fallen into a deep water crisis characterized by very rapid mining of groundwater, extreme water supply shortages in the major cities, and limited access of the population to safe drinking water. WHO reported that only 25% of the population had easy access to safe water (WHO, 2009). Being one of the poorest countries in the Middle East with per capita income of approximately USD510, approximately 42% of Yemen's total population is estimated to be under the national poverty line (World Bank, 2004). The poverty ratio is higher in rural areas where 75% of population lives and only 25% is covered with health care services compared to 80%

of urban areas. This economic imbalance coupled with the current water scarcity have also encouraged or sustained the high prevalence of intestinal protozoan infections in Yemen.

Although intestinal parasitic infections have received attention in Yemen as early as 1950s, most of these efforts were to combat schistosomiasis, unfortunately neglecting the other intestinal parasites (Kuntz *et al*., 1953, Hazza *et al*., 1983). In 1985, a large scale survey by Farag between 1982-1983 involving 37,000 stool specimens showed that 53% of the study population had intestinal parasitic infections with *Trichuris trichiura*, *Ascaris lumbricoides* and *Giardia duodenalis* being at high prevalence (Farag, 1985). In 2000, Raja and his colleagues carried out another survey in seven villages in the rural areas of Ibb governorate. In this study, the prevalence of *G. duodenalis* and *E. histolytica/dispar* was 18% and 14%, respectively (Raja A *et al*., 2000). A recent study carried out on 303 restaurant workers in 58 restaurants in Sana'a City reported high prevalence of *G. duodenalis* (35.3 %) and *E. histolytica/dispar* (48.9%) (Al-shibani., *et al*., 2009a). The infection rates of *G. duodenalis* and *E. histolytica/dispar* were also reported at 18.2% and 52%, respectively, in a similar study conducted on 132 restaurant workers in Almukala City, Hadramowat (Baswaid and Al-Haddad, 2008). The high prevalence of these two intestinal protozoa among restaurant workers is alarming with the possible risk of foodborne outbreak.

Hitherto several studies have focused on parasitic infections among children in Yemen. A cross-sectional study, carried out on 104 children from lowland and highland areas in the south of Yemen showed that the prevalence of *G. duodenalis* in the lowland and highland areas were 34.6% and 35.1%, respectively. Children living in the lowland areas had higher infection rate of *E. histolytica/dispar* (42.3%) compared to those living

in the highland areas (36.8%) (Kopeck *et al*., 1992). In a hospitalized based study carried out on 9,014 children in the pediatric health center in Sana'a City, the prevalence of *G. duodenalis* and *E. histolytica/dispar* was 16.7% and 11.7%, respectively (Azazy and Raja'a, 2003). A recent study which is the first among children in orphanages reported high prevalence of *G. duodenalis* (26.8 %), *E. histolytica/dispar* (13%) and *Cryptosporidium* (24%) (Al-Shibani *et al*., 2009b). Another study conducted in Hadramowat in 2010 on children recorded a prevalence of 19.2% and 16.8% of *Giardia* and *E. histolytica/dispar* infections, respectively. This study highlighted that parasitosis reflected the prevalent hygienic problems and their influences on public health of Hadramowat (Al-Haddad and Baswaid, 2010).

What was alarming in Yemen was that most studies in different localities and different populations have shown high prevalence of intestinal protozoan infections especially with *G. duodenalis*, *E. histolytica/dispar* and *Cryptosporidium* infections and these rates have not indicated any sustainable reduction since the 1980s. Besides the high rates of infections, not much is known about factors contributing to the high prevalence. It should be noted that self-sufficient animal husbandry is a common practice especially in rural and suburban areas of Yemen. Yemeni depend on animals for milk, meat and cash, and the farmers usually keep their animals inside their houses on the ground floor or in small rooms located in the courtyard of the houses. These close contacts with animals increase the opportunity of zoonotic transmission of giardiasis and cryptosporidiosis, which may contribute to the high prevalence of *Giardia* and *Cryptosporidium* infections in the country. However, to date, there has been no genotyping study to provide support for this postulated association.

Thus, this study was aimed at determining the prevalence and factors associated with the high prevalence of intestinal protozoan infections in Yemen and to identify species/genotypes and sub-genotypes of *G. duodenalis* and *Cryptosporidium*. This study will assist in understanding the mode of transmission as well as to accommodate human health practices, which opposes the transmission of protozoan infections. Findings generated from this study will improve our understanding of molecular epidemiology of giardiasis and cryptosporidiosis in Yemen.

1.2 GENERAL OBJECTIVE

To identify species and genotypes of *G. duodenalis* and *Cryptosporidium* isolated from faecal samples collected from patients attending hospitals in Sana'a, the capital city of Yemen.

1.3 SPECIFIC OBJECTIVES

1. To determine the prevalence and risk factors of intestinal protozoan infections in patients attending hospitals in Sana'a, the capital city of Yemen.
2. To identify *G. duodenalis* assemblages and subtypes based on 16S rRNA and *b*-giardin genes.
3. To determine factors associated with infections of *G. duodenalis* assemblages.
4. To identify *Cryptosporidium* species using 18S rRNA gene and subtyping of *C. parvum* and *C. hominis* based on *gp60* gene.
5. To determine factors associated with infections of *Cryptosporidium* species.

CHAPTER II

LITERATURE REVIEW

2.1 *GIARDIA DUODENALIS*

2.1.1 Introduction

The flagellate parasite *Giardia* is a common cause of diarrheal disease; it infects the small intestine of humans and almost all vertebrates worldwide. The transmission route of *Giardia* is by the ingestion of the environmentally resistant cyst. There are two ways of transmission, either direct (fecal-oral) or indirect through the ingestion of contaminated water or food. *Giardia* is considered the most frequent identified etiological agent in outbreaks through ingestion of contaminated surface water.

Giardiasis is an endemic disease common in developed and developing countries causing gastroenteritis. *Giardia* occurs frequently, especially in developing countries (Hellard *et al*., 2000). Clinical presentations of giardiasis range from an asymptomatic cyst passer to cases of diarrhea, which can be acute, chronic, or intermittent (Meyer and Jarroll, 1980). The course of severity of giardiasis depends on host factor (the immunity response) and the virulence of *Giardia* or a combination of both that is still unclear (Thompson, 2000). Giardiasis may result in malnutrition disorder and growth impairment in children (Gendrel *et al*., 2003).

Giardia duodenalis is cosmopolitan and the most frequent intestinal parasite among humans of developing countries. Approximately 200 million people in the world have clinically manifested giardiasis, with 500,000 new cases per year (WHO, 1996b). Studies in different European countries have indicated a prevalence of 1–17%, and up to 100% of the population can be infected in certain highly endemic areas (Plutzer *et al*., 2010). However, *G. duodenalis* has been considered a re-emerging infectious pathogen causing foodborne, waterborne and also diarrhea outbreaks in day-care centers in addition to the high levels of infection seen among domestic animals around the world (Thompson, 2000, Feng and Xiao, 2011).

Genotype analysis of *Giardia* has shown that *Giardia* comprises at least 8 assemblages, A to H. *G. duodenalis* is the only species which has been reported to be able to infect human, although it is also found in other mammals (Thompson *et al*., 2000). Fundamental evidence suggests *G. duodenalis* has a species complexity that cannot be morphologically distinguished but can be genetically differentiated into several major assemblages: Assemblages A and B mainly infect humans but are also found in a wide range of other mammals; C and D have been found in dogs; E has been isolated from livestock (cattle, sheep, and pigs); F and G have been reported from felines and rats, respectively (Monis *et al*., 2003b). More recently, assemblage H has been detected in marine vertebrates (Lasek-Nesselquist *et al*., 2010).

2.1.2 Morphology

Giardia is a unique and ancient eukaryotic single celled organism and seems to share the characteristics of anaerobic prokaryotes. *Giardia* does not have subcellular organelles such as mitochondria, peroxisomes, and apparently Golgi apparatus which are main features of eukaryotic organisms. To compensate for the lack of eukaryotic Golgi apparatus during encystation, there is a developmentally regulated formation of a large secretory compartment containing cyst-wall material. Despite the lack of any morphological similarities, these encystation-specific vesicles (ESVs) show several biochemical characteristics of maturing Golgi cisternae (Marti *et al*., 2003).

A. Trophozoite

The trophozoite is pear-shaped with a broad rounded anterior end and a tapering posterior end. The trophozoite measures about 12 to 15 µm long, 5 to 9 µm wide and 2 to 4 µm thick. The dorsal surface is convex while the ventral surface is concave. The organism is bilaterally symmetrical with a large sucking or adhesive disc on the ventral side. It occupies most of the anterior region of the ventral surface. There are two large anterior vesicular nuclei. Four pairs of flagella are represented. The first pair of flagella emerges anteriorly between the cell edge and dorsal surface, the second or lateral pair is located in the posterio-laterally from a groove between the margin and the dorsal surface, the third or the ventral pair from a groove posterior to the adhesive disc and the fourth pair is caudally from the tapered end. The flagella which run backwards through the middle of the body have been referred to as the median rods or axonemes. Two median bodies composed of bundles of microtubules arranged irregularly in the shape of the claws of a claw- hammer are present. These disappear in

the course of division and it has been suggested to have something to do with the formation of a new adhesive disc. The shape of the median body is used to divide *Giardia* into groups. This is the early description of the trophozoite by (Filice, 1952). The trophozoite has also two nuclei without nucleoli which are located interiorly and symmetrically to the long axis (Figure 2.1). The nuclei contain the same number of gene and the same amount of DNA (Kabnick and Peattie, 1990). Trophozoites inhibit the small intestine of their host using the ventral adhesive disc to attach to the wall of the small intestine. The trophozoites usually encyst before they leave the jejunum. It is not known whether the factors that initiate the encystation are intrinsic or environmently dependent such as changes in the intestinal pH. Most probably, it is a combination of both. The living trophozoite moves in a very characteristic way, like a falling leaf, and this feature is used as a diagnostic criterion to discriminate *Giardia* trophozoites from other protozoan flagellates in fresh stool samples. Encystation into new cysts takes place in the lower part of the small intestine, and the excreted cysts, which are immediately infectious, are also resistant to environmental factors and can survive for a long time under favorable conditions (Ankarklev *et al*., 2010).

B. Cyst

The cysts are ovoid to ellipsoidal and slightly asymmetric, measuring about 5 x 7 - 10µm in diameter and the wall is about 0.3-0.5 µm in thickness. The cysts from the same host often show variations in size and shape. The cysts have four nuclei when mature and a variable number of fibrillar remnants of the organelles of the trophozoite. The cyst wall is smooth without any characteristic markings. The wall composed of an outer filamentous layer and an inner membranous layer with two membranes. Cysts

from different hosts appear to be morphologically indistinguishable (Tombes *et al*., 1979).

2.1.3 Life cycle

The life cycle of *Giardia* is includs excystation and encystations stages (Feely *et al*., 1990, Meyer *et al*., 1994). In the encystation step, the parasite is shed with the faeces as an environmentally resistant cyst (infective stage). When transmission to a new host takes place then the excystation step starts in the duodenum of the new host. Due to the acidic environment, the trophozoite emerges from the cyst and undergoes a mitotic division. One or two pairs of preventral flanges develop to ventral flange (Hetsko *et al*., 1998). The peritrophic space and the preventral flange enlarge as the emerging trophozoite separate from the cyst wall. The trophozoite plays major roles in the attachment process on the epithelial cells of the host that is by means of an adhesive disc which then feeds on the epithelial cells. The trophozoites (the pathogenic stage) detach from the epithelial cells, probably because of the rapid turnover (72 hours) of these cells, and undergo mitotic division in the intestinal lumen. During periods of diarrhea, these trophozoites may be transported with the intestinal contents and excreted, but do not survive long outside the host. Some of the trophozoites encyst during the passage through the intestine and leave the host with the faeces as cysts. In formed stools, cysts are encountered more often than trophozoites.

A. Excystation

Excystation occurs in the proximal small intestine through the acidic environment of the stomach. Excystation of *Giardia in vitro* can be induced by imitating the acidicity of the stomach. Studies have determined the optimum pH to be 1.3, 2.7 or 4.0 (Bingham and

Meyer, 1979, Boucher and Gillin, 1990). The processes of excystation begin within 5 to 10 minutes after being placed in conditions conductive to excystation. Flagellar motion begins and the posterior end of the trophozoite exits through a break in the cyst wall. Cytokinesis begins within 30 minutes, resulting in two binucleate trophozoites (Coggins and Schaefer III, 1986). During excystation, the vacuoles release their contents, leading to the speculation that enzymes from the vacuoles play a role. Small dense vesicles are seen on the surface of the trophozoite as they emerge from the cyst wall.

B. Encystation

Encystation is the process of cyst formation and consists of a series of coordinated events beginning with a response to external stimuli and ending with the formation of an infectious cyst (Gillin *et al*., 1996). *In vitro*, a convenient operational definition of cyst formation is when the encysting organisms become water resistant (Gillin *et al*., 1996, Adam, 1991). This period varies significantly among different *in vitro* encystation techniques and *Giardia* strains (Campbell and Faubert, 1994, Gillin *et al*., 1987, Kane *et al*., 1991) During formation of the cyst wall, trophozoites undergo important biochemical and morphological changes that can be divided into three stages (Lujan *et al*., 1997): (1) reception of the stimulus for encystation and the consequent activation of encystation-specific genes; (2) biogenesis of secretory organelles and the synthesis, packaging, transport and release of cyst wall materials; and (3) assembly of the extracellular cyst wall. The earliest and best-characterized *in vitro* method of inducing encystation and the formation of viable cysts was developed by Gillin and co-workers in 1987 (Gillin *et al*., 1987).

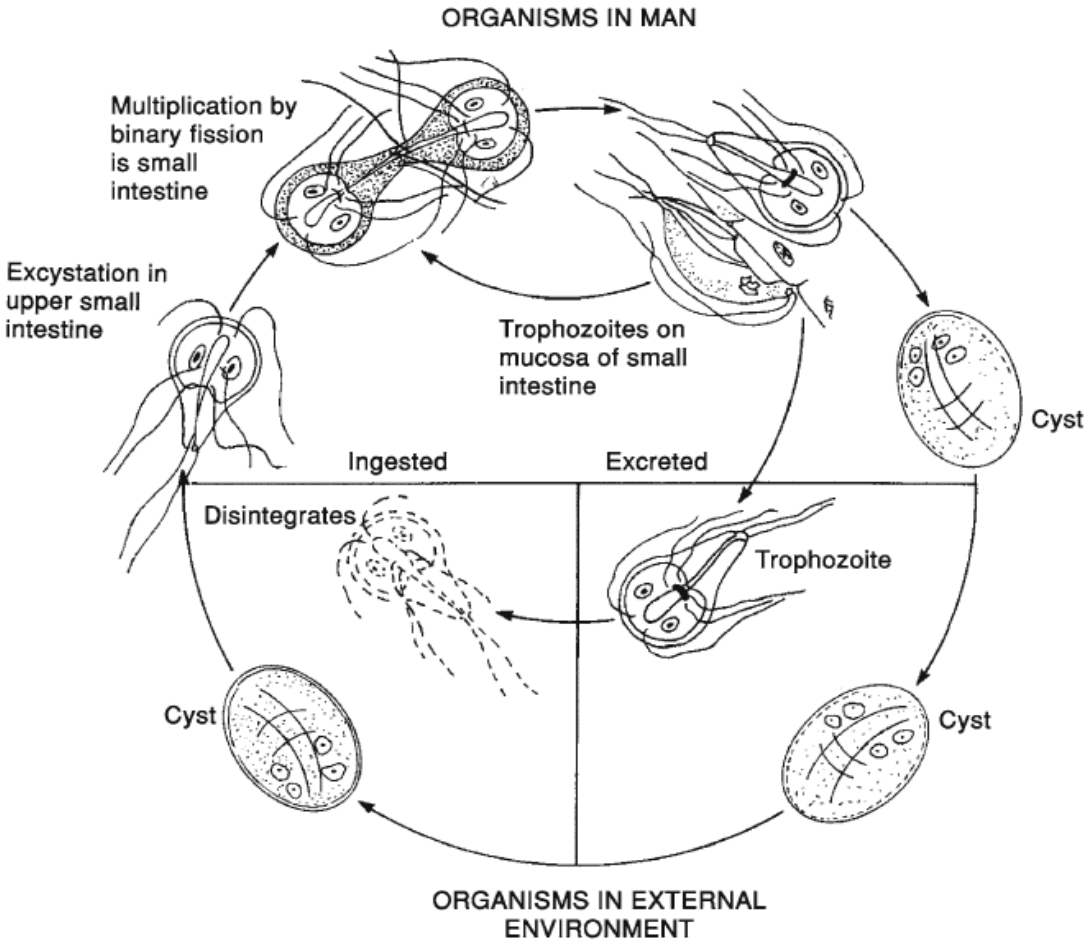


Figure 2.1 Life cycle of *Giardia duodenalis.* Source: Meyer (1980)

2.1.4 Taxonomy

Almost 330 years ago, Antony van Leeuwenhoek examined his own diarrheal stool under a single-lens microscope and was the first to detect a human infection with an intestinal protozoa, describing the organisms he observed as oval animalcules a little bit bigger than blood cells moving around (Dobell, 1920). In November 1681, van Leeuwenhoek sent a letter to the Royal Society in London to report his findings. In the book (Leeuwenhoek and His Little Animals), Dobell concluded that van Leeuwenhoek's description corresponded well with the vegetative (trophozoite) stage of *Giardia*. Lambl in 1859 described *Giardia* in greater detail. Lambl thought the organism belonged to the genus *Cercomonas* and named it *Cercomonas intestinalis* (Lambl, 1859). Thereafter, some have named the genus after him while others have named the species of the human form after him (i.e., *Giardia lamblia*). Grassi in 1879, named a rodent organism now known to be a *Giardia* species, *Dimorphus muris*, apparently not knowing of Lambl's earlier description. The first time *Giardia* was used as a genus name was in 1882 and 1883, Kunstler described an organism in tadpoles (*Giardia agilis*), that he named *Giardia*. In 1888, Blanchard suggested the name *Lamblia intestinalis* (Blanchard, 1888), which Stiles (1902) then changed to *Giardia duodenalis* in 1902. Subsequently, Kofoid and Christiansen proposed the name *G. lamblia* (Kofoid and Christiansen, 1915) and *G. enterica* (Kofoid, 1920).

Mayrhofer *et al.* (1995) defined a major lineage for human *Giardia* isolate and they assigned it to a separate species (*G. lamblia*) that is through analysis of human derived *Giardia*. The designated assemblages A and B, which include all the human isolates and they correspond, respectively, to groups I plus II and III plus IV of

Andrews *et al*. (1998) and Karanis and Ey (1998) to Polish and Belgian genotypes of Homan *et al,* (1992) and to group 1 plus 2 and group 3 of Nash and Keister (1985). Besides assemblages A and B, a number of other distinct assemblages (C–G) have also been identified within the *G. lamblia* morphological group, and each appears to be associated with a single species of mammalian host (Hunter and Thompson, 2005b, Caccio *et al*., 2005). Other non-human host with specific assemblages found in dogs, cats, rats, voles and muskrats are quite distinct from assemblages A and B. In contrast, the assemblages in hoofed livestock and cats are found to be closely related to isolates of the major assemblages identified (Thompson, 2000). The genetically distinct assemblages A-G are shown in (Table 2.1).

Assemblage A has been further classified into A1 which has been isolated from humans and animals (Mowatt *et al*., 1994, Trout *et al*., 2003, Amar *et al*., 2002), A2 has been isolated from humans (Monis *et al*., 1999), animals (Baruch *et al*., 1996, Thompson *et al*., 2000, Sulaiman *et al*., 2003) and water (Feng and Xiao, 2011). While A3 is found exclusively in animals (Cacci *et al*., 2008, Robertson *et al*., 2007b, van der Giessen *et al*., 2006a), *b*-giardin genotyping of assemblage A identified A1 to A6 isolated from humans and animals and A3 in *b*-giardin locus is found in humans (Lalle *et al*., 2005d, Lalle *et al*., 2005a), whilst assemblage B include genetically diverse group of predominantly human and animal isolates (Sulaiman *et al*., 2004, Monis *et al*., 1999, Sulaiman *et al*., 2003, Thompson *et al*., 2000).

Table 2.1 Genotypes of *Giardia duodenalis* groups. Modified from Caccio *et al*., (2005) and Feng and Xiao, (2011).

Genotype assemblages	**Host**
Giardia duodenalis	
Assemblage A	Human, live stock, wild ruminant
Assemblage B	Human
Assemblages C & D	Dog
Assemblage E	Cattle, other hoofed livestock
Assemblage F	Cats
Assemblage G	Rats
Assemblage H	Seals
Giardia muris	Rodents
Giardia agilis	Amphibians
Giardia ardeae	Herons (birds)
Giardia psittaci	Psittacine birds
Giardia microti	Muskrats
Giardia varani	Lizard

2.1.5 Pathology and clinical manifestations

The clinical effects of *Giardia* infection in humans range from a carrier state (the asymptomatic) to a severe malabsorption syndrome. The first time that *Giardia* was recognized to cause pathology was in the late 1970s. Based on symptoms such as malabsorption and the pathology observed in the upper part of the small intestine in patients from whom the organism was isolated. In a clinical study, Kulda and Nohynkova in 1978 concluded that this parasite can cause disease in humans. In 1981, the World Health Organization added *Giardia* to its list of parasitic pathogens (WHO Expert Committee, 1981).

Giardiasis in terms of pathogenesis has not been completely clear. So there are multiple theories that include the direct damage by trophozoites to the intestinal brush border and mucosa, induction of a host immune response that results in the secretion of fluid and damage to the gut, and alteration of bile content or duodenal flora that contributes to diarrhea. Induce apoptosis in small intestinal epithelial cells is an additional hypothesis (Chin *et al*., 2002).

Factors possibly contributing to the variation in clinical manifestations include the virulence of the *Giardia* strain (Aggarwal and Nash, 1987, Muller *et al*., 1996), the number of cysts ingested, the age of the host, and the state of the host immune system at the time of infection. Since symptoms are nonspecific, the clinical diagnoses of giardiasis are difficult and resemble those of a number of other gastrointestinal ailments. Clinical features may range from diarrhea to constipation, nausea, headache, and flatulence (Meyer and Radulescu, 1979, Wolfe and Meyer, 1990). Moreover, the life cycle stage of the parasite affects the variation of the symptoms observed. The incubation period may last 12 to 19 days and is marked by the first detection of cysts in

the faeces (Jokipii, 1985). This period is followed by the acute phase which lasts 3 or 4 days and is characterized by nausea, anorexia and sometimes low grade fever and chills followed by symptoms that include explosive, watery and foul-smelling diarrhea. Upper or mid-epigastric cramps may also occur. The acute phase usually resolves spontaneously and the symptoms will disappear if the immune system of the host is fully developed and healthy. Unfortunately, the acute phase develops into a chronic stage that happens in certain cases, in spite of a healthy and fully developed immune system. In these situations, the symptoms of the disease will reappear for short and recurrent period (Wolfe and Meyer, 1990). During the chronic phase, patient may suffer intermittent diarrhea and persistent mild to moderate symptoms.

The histopathological changes occurring at the mucosal sites range from minimal to severe and enough to cause enteropathy with enterocyte damage, villus atrophy, and crypt hyperplasia (Ferguson *et al*., 1990). The rapid multiplication of the trophozoites by binary fission creates a physical barrier between the intestinal epithelial cells and the lumen of the intestine, interfering with the process of absorption of nutrients causing malnutrition.

The pathogenicity of *G. duodenalis* has been shown to be different in different assemblages and also in subassemblage. However, several studies have linked *G. duodenalis* genotypes with clinical symptoms. The first hypothesis was reported by Homan and Mank (2001b) where assemblage A is correlated with intermittent diarrhea, whereas assemblage B is more associated with persistent diarrhea, and these findings have been reported in Malaysia (Mohammed Mahdy *et al*., 2009b). In contrast there was a strong association between assemblage A and diarrhea among children under 5 years of age that have been reported by Read *et al*. (2002). In this study, diarrhea was more prevalent among patients with assemblage A but this was not significant. On the

other hand, in a study in Spain, the symptoms (e.g., diarrhoea, abdominal pain, rapid weight loss, abdominal cramps, flatulence, nausea) are highly associated with subassemblage A2 among children under 5 years (Sahagun *et al*., 2008). In a study surveillance in south west London between 1999 and 2005, fever was shown to be significant with assemblage A while no difference was noted with other symptoms (Breathnach *et al*., 2010). Wether assemblage A or B is associated with diarrhea or not does not prove its virulence. It is possible to refer that, many factors contribute to the virulence of the infection.

2.1.6 Epidemiology

2.1.6.1 Prevalence and demographic distribution

Giardia duodenalis has a global distribution causing an estimated 2.8×10^8 cases per year (Lane and Lloyd, 2002). In developed countries it is the most common intestinal parasite of humans. In Asia, Africa and Latin America, approximately 200 million people have symptomatic giardiasis with some 500,000 new cases reported every year (WHO, 1996b). In industrialized countries, the prevalence of *Giardia* varies between 2% and 5% and up to 20-30% in developing countries (WHO, 2002) while it reaches up to 35% among children attending daycare centers in the United States in non-outbreak settings (Marshall *et al*., 1997, Ortega and Adam, 1997).

Chronic or repeated infections appear to be common (Gilman *et al*., 1988) and it is variable in the development of immunity. In instances of partial immunity, prolonged carriage may occur. In addition, frequency of asymptomatic infection of *Giardia* infections is not well known. The importance of asymptomatic execrators and symptomatic, undiagnosed execrators in disseminating infection is not known. All age

groups are affected by giardiasis, but in non-endemic areas, a bimodal age distribution is often seen, with the incidence being highest in children aged 0–5 yr, followed by adults aged 31–40 yr. This correlates with reports of disease prevalence being higher than average among children who attend daycare centers together with the family members and daycare workers who care for the infected children (Pickering *et al*., 1984).

Warhurst and Smith (1992) commented upon the global cost of *Giardia* infection, both in human and financial terms in the USA in 1991. Hospital admissions of patients annually costing over US$5 million for giardiasis accounted for 4000 of cases. They emphasized the need to acquire pertinent data to enable strategic targeting of research funds with the ultimate aims being prevention and control. The ability to genotype and subtype *Giardia* fulfils some of these requirements by providing molecular tools to identify sources of infection within populations and routes of transmission.

As mentioned before, there is a variable course of the infection and because of intermittent cyst excretion patterns, there are difficulties in diagnosing infections, tracing source and routes of infection specially with asymptomatic patient that was leading to complexity of *Giardia* epidemiology (Nash *et al*., 1987, Rendtorff, 1954, Rendtorff, 1979).

However recent studies of *Giardia* have identified eight distinct genotypes within *Giardia duodenalis*. Genotyping of a large number of human *Giardia* isolates from different parts of the world has demonstrated that humans are almost exclusively infected with assemblage A or B as reviewed by Thompson (2009). Other assemblages have occasionally been found in humans: C and D (Read *et al*., 2004a, Traub *et al*.,

2004), E (Foronda *et al*., 2008) and F (Gelanew *et al*., 2007a), but these findings require further confirmation. Table 2.2 presents examples of the results of genotyping of human isolates collected worldwide. According to this compilation, assemblage B dominates in Asia, Australia, Europe, and Latin and South America, whereas it seems that assemblage A predominates in Africa. It is not clear whether these data reflect the different populations investigated, the different geographical sites, or the various methods used. Table 2.3 shows the incidence of assemblages in studies using only one marker (data extracted from Table 2.2). According to analysis based on the *tpi* marker, assemblage B is twice as common as assemblage A, whereas the opposite results are obtained using the *b*-giardin marker (i.e., twice as many assemblages A as assemblage B isolates). A similar trend is seen among the sequences deposited in the ZoopNet database (Sprong *et al*., 2009). The reason for these discrepancies is unknown, although it has been speculated that primer sequence mismatches might reduce the ability to amplify certain assemblage B isolates at the *b*-giardin locus (Robertson *et al*., 2007a). Table 2.3 also shows the different subassemblages in various countries.

Giardia is more frequently found in surface waters than *Cryptosporidium*, and it has been associated with at least 132 waterborne outbreaks worldwide (Karanis *et al*., 2007); the most important occurred in Norway in 2004 and affected more than 1,500 persons (Robertson *et al*., 2006). Generally, most human infections are due to the consumption of water from unfiltered surface water sources, shallow wells and during water recreational activities. In United States, since 1971, *Giardia* has been commonly identified as the main pathogen in waterborne outbreaks, with almost 28,000 cases during the period 1965 to 1996 (United States Environmental Protection Agency, 1998).

The study on the probability of *Giardia* infection from drinking water from shallow wells in a peri-urban area in Brazil, showed that *Giardia* cysts recorded an occurrence of 62.5% of the samples (<0.1–36.1 cysts/l). This study indicated the vulnerability of shallow well water supply systems in peri-urban areas (Razzolini *et al*., 2010) which causes contamination with *Giardia duodenalis* cyst. So there are different records of contamination of water around the world, for instance 14% was reported in Pakistan (Ayaz *et al*., 2011).

In New Hampshire, USA, a community giardiasis outbreak occurred and a cohort study to identify risk factors for giardiasis was conducted. Stool and environmental samples were analysed. This study reported that consuming tap water was significantly associated with illness and *Giardia* cyst was recovered in a home water filter. This outbreak, which caused illness in 31 persons, represents the largest community drinking-water-associated giardiasis outbreak in the USA in 10 years (Daly *et al*., 2010).

The variety of *G. duodenalis* transmissions that can be maintained in independent cycles involving wildlife or domestic animals is shown in Figure 2.2. The transmission cycle is independent and not requiring interaction between them. The circumstances under which such cycles may interact and where zoonotic transfer occurs are not completely understood (Hunter and Thompson, 2005a). The epidemiology of *Giardia* is actually affected by the environmental and socioeconomic conditions. The inadequate hygiene practice and drinking untreated water was highlighted in this study as risk factors for *Giardia* cysts which indicated the possibility of transmission by the faecal–oral route, either direct or indirect. Potential mechanisms of transmission include: person to person, animal to animal, zoonotic (animal to human, human to animal), waterborne from humans or animals through drinking water or recreational

contact such as in swimming and foodborne from contamination of water used in food preparation and manufacturing or from food handlers (Porter *et al*., 1990, Shields *et al*., 2008a, Takizawa *et al*., 2009, Karanis *et al*., 2007).

Table 2.2 Distribution of assemblages A and B in 2471 human cases of giardiasis. Modified from Feng and Xiao (2011) and Caccio *et al.* (2005).

Origin	N	Loci tested	Assemblages			References
			A	B	A&B	
Argentina	28	*tpi*	0	28	0	(Molina *et al*., 2007)
Argentina	43	*tpi*	3	40	0	(Minvielle *et al*., 2008)
Argentina	70	*tpi*	22	46	2	(Molina *et al*., 2011)
Brazil	62	*b*-giardin	62	0	0	(Volotao *et al*., 2007)
Brazil	37	*gdh*	29	8	0	(Souza *et al*., 2007)
Brazil	58	ssu rRNA	9	43	6	(Kohli *et al*., 2008a)
Cuba	20	*b*-giardin, *gdh*	9	11	0	(Pelayo *et al*., 2003)
Mexico	9	*b*-giardin	9	0	0	(Lalle *et al*., 2005b)
Nicaragua	119	*b*-giardin, *gdh*	25	92	2	(Lebbad *et al*., 2008)
Peru	16	*gdh*	10	6	0	(Peréz Cordń *et al*., 2008)
Peru	25	*tpi*	6	19	0	(Sulaiman *et al*., 2003)
Canada	15	*b*-giardin	3	9	3	(Guy *et al*., 2004)
Total in America	**502**		**187**	**302**	**13**	
Australia	8	*gdh*, ssu rRNA	2	6	0	(Read *et al*., 2004a)
Australia	23	ssu rRNA	7	16	0	(Read *et al*., 2002)
Australia	124	*gdh*, ssu rRNA	31	93	0	(Yang *et al*., 2010_a)
New Zealand	30	*b*-giardin	23	7	0	(Winkworth *et al*., 2008)
Total in Australia and New Zealand	**185**		**63**	**122**	**0**	
Bangladesh*	267	ssu rRNA, *tpi*	20	231	16	(Haque *et al*., 2005b)
India	101	*tpi*	7	88	6	(Ajjampur *et al*., 2009)
India	16	*tpi*	5	8	3	(Traub *et al*., 2004)

Table 2.2 continued

Origin	N	Loci tested	Assemblages			References
			A	B	A&B	
Malaysia	42	ssu rRNA	1	41	0	(Mohammed Mahdy *et al*., 2009a)
Nepal	35	ssu rRNA, *tpi*	7	26	2	(Singh *et al*., 2009)
Palestine	8	*gdh*	5	3	0	(Hussein *et al*., 2009)
The Philippines	133	*tpi*	18	83	32	(Yason and Rivera, 2007)
Thailand	61	*b*-giardin, *gdh*, *tpi*	5	31	25	(Tungtrongchitr *et al*., 2010)
Thailand	12	*gdh*	5	7	0	(Ratanapo *et al*., 2008)
Thailand	30	*b*-giardin	12	17	1	(Kosuwin *et al*., 2010)
Thailand*	10	*gdh*	3	7	0	(Boontanom *et al*., 2010)
China	18	*tpi*	12	6	0	(Wang *et al*., 2010)
Korea	5	ssu rRNA	5	0	0	(Caccio *et al*., 2005)
Saudi Arabia	24		15	9	2	(Al-Mohammed, 2011)
Total in Asia	**705**		**120**	**557**	**87**	
Egypt	41	*tpi*	31	8	2	(Helmy *et al*., 2009a)
Egypt	17	*tpi*	1	16	0	(Foronda *et al*., 2008)
Egypt	87	*tpi*	58	10	19	(Abdel-Moneim and Sultan, 2008)
Ethiopia	52	*b*-giardin	31	13	8	(Gelanew *et al*., 2007a)
Ivory Coast	14	*tpi*, *gdh*	0	14	0	(Bertrand *et al*., 2005)
Western Sahara	36	*tpi*, *gdh*	16	18	2	(Lalle *et al*., 2005c)
Total in Africa	**247**		**137**	**79**	**31**	
Albania	22	ssu rRNA	10	12	0	(Berrilli *et al*., 2006)
Belgium	72	*b*-giardin, *gdh*, *tpi*	16	30	26	(Geurden *et al*., 2009)

Table 2.2continued

Origin	N	Loci tested	Assemblages			References
			A	B	A&B	
France	25	*tpi*, *gdh*	9	16	0	(Bertrand *et al*., 2005)
Italy	11	*b*-giardin	5	5	1	(Giangaspero *et al*., 2007)
Italy	30	*b*-giardin	24	6	0	(Caccio *et al*., 2002a)
Italy	37	*b*-giardin	17	15	5	(Lalle *et al*., 2005c)
Italy	42	ssu rRNA	19	13	10	(Giangaspero *et al*., 2007)
Italy	68	ssu rRNA, *b*-giardin	23	45	0	(Calderaro *et al*., 2010)
Norway	63	*b*-giardin, *gdh*,	4	59	0	(Robertson *et al*., 2007a)
Portugal	7	*b*-giardin	2	5	0	(ALMEIDA *et al*., 2006)
Portugal	25	*tpi*, *b*-giardin	25	0	0	(Sousa *et al*., 2006)
Spain	108	*tpi*	43	61	4	(Sahagun *et al*., 2008)
The Netherlands	98	ssu rRNA, *gdh*	32	64	--	(van der Giessen *et al*., 2006a)
The Netherlands	18	*gdh*	9	9	0	(Homan and Mank, 2001b)
Turkey	44	*tpi*	19	25	0	(Aydin *et al*., 2004)
UK	199	ssu rRNA, *tpi*	48	145	6	(Breathnach *et al*., 2010)
UK	33	*tpi*	9	21	3	(Amar *et al*., 2003)
Total in Europe	**902**		**314**	**531**	**57**	

N= the total of genotyped samples

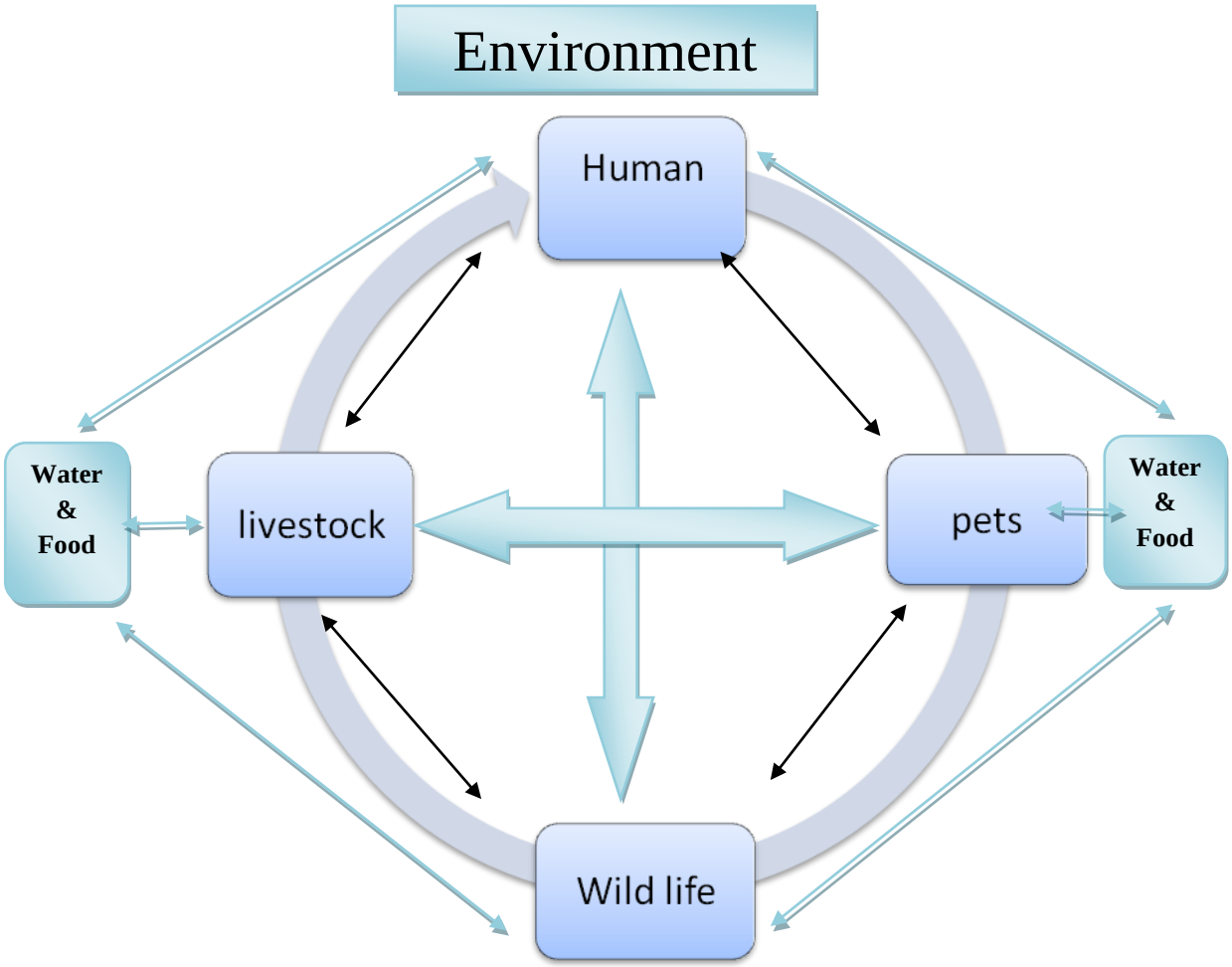


Figure 2.2 The independent cycle shows the interaction between protozoans with the surrounding environment.

Table 2.3 Subassemblage of *Giardia* found in different countries. Modified from Caccio *et al.* (2005).

Origin	locus	A	B	References
Thailand	*b-giardin*	A1 (12%) A2 (88%)	B3 (45.5%) B4 (54.5%)	(Tungtrongchitr *et al*., 2010)
Saudi Arabia	*Igs*	A1 (30%) A2 (27.5%) A1+A2+B (5%)	B (37.5%)	(Al-Mohammed, 2011)
Italy	*b-giardin*	A1(1) A2(7) A3(3) A4(2) A5(1) A6(1) A7 (1) A1+A2 (1) A+B (5)	B1(1) B3(4) B4(5) B1+B3(3) B3+B4(2)	(Lalle *et al*., 2005c)
Italy	*b-giardin*	A1 (10) A2 (10) A3 (1) A2+A3(1)	B1(3) B3(1) B3(2)	(Caccio *et al*., 2002a)
England	*Tpi*	A2(9)	B(21)	(Amar *et al*., 2002)
France	*tpi, gdh*	A2 (8)	B3(8) B4(5)	(Bertrand *et al*., 2005)
Mexico	*Gdh*	A2 (19)		(Ponce-Macotela *et al*., 2002)
Mexico	*b-giardin*	A1 (15) A3 (2)		(Lalle *et al*., 2005a)
Nicaragua	*b-giardin*	A2 (3) A3 (13)	B(94)	(Lebbad *et al*., 2008)
Brazil	*b-giardin*	A1 (60) A2 (2)		(Volotao *et al*., 2007)
Brazil	*Gdh*	A2 (29)	B(8)	(Souza *et al*., 2007)
Peru	*Tpi*	A2(6)	B(19)	(Sulaiman *et al*., 2003)
Korea	*Igs*	A2(3)	B(10)	(Lee *et al*., 2006)
Bangladesh	ssrRNA, *tpi*	A1 (8) A2 (20) A1+A2 (1)	B(231)	(Haque *et al*., 2005b)
Argentina	*Tpi*	A2 (22)	B(46)	(Molina *et al*., 2011)
Philippines	*Tpi*	A1 (3) A2 (47)	B(115)	(Yason and Rivera, 2007)
Australia	*Gdh*	A1 (4) A2 (5)	B4(6)	(Read *et al*., 2004b)
Ethiopia	*b-giardin*	A1 (1) A2 (5) A3 (16)	B (8) B3(1) B7(1) B8 (1) B19(2) A+B(7)	(Gelanew *et al*., 2007b)

2.1.6.2 Transmission

A. Person-to-person transmission

The accidental ingestion of cyst in contaminated water or food, or through environment where hygiene levels may be compromised in this case the faecal-oral is the most common route of transmission (Núñez *et al*., 2003). This study in Havana, Cuba indicated the environments where the frequency of transmission is high and/or conditions are conducive to direct person-to-person transfer are such as localized endemic communities or institutional settings (i.e., day care centers). It would be expected that competitive interactions might result in the predominance of particular genotypes. Diarrhea is a common disease in this type of institution and *Giardia* is considered the main faecal-oral pathogen causing diarrhea especially among children who are not toilet trained and toddlers (Thompson and Monis, 2004). On account of this, day care centers may also act as focus points for *Giardia* infection which may spread to day care staff, parents and community and 20-25 % of staff and family members who were in close contact with the infected children have been reported to be asymptomatically infected (CDC, 1994).

The asymptomatic child acts as hidden source of infection. A study among children in day-care centers in Perth, Western Australia, found that children infected with isolates of *Giardia* genotype assemblage A were 26 times more likely to have diarrhea than children infected with assemblage B isolates (Read *et al*., 2002). The children infected with *Giardia* assemblage B were acting as a hidden source of infection since most of them did not have diarrhea. This would explain why infections with assemblage B are more common in such environments. Children with such infections

are likely not treated which also raise questions about the long term consequences of such untreated chronic infections (Thompson, 2004).

Giardia contributes to nutritional disorders and poor growth and is reported among Aboriginal communities in northern Australia. In such communities, infections with isolates of *Giardia* from assemblage B are more common than those with assemblage A (Meloni *et al*., 1995, Thompson, 2000).

The risk of asymptomatic patient is not limited to children but also in restaurant workers where they could be a source of protozoan diseases in the community. An outbreak of giardiasis occurred among staff of a job training center after a meeting at a restaurant where twenty-seven (75%) of 36 attendees became ill (Quick *et al*., 1992). In a study in Yemen, 18.2% of restaurant workers have *Giardia* (Baswaid and Al-Haddad, 2008). The most frequently reported factor associated with infected worker was bare hand contact with the food followed by failure to properly wash hands, inadequate cleaning of processing or preparation of equipment or utensils, cross-contamination of ready-to-eat foods by contaminated raw ingredients (Todd *et al*., 2007).

In addition to that, water source could be contaminated by *Giardia* cyst through human faeces (Heitman *et al*., 2002) especially if the source is surface water as *Giardia* cyst is resistant to environmental situation and this is seen in rural area and even in sub-urban area in Yemen and the insufficient sewage disposal system in those areas could be a risk of contamination of water sources.

B. Waterborne transmission

From the beginning of the previous century till 2004, more than 100 waterborne giardiasis outbreaks have been reported worldwide and were systematically reviewed by Karanis *et al*. (2007). In New Hampshire (USA), a water fountain was associated with giardiasis outbreak, and drinking water associated outbreaks in Nokia (Finland) were reported (Daly *et al*., 2010, Rimhanen-Finne *et al*., 2007, Eisenstein *et al*., 2008). The largest drinking water related outbreak described to date occurred in Norway in 2004 affecting around 1500 people was caused by *G. duodenalis* assemblage B (Robertson *et al*., 2006). The most frequently cited reasons for giardiasis outbreaks are deficiencies in the drinking water treatment process including insufficient barriers, inadequate or poorly operated treatment (Karanis *et al*., 2007).

The possible way of contamination of surface water was through discharge of untreated and treated human sewage and/or from urban or rural land drainage containing animal faeces. The relative significance of these sources differs between watersheds. Large rivers and lakes often receive both agricultural run-off and treated and untreated domestic wastewater (Hunter and Thompson, 2005a).

Giardia actually has some features that enhance the possibility of the waterborne transmission; the small cyst is able to penetrate the physical barriers of water treatment and is insensitive to some disinfectants used in water industry and cyst can survive in water for 1-2 months. The low host specificity and the ability of *Giardia* to infect wide spectrum of domestic and wild life animals increase the source of water contamination. Hence the large numbers of excreted cyst by infected hosts and the low infectious dose of cysts are other factors that increase the risk of waterborne transmission. Genotyping

of *Giardia* isolated from waterborne outbreaks could help water authority in determining the sources of infection (Caccio *et al*., 2005).

C. Foodborne transmission

The contamination of fruits, vegetables and shellfish with *Giardia* cysts is an important public health concern, because these products are frequently consumed raw without thermal processing (Blasi *et al*., 2008, Pozio, 2008). Filter feeders such as oysters (*Crassostrea* spp.) and clam species (*Macoma* spp., *Corbicula fluminea, Dreissena polymorpha, Mytilus galloprovincialis, and Anadonta piscinalis*) (Graczyk *et al*., 1999b, Graczyk *et al*., 1999a, Graczyk *et al*., 2003, Gomez-Couso *et al*., 2005, Hänninen *et al*., 2005), have been shown to accumulate *Giardia* cysts (Schets *et al*., 2007, Graczyk *et al*., 2006, Graczyk *et al*., 1998). These data suggested that consumption of raw bivalve molluscs may lead to cases of gastro-intestinal illness. Cook *et al*. (2007) found one *Giardia* cyst in 50 g on examining a "natural food" sample of organic watercress, spinach, and rocket salad as a surface contaminant. Dill, lettuce, mung bean sprouts, radish sprouts and strawberries have also been found contaminated by *Giardia* cysts (mean 3 cysts per 100 g) as reported by (Robertson and Gjerde, 2001).

Poor personal hygiene of food handlers causing giardiasis outbreak has been reported previously (Mintz *et al*., 1993, Porter *et al*., 1990, Smith *et al*., 2007). In the 27 Member States of the European Union, zoonotic parasites transmitted by food are circulating with different prevalence according to the country, the environmental conditions, the human behavior, and the socio-economic level (Pozio, 2008).

D. Zoonotic transmission

Zoonotic transmission of *Giardia* is in the forefront of public health interest and has been controversial for several years. Likewise, the zoonotic transmission is shown to be possible in several studies using *Giardia* cysts isolated from both humans and animals (Monis and Thompson, 2003). Over 25 years ago, WHO initially listed giardiasis as a zoonosis as a result of epidemiological observations suggesting giardiasis in campers in Canada was caused by drinking stream water contaminated with *G. duodenalis* from beavers (Thompson, 2004).

The zoonotic potential of *G. duodenalis* has been discussed by various authors (van Keulen *et al*., 2002, Traub *et al*., 2004, Lalle *et al*., 2005a, Savioli *et al*., 2006), however the real clinical significance of zoonotic potential is still not clear. Genotypes of *Giardia* in assemblage A have the major zoonotic risk while to a lesser extent, genotypes in assemblage B (Thompson, 2000) have this potential risk. However, genotypes using a multilocus sequence typing define only assemblage A and not assemblage B to have a zoonotic potential (Lebbad *et al*., 2010, Sprong *et al*., 2009).

Humans, dogs, cats, domestic livestock (cattle, sheep, pig, horse, goat) and certain species of wildlife (Table 2.4) have been described as natural hosts of *G. duodenalis* assemblage A. In addition most infected animals remain asymptomatic and if clinical disease occurs, it is usually associated with young animals and those in kennel or cattery (Robertson *et al*., 2000). *Giardia duodenalis* assemblages A and B have been recovered in marine animals too, such as porpoises, dolphins, seals (ringed seal), common eiders, and thresher shark. The possibility of human infection from these animals is very low. However, they can contaminate water that is used by humans for recreational activities (Lasek-Nesselquist *et al*., 2008, Dixon *et al*., 2008, Yang *et al*.).

Furthermore, *Giardia* cysts have been detected in flies and in the faecal droppings of wild birds in prevalence of 7.3% and 5–49% respectively, identifying both assemblages A and B, indicating the potential role of such vectors in the transmission of intestinal parasites (Szostakowska *et al*., 2004, Kuhn *et al*., 2002, Plutzer and Tomor, 2009, Foronda *et al*., 2008). *G. duodenalis* assemblage E has been detected in human stool samples in Egypt based on the *tpi* gene. However, further analyses of a second locus are required to confirm this result. The zoonotic distributions of assemblages A and B in several studies is shown in Table 2.4.

Table 2.4 The reported natural wild hosts of *Giardia duodenalis* assemblages A and B. Modified from Plutzer *et al*., (2010).

Giardia duodenalis assemblage	Natural host	References
Assemblage A	Fallow deer	(Cacci *et al*., 2008, Lalle *et al*., 2007)
	White tailed deer	(Trout *et al*., 2003)
	Reindeer	(Robertson *et al*., 2007b)
	Coyote	(Thompson *et al*., 2009)
	Fox, Norwegian wild red fox	(Hamnes *et al*., 2007, McCarthy *et al*., 2008)
	Australian house mice	(Moro *et al*., 2003)
	Wild moose	(Robertson *et al*., 2007b)
	Muskoxen	(Kutz *et al*., 2008)
	Southern brown howler monkey	(Volotao *et al*., 2008)
	Black howler monkey	(Vitazkova and Wade, 2006)
	Alpaca	(Trout *et al*., 2008)
	Water buffalo	(Caccio *et al*., 2007)
	Wild boar	(Cacci *et al*., 2008)
	Western grey kangaroo	(McCarthy *et al*., 2008, Thompson *et al*., 2008)
	Common brushtail possum	(Thompson *et al*., 2008)
	Mountain brushtail possum	(Thompson *et al*., 2008)
	Swamp wallaby	(Thompson *et al*., 2008)
	Koala	(Thompson *et al*., 2008)
	Domestic ferret	(Abe *et al*., 2005, Abe *et al*., 2010)
	Red deer	(Bajer, 2008)
	Roe deer	(van der Giessen *et al*., 2006a)
	Marmoset	(van der Giessen *et al*., 2006a)
	Common planigale	(Thompson *et al*., 2010)
	Quenda	(Thompson *et al*., 2010)
Assemblage B	Beaver	(Fayer *et al*., 2006a)
	Mandrill	(Cacci *et al*., 2008)
	Macaque	(Cacci *et al*., 2008, Itagaki *et al*., 2005)
	Chimp	(Cacci *et al*., 2008)
	Black howler monkey	(Vitazkova and Wade, 2006)
	Coyote	(Trout *et al*., 2008)
	Western grey kangaroo	(Thompson *et al*., 2008)
	Norwegian wild red fox	(Hamnes *et al*., 2007)
	Chinchilla	(van der Giessen *et al*., 2006a)

2.1.6.3 Risk factors

The risk factors for clinical giardiasis, particularly in humans, have yet to be resolved, but clearly involve host and environmental factors as well as the 'strain'/ genotype/assemblage of the parasite (Buret, 2007). For instance, the epidemiology of giardiasis is different from high income and low income countries. Several studies have been done on risk factors of *Giardia* in developing countries Table 2.5. The sharing risk on developing and developed countries is related to water from improper sewage disposal in developing countries and drinking untreated water in both developing and developed countries (Table 2.5 and Table 2.6).

In developing countries most of the risk factors are related to personal hygiene such as not using toilet (Mahmud *et al*., 1995), not washing hands, large family size (Norhayati *et al*., 1998), environmental sanitation like lacking of sewage disposal (Cifuentes *et al*., 2000, Prado *et al*., 2003, Al Shammari *et al*., 2001) and contact with animals in household.

In the same way, current epidemiological evidence indicates that hand washing with soap (HWWS) prevents about 30–47% of child diarrheas (Curtis and Cairncross, 2003, Ejemot *et al*., 2008). A recent comprehensive review ranked hygiene promotion, including hand washing, as the most cost-effective intervention to prevent disease, at a cost of $3.4 for each disability-adjusted life year saved (Jamison and Bank, 2006). Therefore, the striking effect of hand washing with soap is consistent across various study designs and pathogens, though it depends on access to safe water and this problem is faced by many countries including Yemen where the access to safe water cover only 40% of the community.

In developed countries, the most risk factors reported were drinking untreated water and swimming, these two risk factors were showed in a case control study in UK through the accidental swallowing of water while swimming and the contact with recreational water and eating contaminated lettuce were also reported as risk factors (Stuart *et al*., 2003). Surprisingly, drinking treated water from New Zealand was 7 times higher risk than drinking water from sources outside New Zealand. Also travelling and swimming were recorded as risk factors (Hoque *et al*., 2002). In addition to these factors, children wearing nappies in New Zealand were significantly infected with *Giardia* (Hoque *et al*., 2002, Hoque *et al*., 2001). Presence of animals in household has been reported as a risk factor in UK by Waburton (1994). The risk factors in several developed countries are shown in Table 2.6.

Table 2.5 The main risk factors of giardiasis reported in developing countries.

Risk factors	OR	95%CI	Country	References
Improper sewage	1.2	1.0-1.5	Mexico	(Cifuentes *et al*., 2000)
Disposal	1.9	1.2-3.2	Brazil	(Prado *et al*., 2003)
	2.4	1.54–3.84	Saudi Arabia	(Al Shammari *et al*., 2001)
	1.19	0.97-1.45	Mexico	(Cifuentes *et al*., 2000)
Living in rural area	5.4	1.5-20.1	Brazil	(Pereira *et al*., 2007)
	3·01	1·23-7·35	Cuba	(Bello *et al*., 2011)
Large family size	2.1	1.3-3.3	Brazil	(Prado *et al*., 2003)
	3.8	2.5-5.7	Malaysia	(Norhayati *et al*., 1998)
Unsafe food hygiene	2.9	1.34-6.43	Brazil	(Pereira *et al*., 2007)
Drinking untreated water	1.2	1.07-1.42	Saudi Arabia	(Al Shammari *et al*., 2001)
	1.76	0.95-3.23	Mexico	(Cifuentes *et al*., 2000)
	3·64	2·14-6·26	Cuba	(Bello *et al*., 2011)
Poor hand washing	2.3	1.0-5.2	Mexico	(Cifuentes *et al*., 2004)
Not using toilet	2.63	1.4-4.9	Egypt	(Mahmud *et al*., 1995)
Eating raw vegetables	4·84	2·33-10·14	Cuba	(Bello *et al*., 2011)
Eating fresh fruits	7.78	1.01–60	Malaysia	(Mohammed Mahdy *et al*., 2009b)
Children ≤ 12 years	3.56	1.79–102	Malaysia	(Mohammed Mahdy *et al*., 2009b)
Contact with animals	2.5	1.1–5.6	Egypt	(Mahmud *et al*., 1995)

Table 2.6 The main risk factors of giardiasis reported in developed countries. Modified from Hunter and Thompson (2005).

Risk factors	OR	95%CI	Country	References
Drink treated water	1.3	1.1-1.5	UK	(Stuart *et al*., 2003)
	7.97	4.2-15.1	New Zealand	(Hoque *et al*., 2003)
Drinking untreated water	12	5.9-24.7	British Columbia	(Isaac-Renton and Philion, 1992)
	3.4	2.1-5.5	New Hampshire	(Dennis *et al*., 1993)
Swimming	2.4	1.1-5.3	New Zealand	(Hoque *et al*., 2003)
	2.04	1.33-3.1	New Zealand	(Hoque *et al*., 2002)
	5.3	1.9-14.8	UK	(Stuart *et al*., 2003)
	2.4	1.0-6.1	UK	(Gray *et al*., 1994)
	4.6	2.4-86.0	New Hampshire	(Dennis *et al*., 1993)
Travelling	7.5	4.0-14.2	New Zealand	(Hoque *et al*., 2002)
	2.5	1.03-6.0	New Zealand	(Hoque *et al*., 2002)
	7.6	0.8-70.1	UK	(Gray *et al*., 1994)
	6.3	1.1-35.8	British Columbia	(Isaac-Renton and Philion, 1992)
Eating raw vegetables	2.2	1.2-4.3	UK	(Stuart *et al*., 2003)
Nappy handling	4	-	New Zealand	(Hoque *et al*., 2001)
	2.1	1.4-17.4	New Zealand	(Hoque *et al*., 2002)
Contact with farm animals	4.8	1.3-17.4	UK	(Warburton *et al*., 1994)
Contact with pets	14.6	4.2-50.6	UK	(Warburton *et al*., 1994)

2.1.7 Genotyping

Recently, genotyping tools marked a new phase in the epidemiology of *Giardia*. The source of infection could easily be identified by genotyping. Otherwise there are limitations in cross transmission study between human and animal and vice versa. Zoonotic transmission has been proven by genotyping and where humans live in close contact to animals. Genotyping also helps health authorities to detect the sources of water contamination in many waterborne outbreaks, whether from animals or humans.

Molecular tools provide the techniques for accurate diagnosis and gene sequencing-generated data which allows the construction of molecular phylogenies mapping the evolutionary relationships between individual species and isolates. All these efforts lead to the reopening of the question: 'Is the number of species currently recognized sufficient, or do we need to define more?'(Lindsay, 2010).

Until now the genotyping of *Giardia* revealed seven genetically distinct, morphologically identical lineages from A to G but recently a new genotype (H) have been reported in marine vertebrates (Lasek-Nesselquist *et al*., 2010). There is a wide range of biological diversity in genotypes of *Giardia*; each genotype has different ability to infect one or multiple host species and each varying in prevalence of infection between and within countries. However the development of genotyping technique is useful to improve the epidemiological information of the risk factors (socioeconomic or environmental condition) of *Giardia* even in pathogeneses approach. Likewise to distinguish species, genotypes and their zoonotic potential in addition to discovering new genotypes, it is necessary to use molecular analysis at informative loci.

Genotyping techniques for *Giardia* spp until now are not particularly advanced and the vast majority of studies have relied on the analysis of the small subunit

ribosomal RNA (ssu rRNA), *b*-giardin (*bg*), glutamate dehydrogenase (*gdh*), elongation factor 1-alpha (*ef-1a*), triose phosphate isomerase (*tpi*), *Giardia duodenalis* open reading frame the (C4) GLORF-C4 genes (Caccio *et al*., 2005) and recently, the inter-genomic rRNA spacer region (IGS) (Lee *et al*., 2006). The different protocols for genotyping of *Giardia* are shown in Table 2.7. As the genome of a *G. duodenalis* isolate (WB, assemblage A, subgroup A1) has been completely sequenced (Morrison *et al*., 2007), it is possible to locate those genes on chromosomes or on large contigs. However, the genes mentioned above are unlinked in the *G. duodenalis* genome, at least in the assemblage A genome, which is a desirable property for genetic studies (Plutzer *et al*., 2010).

Based on hybridization studies on chromosomes separated by pulsed-field gel electrophoresis, the *tpi* gene has been mapped to chromosome 5, the *gdh* and *b*-giardin were mapped to chromosome 4, and the majority of ssu rRNA gene copies were mapped to chromosome 1 that was reviewed by (Adam, 2001).

The ssu rRNA gene and the *ef-1a* gene can be used to distinguish major assemblages, whereas the *tpi* gene, *b*-giardin gene and the *gdh* gene allow different subgenotypes to be identified within each assemblage. In addition, restriction fragment length polymorphism (RFLP) genotyping has been developed for *tpi* (Amar *et al*., 2002), *gdh* (Read *et al*., 2004a) and *b*-giardin (Caccio *et al*., 2002b).

A large number of sequences have been obtained from each of these genes from isolates of humans and animals origin collected worldwide (i.e., 151 for ssu rRNA, 210 for *bg*, 134 for *gdh*, 14 for *ef-1a*, 70 for *tpi*, and 3 for C4, as from a search of the November 2007 GenBank release). In terms of their polymorphisms, these genes are quite different, with *tpi* and *gdh* genes being the more variable, followed by *b*-

giardin and C4 genes, then by the much more conserved *ef-1a* and ssu rRNA genes (Monis *et al*., 1999). This is also reflected by differences in substitution patterns, with the *b*-giardin and *ef-1a* genes showing few, if any, non-synonymous changes, whereas the *tpi* and *gdh* genes appear to tolerate amino acid replacements. A comprehensive review of the utility of these genotyping loci to distinguish *G. duodenalis* assemblages and sub-assemblages and to infer their phylogenetic relationship has been recently published (Wielinga and Thompson, 2007).

Two subgroups in assemblage A (A1 and A2) and in assemblage B (B3 and B4) have shown the existence by DNA sequence analyses (Monis *et al*., 1999). It is clear, however, that more variability is present in these as well as in isolates from the other *G. duodenalis* assemblages (Monis *et al*., 2003b). The subtyping which involved within these assemblages from the same host species formed a sub-cluster, which may correspond to as yet undefined subgroups (Monis *et al*., 2003b, Sprong *et al*., 2009).

DNA-based genotyping studies have identified more subtypes from both humans and animals isolates, e.g., (Lalle *et al*., 2005d) and (Sulaiman *et al*., 2003), and have confirmed sub-structuring within major *G. duodenalis* assemblages. In these studies, a different terminology was used to describe subtypes, such as A3, A4, A5, or B1, B2, B3 at the *bg* locus (Lalle *et al*., 2005d), B S1, B S2, B S3, or C S1, C S2, C S3 at the *tpi* locus (Sulaiman *et al*., 2003), or even B0, B1, B2 at the *gdh* locus. This has generated a certain degree of confusion, particularly when different subtypes were found by sequencing different genetic markers, i.e., when there was a lack of concurrence between the results for these genes (Robertson *et al*., 2006).

The sensitivity of these techniques depends on DNA extraction methods, primers and type of targeted gene. Another aspect that has received little attention is the

fact that the developed primers do not consistently amplify *G. duodenalis* DNA. It is quite easy to explain that ssu rRNA has the highest sensitivity, because it has multicopy nature, and specificity, due to the strong sequence conservation. It has been reported that certain isolates could be amplified at one locus but not at another, whereas other isolates may just show the opposite behavior (van der Giessen *et al*., 2006a, Robertson *et al*., 2006, Robertson *et al*., 2007a, Volotao *et al*., 2007). Thus, greater sequence variability may be present in those genes, and excessive mismatches in the binding regions of the primer(s) can prevent their successful amplification.

The presence of DNA polymerase inhibitors and degraded DNA in faecal material may cause problems in PCR amplification for clinical samples, in addition to non-specific binding of primers and intermittent parasite shedding (Caccio and Ryan, 2008, Nantavisai *et al*., 2006, Traub *et al*., 2005). So to overcome PCR inhibition, several protocols for concentration prior to DNA extraction have been developed; however, it is still unclear what combination of molecular protocols is optimal for *Giardia* (Hopkins *et al*., 1997, Pelayo *et al.*, 2008, Read *et al.*, 2004a). Creating a reproducible and highly sensitive technique to differentiate *G. duodenalis* strains is essential for comparisons of regional data and global human infection (Smith *et al*., 2006). DNA extraction by using FTA filter paper (Whatman Bioscience, Cambridge, UK) and 18S rRNA amplification have been previously reported as the most sensitive method for *G. duodenalis* detection in faecal samples (Nantavisai *et al*., 2006) while recently direct DNA extraction using the ISOLATE Fecal DNA Kit generated the highest DNA concentration measurements in *gdh* amplicons than PrepGEM DNA extraction (ZyGEM Corporation Ltd., Hamilton, New Zealand)., following IMS (Asher *et al*., 2011).

These findings could not be generalized because many techniques were not included.

Table 2.7 *Giardia* genotype protocols.

Target gene	Primers	PCR assay	References
16S rRNA	RH-4,RH-11	PCR	(Hopkins *et al*., 1997)
	RH4, RH11+GiaR, GiarF	Nested PCR	(Read *et al*., 2002)
	AL4303,AL4305+ Al4304, AL4306	Nested PCR	(Sulaiman *et al*., 2003)
Gdh	GDH1,GDH4 GDHeF, GDHiR+ GDHiF	Nested PCR	(Read *et al*., 2004b)
b-giardin	G7,G759	PCR	(Giangaspero *et al*., 2007)
	G7,G759+ forword, Revers	Nested PCR	(Lalle *et al*., 2005b)
Tpi	AL3543, AL3546 AL3544,AL3545	Nested PCR	(Read *et al*., 2002)
ef-la	EFlAR/GLONGF and RTefl-F/ RTef-R	Nested PCR	(Monis *et al*., 1999)
Igs	GLF,GSR+GA1F, GA2F, GBF,GBR	Real time PCR	(Lee *et al*., 2006)

2.1.8.Diagnosis

Microscopic diagnosis is still the cornerstone and gold standard for detecting intestinal parasites in stool samples in both humans and animals. However, progress has been made to develop non-morphologically based diagnostic tests for intestinal parasite antigens. Immunofluorescence microscopy (IF), enzyme linked immunosorbent assays (ELISA), parasite DNA polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), and real time PCR (RT-PCR) have all been utilized for *Giardia* diagnosis.

Traditionally fresh or preserved stool samples are microscopically examined directly or with trichrome staining, with or without concentration. Unfortunately, the sensitivity of this conventional ovum-and-parasite (O&P) examination on a single stool sample for *Giardia* is found to be less than optimal and was determined by Cartwright to be only 74% (Cartwright, 1999).

The low-level of parasite shedding and the variable excretion in both symptomatic and asymptomatic patients cause poor sensitivity of a single parasitological stool examination for diagnosing giardiasis. Parasites are detected in the stool, hence repeated examinations are necessary until morphological forms are seen, as is well described and recommended in many parasitology textbooks. To overcome sampling issues, the Triple-Faeces-Test (TFT) was recently developed in the Netherlands as all round test for the laboratory diagnosis of intestinal parasites. The advantages of both fixatives, permanent staining methods and multiple sampling are combined in this test (Van Gool *et al*., 2003).

Since the early nineties, enzyme immunosorbent assays (EIAs) for detecting *Giardia*-specific antigens in stool samples have become commercially available. A number of clinical evaluations of *Giardia* EIAs have been reported in the literature; the sensitivity of EIA has been found to be either similar, or in most cases slightly superior to single, as well as to multiple, microscopic stool examinations (Mank *et al*., 1997, Zimmerman and Needham, 1995). Given these findings, and due to the fact that *G. duodenalis* is by far the most commonly found enteric pathogen in general practice, EIA is an attractive alternative to conventional O&P (ovum and parasite) examination. However, in the case of persistent symptoms with a negative test result, a permanent stained smear from a preserved stool sample should also be performed.

PCR assays for specific genera/genotypes of intestinal parasites are useful for surveys, but not for the clinical diagnostic laboratory. Stools must be screened for a variety of pathogens and the cost of different PCR assays would be too high. RT-PCR may have a role in diagnostic and reference laboratories, as several targets could be combined in one multiplex RT-PCR assay, allowing the simultaneous detection of multiple infections such as *Entamoebia. histolytica, G. duodenalis* and *Cryptosporidium* species and genotypes infectious to humans.

The use of the new approaches in routine parasitological stool examinations, a substantial increase in sensitivity and specificity in the laboratory diagnosis of intestinal protozoan infections can be achieved. Nevertheless it is still extremely important to familiarise physicians with the clinical relevance and epidemiology of these infections.

2.1.9 Treatment

Despite the fact that giardiasis is an important human disease, there have been relatively few chemotherapeutic agents used for its therapy. Quinacrine was the first used anti-giardial drug in the mid-1930s. Initially introduced as an anti-malarial (Hartman and Kyser, 1941), it was used as an antigiardial drug until it was replaced by metronidazole. Nitroimidazoles, the main agents used against giardiasis are metronidazole, tinidazole, ornidazole and secnidazole, which have been used for the treatment of giardiasis since the mid-1950s. Following its discovery in the mid-1950s, metronidazole was used to treat *Trichomonas vaginalis* and *Entamoeba histolytica*, and in 1962, Darbon *et al* found it useful for treating giardiasis. Since then, clinicians have used metronidazole and other nitroimidazoles as the mainstay of therapy for giardiasis.

Metronidazole is the most commonly used drug worldwide. The drug is activated by reduction of its nitro group. Reduced metronidazole serves as a terminal electron acceptor which binds to DNA macromolecules, damaging the DNA helical structure. However, metronidazole does not affect cyst viability (Paget *et al*., 1989). Its median reported efficacy is 92% (Levi *et al*., 1977, Cimerman *et al*., 1989, Gorbea Robles *et al*., 1989, Kalayci *et al*., 1995).

Metronidazole is prescribed as a 250 mg/dose 2–3 times daily for 7–10 days or as a 3-day course, daily single-dose therapy of 2.0 or 2.4 g/dose, or a single dose that increases compliance but reduces efficacy and carries strong adverse effects. Tinidazole, another nitroimidazole derivative, is given as a 2 g single dose for adults or equivalent for children. Tinidazole has a clinical efficacy of 80–100% with a median efficacy of 92% (Jokipii and Jokipii, 1979, Jokipii and Jokipii, 1982).

Ornidazole is another nitroimidazole with few clinical trials, but studies indicate an efficacy similar to tinidazole (92–100%) when given over several days or as a single dose (Sabchareon *et al*., 1980, Bassily *et al*., 1987). Another study in Turkey showed single dose showed 94-97% efficacy among children (Özbilgin *et al*., 2002). Secnidazole is also a member of the nitroimidazole family similar to tinidazole and ornidazole and usually given in a single dose. At the molecular level, resistance to metronidazole is associated with changes in DNA. DNA probes that hybridize with specific chromosomes and repetitive sequences indicate that rearrangements both at the chromosome and repetitive DNA level occurred concurrently with the development of metronidazole resistance (Upcroft *et al*., 1990).

Paramomycin inhibits protein synthesis in *G. duodenalis* by interacting with the 50S and 30S ribosomal subunits, resulting in misreading of mRNA codons. It is poorly absorbed, with about 60–70% efficacy against *G. duodenalis*. Paramomycin has been considered a possible drug for *Giardia* infections in pregnant patients as it is excreted in faeces without being metabolized (Mineno and Avery, 2003). Nitazoxanide appears to be the drug of choice in treatment of *Giardia* even for cases resistant to metronidazole and albendazole therapy (Abboud *et al*., 2001).

2.2 *CRYPTOSPORIDIUM*

2.2.1 Introduction

Cryptosporidium is an opportunistic coccidian protozoan parasite which is one of the most prevalent enteropathogens worldwide and the etiological agent of a diarrheal disease dependent largely on the immunological status of the affected individual. It can also infect farm animals, companion animals and wild animals (Fayer R, 2004).

Cryptosporidium has attracted a great deal of attention since the late 1970s as an organism for epidemiological and molecular biological studies. The first time that *Cryptosporidium* was identified between 1907 and 1912, by Ernest Edward Tyzzer, he reported novel coccidia-like organisms in the gastric glands (Tyzzer, 1907, Tyzzer, 1910) or small intestine (Tyzzer, 1912) of laboratory mice and he gave an accurate description of the parasites' life cycle. Tyzzer also divided these protozoans as two species and gave the names *Cryptosporidium muris* for the gastric and *Cryptosporidium parvum* for the intestinal parasites. The description of *Cryptosporidium meleagridis* in turkeys in 1955 (Slavin, 1955) and the identification of cryptosporidia in diarrheric calves in 1971 (Panciera *et al*., 1971) provided the first indications that infection with *Cryptosporidium* spp. might be associated with morbidity and mortality.

The first two cases reported in human were described in 1976 in an immunocompromised patient and a 3 year old girl who vomited everything taken by mouth and had severe watery diarrhea (Nime *et al*., 1976, Meisel *et al*., 1976, Crawford *et al*., 1988). Latter in 1980s, *Cryptosporidium* was reported as a cause of death in AIDS patients, highlighting the public health significance of this parasite (Current *et al*., 1983). In immunocompetent subjects, infections are usually self-limiting, lasting 1±2 weeks, while immunocompromised individuals frequently experience a severe and

unremitting watery diarrhea that can lead to death (Petersen, 1992, Flanigan *et al*., 1992). In the early childhood, infection with *Cryptosporidium* is associated with malnutrition disorder (Agnew *et al*., 1998, Sallon *et al*., 1988, Checkley *et al*., 1998).

Cryptosporidium is common in developed and developing countries causing a major public health problem. The mode of transmission in developed countries is shown to be more related to consuming contaminated water and food especially, through recreational water activities, and close person-to-person contact, e.g. hospital cross infections, and through zoonotic sources (Koch *et al*., 1985). Furthermore, there have been numerous reported waterborne and day care outbreaks (Fayer R, 2004). *Cryptosporidium* is of great public health interest after the large human waterborne outbreak in Milwaukee in 1993 (Mac Kenzie *et al*., 1994). These outbreaks lead to the recognition that *Cryptosporidium* was one of the most serious and difficult to control waterborne pathogens to date.

In developing countries, many laboratories do not include *Cryptosporidium* in routine test except in cases where doctors specifically request for the test, since *Cryptosporidium* is not considered out of immunocompetent patients (CDC Summary of Notifiable Diseases United States, 1999).

Cryptosporidium oocysts can remain viable in water for over 140 days (Hooda *et al*., 2000) and have the ability to resist most common disinfectants (Campbell *et al*., 1982), making them difficult to destroy by conventional chlorination treatment. Outbreaks have been associated with contamination of surface waters, well waters, swimming pools, and public water supplies (CDC 1998, CDC, 2001). In the US, the parasite has been identified in 80–97% of all surface waters (rivers, lakes, ponds, etc.) (LeChevallier *et al*., 1991).

The fact that *Cryptosporidium* is even when treated (filtered and chlorinated), water may still contain low numbers of *Cryptosporidium* oocysts (LeChevallier *et al.*, 1991). This is because the oocyst is very tolerant to environmental and physical changes. Besides that, sewage or faeces of infected animals or humans can cause the contamination of the recreational waters like swimming pools, jacuzzis, ponds and fountains (Kramer *et al.*, 1998).

On the other hand, cattle and agricultural activities can be a potential source of waterborne outbreaks. *Cryptosporidium* among cattle and calves is found to be of high prevalence, but these implications have rarely been confirmed during outbreak investigations. There is improvement in molecular techniques which aids in detecting the source and causes of *Cryptosporidium* outbreaks that is achieved by the development of highly sensitive detection and genotyping methods.

Wildlife and companion animals can also be sources of *Cryptosporidium* infections. Early reported potential risk was among infected dogs and cats (El-Ahraf *et al*., 1991). *Cryptosporidium* infection has also been found in a wide range of wild animals, and these animals can be significant risk to human and livestock through contamination of the environment (Simpson, 2002, Sturdee *et al*., 1999). The molecular tools that could help to detect the source of infection are expensive to conduct, and the traditional techniques do not provide enough information to allow the identification of modes of transmission. Due to these challenges, the epidemiology of *Cryptosporidium* is still not entirely clear**.** There are several molecular techniques that have been developed to detect and differentiate *Cryptosporidium spp*. designed to detect genotypes and subgenotypes.

Currently there are 23 recognized species in various kind of animals (Xiao *et al*., 2001, Cama *et al*., 2003, Gatei *et al*., 2006, Fayer, 2010b, Traversa, 2010). The classification of *Cryptosporidium* depends on the host occurrence, parasite morphology, host predilection and site of infection. There are more than 44 genotypes found in the environment, water and food concentrates, around the world. Six *Cryptosporidium* species /genotypes are responsible for most human cryptosporidiosis cases, including *C. hominis, C. parvum, C. meleagridis, C felis, C. canis* and *C. suis* (Xiao and Feng, 2008). Among them, *C. hominis* and *C. parvum* are the most common in humans. Humans have been considered the main source of infection and animals have been implicated as the sources of water contamination and human infections.

2.2.2 Morphology

The small size of *Cryptosporidium* oocysts makes them indistinguishable at the species level based on morphology by light microscope (Fall *et al*., 2003). The oocysts are spherical or ovoid in appearance and contain 4 naked parallel sporozoites surrounded by a smooth oocyst wall. At the wall, a faint suture can be seen through which the sporozoites exist during excystation (Morgan-Ryan *et al*., 2002). There is some variation from species to species. The length of oocyst ranges from 4.5 to 7.5 µm and the width from 4.2 to 5.7 µm (Marquardt and Speer, 2000). At the electron microscope level, the zoites of *Cryptosporidium* show some of the elements of the apical complex; such as electron dense collar similar to a conoid, micronemes and electron-dense bodies that may be similar to rhoptries (Marquardt and Speer, 2000). The two apical rings can also be seen at the tip of the zoite. The pellicle is similar to that of other apicomplexans

and subpellicular tubules are also present. They lack a true conoid, perhaps rhoptries, and mitochondria (Marquardt and Speer, 2000). *Cryptosporidium* have a few life cycle stages; oocyst, trophozoite, schizont, merozoit and sexual stages including micro- and macrogamonts.

2.2.3 Life cycle

The life cycle of *Cryptosporidium* is shown in Figure 2.3. The life cycle begins when oocyst is ingested by the host. Sporozoites released adhere directly to the intestinal epithelial cells of the host. Cell invasion by sporozoite is followed by intracellular development to trophozoite. Trophozoites undergo merogony to form meronts. Asexual replication occurs by re-infection of merozoites, released by type I schizont. Development of type II from type I meronts is the initial step of the asexual reproductive cycle. Merozoites are released from type II meronts and re-infect neighbouring cells where they develop into microgametocytes (male) or macrogametocytes (female). The macrogametocyte is fertilised by released microgametes and matures into a zygote, which undergoes further development into an oocyst (two sexual divisions) with each oocyst containing four sporozoites (Tzipori and Ward, 2002). Two types of oocysts are released: (I) thick-walled oocysts, which are excreted in the faeces, or, (II) thin walled oocysts for endogenous reinfection (auto-infection). Auto-infection explains the persistence infection in AIDS patients in the absence of successive oocyst exposure (Current and Garcia, 1991). The prepatent period ranges from one to three weeks, whereas the patent period may persist for years, depending on the parasite species and the immune status of the hosts (Sturdee *et al*., 1999).

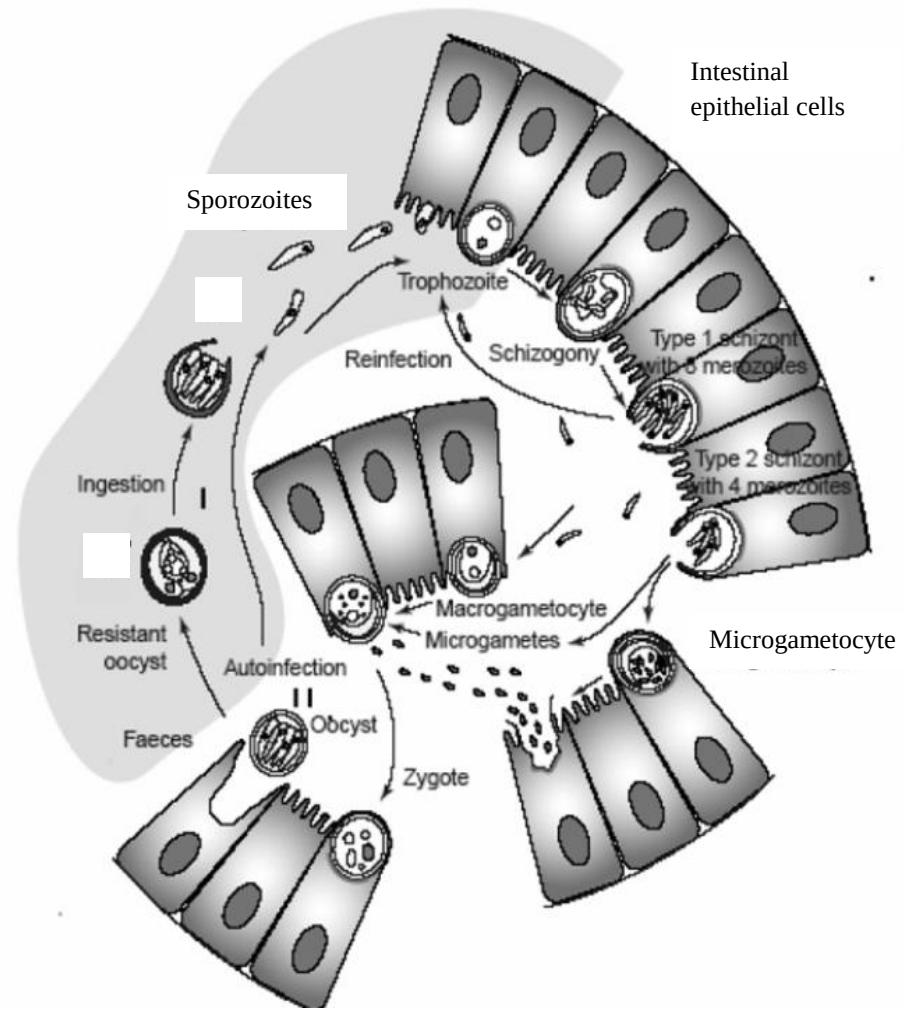


Figure 2.3

The life cycle stages of *Cryptosporidium* showing excysted and nonexcysted stages.

Sources: (Sunnotel *et al*., 2006, Smith *et al*., 2005, Tetley *et al*., 1998).

.2.4 Taxonomy

The taxonomy of *Cryptosporidium* depends on phenotypic characteristics such as host specificity, location of endogenous stages and oocyst morphology of endogenous or exogenous stages. For example, oocysts found in sheep feaces were named *Cryptosporidium agni* (Barker and Carbonell, 1974), others in human feces were named *Cryptosporidium garnhami* (Bird, 1981) or *Cryptosporidium enteriditis* (Qadripur and Klose, 1985). These and several other named species lacked taxonomic data that could clearly distinguish these species from all others (morphological, biological, and/or molecular data) and they became non-valid names. Morphological identification is difficult since *Cryptosporidium* species is identical. There are a large number of species being described with a history of confusion and controversy (Thompson, 2002). However currently, there are 26 species (Xiao *et al*., 2001, Cama *et al*., 2003, Gatei *et al*., 2006, Fayer, 2010b, Traversa, 2010, Bass *et al*., 2011, Kik *et al*., 2011) of *Cryptosporidium* that have thus far been identified and Table 2.8 shows the taxonomy with regards to host specificity.

Previously, *Cryptosporidium parvum* is thought to be the cause of all cryptosporidiosis in mammals (Tzipori *et al*., 1980). During this time, *C. parvum, C. parvum*-like or simply *Cryptosporidium* oocysts were isolated from many different animal species and humans. But the attention was focused following the onset of HIV AIDS reports which consider *Cryptosporidium* as opportunistic pathogens in humans.

On other hand, the genotyping tools recognized every species as unique and distinct and species name and status were also acquired. The canine genotype became *Cryptosporidium canis*, cat genotype *Cryptosporidium felis* pig genotype I

became *Cryptosporidium suis*, bovine B genotype became *Cryptosporidium bovis*, and the deer-like genotype became *Cryptosporidium ryanae*. This trend is strongly supported by genetic data and is expected to continue (Fayer, 2010b).

Table 2.8 *Cryptosporidium* species and their hosts. Adapted from Xiao and Fayer (2008)

Species	Hosts	References
Cryptosporidium parvum	Cattle, other ruminants, humans	(Tyzzer, 1912)
Cryptosporidium hominis	Human, monkey	(Morgan-Ryan *et al*., 2002)
Cryptosporidium meleagridis	Turkey, human	(Slavin, 1955)
Cryptosporidium cuniculus	Rabbit, human	(Inman and Takeuchi, 1979)
Cryptosporidium canis	Dogs	(Fayer *et al*., 2001)
Cryptosporidium felis	Cats	(Iseki, 1979)
Cryptosporidium suis	Pigs	(Ryan *et al*., 2004)
Cryptosporidium fayeri	Red kangaroo	(Ryan *et al*., 2008)
Cryptosporidium pestis	Cattle	(Šlapeta, 2006)
Cryptosporidium muris	Rodents	(Tyzzer, 1907)
Cryptosporidium andersoni	Cattle	(Lindsay *et al*., 2000)
Cryptosporidium wrairi	Guinea pigs	(Vetterling *et al*., 1971)
Cryptosporidium bailey	Poultry	(Current *et al*., 1986)
Cryptosporidium serpentis	Reptiles	(Levine, 1980)
Cryptosporidium galli	Birds	(Pavlasek, 1991)
Cryptosporidium saurophilum	Lizard	(Koudela and Modry, 1998)
Cryptosporidium molnari	Fish	(Alvarez-Pellitero and Sitj - Bobadilla, 2002)
Cryptosporidium ryanae	Deer	(Fayer, 2010b)
Cryptosporidium bovis	Calf	(Fayer *et al*., 2005, Barker and Carbonell, 1974)
Cryptosporidium scophthalmi	Fish	(Ryan *et al*., 2003)
Cryptosporidium macropodum	Kangaroo	(Fayer *et al*., 2009, Power and Ryan, 2008)
Cryptosporidium fragile	Frog	(Jirku *et al*., 2008)
Cryptosporidium reichenbachklinkei	Fish	(Paperna and Vilenkin, 1996)
Cryptosporidium nasoris	Fish	(Hoover *et al*., 1981)
Cryptosporidium varanii	Lizard	(Ryan *et al*., 2003)
Cryptosporidium cichlidis	Fish	(Paperna and Vilenkin, 1996)

2.2.5 Pathology and clinical manifestations

The developmental stages of *Cryptosporidium* can be identified at all levels of gastrointestinal tract. Although the jejunum is usually the most heavily infected site, the parasite can be found throughout the length of the colon, as well as the duodenum including the ampulla of vater, ileum, gall bladder, bile duct, and pancreatic duct. In immunocompromised patients, it causes severe and life threatening diarrhea. It also induces diarrhea in immunocompetent persons. In the small intestine and colon, the parasite may cause no histological abnormality but in some people it may be associated with histological acute enteritis or colitis with increase inflammatory cells in the lamina propria, or small intestinal villous blunting. Other clinical manifestations include nausea and vomiting, abdominal cramps, and fever. Hepatobiliary tract infection is a frequent complication of cryptosporidiosis and the spread of the parasite to hepatobiliary and the pancreatic ducts cause cholangiohepatitis, cholecystitis, choledochitis or pancreatitis. In children, cryptosporidiosis may be associated with subsequent impaired physical and cognitive development (Guerrant *et al*., 1999) even in the absence of diarrhea (Checkley *et al*., 1998, Checkley *et al*., 1997, Molbak, 1994).

In AIDS patients, cryptosporidiosis is one of the most serious opportunistic infections. An AIDS patient with CD4 T-cells counts of <150/ml, infected with *C. parvum* can develop persistent infection. The most severe extraintestinal complication of cryptosporidiosis in AIDS patients is secondary infection of the gall bladder, biliary tract, and pancreatic ducts. It has been estimated that 15% of AIDS patients with *Cryptosporidium* have hepatobiliary tract infection (Mahajani and Uzer, 1999). Cryptosporidial infection of the gall bladder is often diagnosed following

cholecystectomy for acalculous cholecystitis. In AIDS patients with chronic cryptosporidiosis, crypt abscess of the gut has been reported (Tzipori *et al*., 1995). *Cryptosporidium parvum* also seems to induce apoptosis in host cells (Certad *et al*., 2007).

Actually the immune response controls the severity and duration of clinical symptoms. The mechanism which causes and induces diarrhea by *Cryptosporidium* is still not clear. One of the possible causes of diarrhea i the impaired intestinal absorption and digestion due to disruption of microvillous (Bird and Smith, 1980).

The correlation between *C. hominis* and *C. parvum* in terms of symptoms showed no difference in children in Brazil (Bushen *et al*., 2007). It also reported that symptomatic children with *C. hominis* had greater long-term growth short falls (Bushen *et al*., 2007). Whilst another report showed variation in duration of symptoms and severity and was significantly greater with *C. hominis* than *C. parvum* (Hunter *et al*., 2004). A study in Peru found that children with *C. parvum* are more likely to have diarrhea, nausea, and vomiting than other *Cryptosporidium* species (Cama *et al*., 2008). These results demonstrate that different *Cryptosporidium* spp. are linked to different clinical manifestations in different human populations.

2.2.6 Epidemiology

2.2.6.1 Prevalence and demographic distribution

Cryptosporidiosis is reported to be widespread and the infection in developed countries ranged from <1 – 4.5% of individuals surveyed by stool examination while in developing countries the infection seemed to be much higher. The range in developing countries ranged from 5% in Asia to 10% in Africa (Bushen *et al*., 2006, Current, 1994).

Cryptosporidiosis among AIDS patients is the most common cause of persistent diarrhea in the U.S., but it has become less of a problem since the introduction of anti-retroviral therapy. Those with high risk of being infected by *Cryptosporidium* are children under two years of age, animal handlers, travelers to endemic areas, men who have sex with men, and close contacts of infected individuals. Drinking contaminated water was reported to be associated with outbreaks in daycare centers (Alpert *et al*., 1986). Besides that, outbreaks have been reported through contaminated swimming pools, lakes and ponds, and with drinking unpasteurized cider made from apples contaminated with cow manure (Millard *et al*., 1994). Dairy calves shed oocysts and the reported prevalence was 50% while more than 90% of dairy farms (Sischo *et al*., 2000) had *Cryptosporidium.* In developing nations, *Cryptosporidium* occurs mostly in children younger than 5 years (Samie *et al*., 2006, Sulaiman *et al*., 2005, Simango and Mutikani, 2004, Xiao *et al*., 2001, Abd El Kader *et al*., 2011). This could be attributed to the short-lived or incomplete acquired immunity to *Cryptosporidium* infection (Xiao *et al*., 2001, Newman *et al*., 1999).

In most developing countries, *C. parvum* and *C. hominis* account for 90% of human cases, with the remainder attributable to *C. meleagridis*, *C. canis* and *C. felis*.

Some areas, however, have a high prevalence of these unusual species. In Lima (Peru) and Bangkok (Thailand), *C. meleagridis* is as prevalent in humans as *C. parvum*, being responsible for 10–20% of human cryptosporidiosis cases (Xiao *et al*., 2001, Gatei *et al*., 2002, Cama *et al*., 2003, Cama *et al*., 2008).

The unusual species probably have anthroponotic transmission due to the concurrent presence of *C. hominis* in some of the *C. meleagridis, C. canis* or *C. felis* as well as *C. cuniculus* infected individuals (Chalmers *et al*., 2011a). A few other *Cryptosporidium* species and genotypes are occasionally found in humans, including *C. muris*, *C. suis, C. andersoni* and *Cryptosporidium cervine*, horse, rabbit, skunk and chipmunk I genotypes (Robinson *et al*., 2008, Xiao and Feng, 2008, Xiao *et al*., 2007b).

On the other hand, zoonotic transmission by *C. canis* between two siblings and a dog occurred in a household in a slum in Lima, Peru, however, the route of transmission was not clear (Xiao *et al*., 2007a). Cryptosporidiosis varies from one country to another where *C. hominis* is more prevalent in humans in developing country (Cordova Paz Soldan *et al*., 2006, Muthusamy *et al*., 2006, Samie *et al*., 2006, Ajjampur *et al*., 2007, Hung *et al*., 2007, Jex *et al*., 2008), whereas both *C. parvum* and *C. hominis* in Europe (Zintl *et al*., 2009, Šoba *et al*., 2006a, Llorente *et al*., 2007, Wolska-Kusnierz *et al*., 2007, Bajer *et al*., 2008, Savin *et al*., 2008, Wielinga *et al*., 2008, Chalmers *et al*., 2009, Leoni *et al*., 2006, Nichols *et al*., 2006). In the Middle East, *C. parvum* is the dominant species in humans (Sulaiman *et al*., 2005, Meamar *et al*., 2007, Tamer *et al*., 2007, Pirestani *et al*., 2008, Nazemalhosseini-Mojarad *et al*., 2011, Al-Brikan *et al*., 2008, Hijjawi *et al*., 2010). Geographic variations in the distribution of *C. parvum* and *C. hominis* can also occur within a country. For example, *C. parvum* is more common than *C. hominis* in rural states in the United States and Ireland (Zintl *et al*., 2009, Feltus *et al*., 2006).

There is also age-associated variation in the disease burdens between *C. parvum* and *C. hominis* (Chalmers *et al*., 2009).. In the Netherlands, *C. hominis* was more commonly found in children and *C. parvum* more in adults (Wielinga *et al*., 2008). In the United Kingdom, *C. hominis* was more prevalent in infants less than one year, females aged 15–44 years and international travelers, and there has been a decline in *C. parvum* cases since 2001 (Chalmers *et al*., 2009, Chalmers *et al*., 2008). There is also seasonal variation like in the earlier observations in the United Kingdom and New Zealand, *C. hominis* was more prevalent in autumn (in the Netherlands) and *C. parvum* was more prevalent in spring (in Ireland) in some recent studies (Wielinga *et al*., 2008, Zintl *et al*., 2009).

Most outbreaks of cryptosporidiosis related to waterborne, foodborne and direct contact are caused by *C. parvum* and *C. hominis* (Xiao *et al*., 2007b). Recently a report of *Cryptosporidium* rabbit genotype from England caused a waterborne outbreak (Chalmers *et al*., 2009b). In China, analysis of 50 sources of water samples, 32% were positive for *Cryptosporidium* and 18% for *Giardia* while *Cryptosporidium* was found in 2 tap water samples and molecular analysis showed *C. suis* , *C. baileyi, C. meleagridis* and *C. hominis* in water sources (Feng *et al*., 2011). They found farm animals, especially cattle and pigs, were the major sources of water contamination in Shanghai.

C. hominis and *C. parvum* are subdivided into different subtypes. *C. hominis* is subdivided into Ia–Ig by *gp60*. The subtype family Ib is dominant and broadly distributed while subtype families If and Ig are rare. *C. parvum* is subdivided into lla-llk and the most dominant is lla. Figure 2.9 shows the diversity of each subtype among humans. Among the subtypes, there are also richness in diversity and distribution variation in the number of TCA microsatellite region nucleotide repeat. *C. hominis*

subtype IbA10G2R2 is distributed globally (reported from 28 countries, representing all inhabited continents, except South America). The next most common recorded Ib subtype is *C. hominis* IbA9G3R2 (n=39: 6.1% of all published records for Ib). The subtype IbA9G3R2 has been reported from nine countries (all inhabited continents, except South America), suggesting that this subtype is also global in distribution. In contrast, the other *C. hominis* Ib subtypes identified to date are rare, based on available data that was reviewed by (Jex and Gasser, 2010). The subtypes la and ld are also rich in diversity and the global distribution of IaA12G1R1 is isolated from Japan, Nepal, Pakistan and Peru (Abe *et al*., 2006, Wu *et al*., 2003, Chalmers *et al*., 2008, Cama *et al*., 2008). Subtype IaA21G1R1 has been reported from Egypt, India, Malawi, South Africa, and Spain (Jex and Gasser, 2008, Peng *et al*., 2003, Leav *et al*., 2002). Within *C. hominis* subtype family Id, the most commonly recorded subtype is IdA15G1R1 reported from Australia, Europe, India and Pakistan (Jex and Gasser, 2010). The remaining subtypes of family Id are rare. The subtype Ie is of low subtypic richness and diversity. It has been reported 69 times, 3 subtypes have been described but 89.8% of all Ie reports are subtype IeA11G3T3R1. In addition to being the dominant Ie subtype, Ie A11G3T3R1 appears to be globally distributed (Jex and Gasser, 2010).

The subtype families of *C. parvum* display a greater richness and diversity than *C. hominis*. The subtype family IIa is the most commonly recorded for *C. parvum* (n=1541; 57.8% of all records) and is the second recorded in humans overall (n=345; 25.5% of all *gp60* sequence data linked to humans). Current data indicated that *C. parvum* subtype family IIa is globally distributed (reported from 26 countries). The subtype families IIc and IId are also common (58 and 160 records, respectively) and broadly distributed (reported from 11 and 13 countries, respectively). The other known

gp60 subtype families, namely IIb and IIe–k, are rare based on present datasets, each represented by a few records.

The subtype IIaA16G2R1 is the most reported subtype with prevalence of 24.6% of all IIa records and it is also reported from animals beside IIaA19G3R1 indicating a potential zoonotic transmission. Subtype IIaA17G1R1 is found in humans and cattle and IIaA18G2R1 has been recovered from humans or non-humans in different regions, and have been reviewed by Jex *et al.* (2010). Subtype IIcA5G3R2 is recorded only among humans. The *C. parvum* subtype families IIb and IIe–k have low 'subtypic richness' and have been reported exclusively from humans. A summary of the global diversity of *gp60* subtype families and subtypes for *C hominis* and *C. parvum*, and their relative prevalence in all hosts and in humans is specifically shown in Table 2.9.

Table 2.9 A summary of the global diversity of *gp60* subtype families and subtypes for *C. hominis* and *C. parvum*, and their relative prevalence in all hosts and in humans specifically. Modified from Jex *et al.* (2010).

Species	Subtype Family	Reports (n)	Subtypes (n)	Countries (n)	Dominance in humans(%)	Overall dominance (%)
C. hominis	Ia	89	25	19	3.3	6.6
	Ib	641	12	36	24.1	47.4
	Id	75	23	18	2.8	5.6
	Ie	69	3	15	2.6	5.1
	If	15	9	6	0.6	1.1
	Ig	2	1	2	0.1	0.1
C. parvum	IIa	1541	50	26	57.8	25.5
	IIb	3	2	1	0.1	0.2
	IIc	58	1a	11	2.2	4.3
	IId	160	17	13	6.1	3.6
	IIe	2	2	2	0.1	0.1
	IIf	1	1	1	<0.1	<0.1
	IIh	1	1	1	<0.1	<0.1
	IIi	3	2	1	0.1	0.2
	IIj	2	2	2	0.1	
	IIk	1	1	1	<0.1	

n: number of cases

Table 2.10 Distribution of *C. parvum* subtypes in different countries modified from Xiao, (2010)

Country	IIa	IIc	IIb	IId	IIe	Others	Total	Reference
South Africa	0	5	0	0	0	0		(Leav *et al*., 2002)
Malawi	0	1	0	0	1	0		(Peng *et al*., 2003)
Uganda	0	10	0	0	0	5		(Akiyoshi *et al*., 2006)
Madagascar	0	1	0	0	0	0		(Areeshi *et al*., 2008)
Ethiopia	6	0	0	0	0	0		(Gelanew *et al*., 2007b)
Nigeria	0	2	0	0	0	0		(Ayinmode *et al*., 2012)
Total in Africa	**6**	**19**	**0**	**0**	**1**	**5**	**31**	
India	0	3	5	0	0	1		(Muthusamy *et al*., 2006)
India	0	7	0	0	0	0		(Ajjampur *et al*., 2007)
Bangladesh	0	0	0	0	0	2		(Hira *et al*., 2011)
Malaysia	0	0	0	1	0	0		(Lim *et al*., 2011)
Malaysia	12	0	0	1	0	0		(Iqbal *et al*., 2012)
Australia	6	0	0	0	0	0		(Chalmers *et al*., 2005)
Australia	23	1	0	0	0	0		(Jex *et al*., 2008)
Australia	18	5	0	0	0	0		(Jex *et al*., 2007)
Australia	4	0	0	0	0	0		(Ng *et al*., 2008)
Australia	8	1	0	0	0	0		(O'Brien *et al*., 2008)
Australia	30	1	0	1	0	0		(Waldron *et al*., 2009)
Australia	5	0	0	1	0	1		(Ng *et al*., 2012)
Japan	1	1	0	0	0	0		(Abe *et al*., 2006)
Kuwait	28	2	0	0	29	1		(Sulaiman *et al*., 2005)
Kuwait	29	10	0	19	0	3		(Iqbal *et al*., 2011)
Jordan	3	0	2	8	0	1		(Hijjawi *et al*., 2010)
Iran	6	0	0	17	0	0		(Taghipour *et al*., 2011)
Iran	7	0	0	15	0	0		(Nazemalhosseini-Mojarad *et al*., 2011)
Saudi Arabia	1	2	0	34	0	0		(Nazemalhosseini-Mojarad *et al*., 2012)
Pakistan	0	0	1	0	0	0		(Jex & Gasser, 2008)
Total in Asia	**181**	**33**	**8**	**97**	**29**	**9**	**357**	

Continued Table 2.10

Country	IIa	IIc	IIb	IId	IIe	Others	Total	Reference
Italy	4	4	0	0	0	0		(Del Chierico *et al*., 2011)
Ireland	78	0	0	2	0	0		(Zintl *et al*., 2009)
Slovinia	29	1	0	0	0	1		(Soba and Logar, 2008)
Portugal	9	7	1	8	0	0		(Alves *et al*., 2006)
Portugal	3	0	1	4	0	0		(Alves *et al*., 2003)
UK	4	0	0	0	0	0		(Ng *et al*., 2008)
UK	56	0	1	9	0	0		(Chalmers *et al*., 2011b)
Belgium	4	0	1	1	0	5		(Geurden *et al*., 2009)
The Netherlands	13	9	1	0	3	0		(Wielinga *et al*., 2008)
Spain	1	0	1	1	0	0		(Jex & Gasser, 2008)
Total in Europe	**201**	**21**	**6**	**25**	**0**	**6**	**259**	
Jamaica	0	7	0	0	0	0		(Gatei *et al*., 2008)
Guatemala	0	1	0	0	0	0		(Xiao *et al*., 2004a)
Peru	0	22	0	0	0	0		Cama *et al.* 2007
Peru	0	15	0	0	0	0		(Cama *et al*., 2008)
Total in South America	**1**	**45**	**0**	**0**	**0**	**0**	46	
Canada	4	0	0	0	0	0		(Trotz-Williams *et al*., 2006)
USA	30	0	0	0	0	0		(Feltus *et al*., 2006)
USA	1	5	0	0	0	0		(Xiao *et al*., 2004b)
USA	1	0	0	0	0	0		(Jex & Gasser, 2008)
Total in North America	**36**	**5**	**0**	**0**	**0**	**0**	41	

*Data represent the open access of online articles and reviews.

Table 2.11 Distribution of *C. hominis* in different regions*

Country	Ia	Ib	Ic	Id	Ie	If	Ig	Total	Reference
South Africa	0	3	0	5	5	0	0		(Leav *et al.* 2002)
Malawi	4	5	1	11	6	0	0		(Peng *et al*., 2003)
Uganda	4	1	0	1	7	0	0		(Akiyoshi *et al*., 2006)
Ethiopia	0	1	0	0	0	0	0		(Gelanew *et al*., 2007b)
Nigeria	2	1	0	0	0	0	0		(Ayinmode *et al*., 2012)
Egypt	1	0	0	0	0	0	0		(Jex & Gasser, 2008)
Total in Africa	**11**	**11**	**1**	**17**	**18**	**0**	**0**	**58**	(Jex & Gasser, 2008)
India	6	8	3	4	0	8	0		(Muthusamy *et al.* 2006 and Rao *et al.* 2007)
India	35	0	3	8	0	0	0		(Ajjampur *et al.*, 2007)
Pakistan	1	1	0	3	4	1	0		(Jex & Gasser, 2008)
China	1	6	0	2	0	0	0		(Wang *et al*., 2010)
Japan	1	1	0	0	1	0	0		(Abe *et al*., 2006)
Australia	0	53	0	6	1	1	3		(Ng *et al*., 2008)
Australia	2	28	0	5	1	1	0		(Waldron *et al*., 2009)
Malaysia	2	3	0	0	0	0	0		(Lim *et al*., 2011)
Malaysia	3	2	0	0	0	0	0		(Iqbal *et al*., 2012)
Bangladesh	5	4	0	2	9	4	0		(Hira *et al*., 2011)
Jordan	0	8	0	7	0	0	0		(Hijjawi *et al*., 2010)
Iran	0	0	0	0	0	2	0		(Taghipour *et al*., 2011)
Iran	0	0	0	1	0	1	0		(Nazemalhosseini-Mojarad *et al*., 2011)
Kuwait	0	2	0	1	0	0	0		(Sulaiman *et al*., 2005)
Turkey	0	3	0	0	0	0	0		(Jex & Gasser, 2008)
Total in Asia	**56**	**119**	**6**	**39**	**16**	**18**	**3**	**255**	

Table 2.11 Continued

Country	Ia	Ib	Ic	Id	Ie	If	Ig	Total	Reference
Portugal	1	1	0	1	1	1	0		Alves *et al.* 2006
Portugal	0	5	5	0	1	1	0		Alves *et al.* 2003
UK	0	2	0	1	0	0	0		(Ng *et al.*, 2008)
Peru	0	22	0	0	3	0	0		Cama *et al.* 2007
Denmark	0	1	0	0	0	0	0		(Jex & Gasser, 2008)
UK	1	13	0	1	0	0	0		(Jex & Gasser, 2008)
France	0	2	0	0	0	0	0		(Jex & Gasser, 2008)
Greece	0	5	0	0	0	0	0		(Jex & Gasser, 2008)
Holland	0	1	0	0	0	0	0		(Jex & Gasser, 2008)
Spain	1	15	0	0	0	0	0		(Jex & Gasser, 2008)
Turkey	0	3	0	0	0	0	0		(Jex & Gasser, 2008)
Total in Europe	**3**	**48**	**5**	**3**	**1**	**2**	**0**	**62**	
Peru	0	22	0	0	3	0	0		Cama *et al.* 2007
Total in South America	2	22	0	0	3	0	0		
Canada	2	0	0	1	1	0	0		(Trotz-Williams *et al.*, 2006)
USA	0	2	0	0	0	0	0		(Jex & Gasser, 2008)
Total in North America	2	2	0	1	1	0	0	**6**	

*Data represent the open access of online articles and reviews.

2.2.6.2 Transmission

The transmission routes of *Cryptosporidium* to humans include several ways, direct contact with infected persons (person-to-person transmission) or animals (zoonotic transmission) and ingestion of contaminated food (foodborne transmission) and water (waterborne transmission). *Cryptosporidium* has the ability of infecting various animals and the ubiquitous presence of oocysts in the environments. Interestingly, a study in Egypt on the currency coins and banknotes found that 60.2% of 103 banknotes and 56.6% of 99 coins obtained from food-related workers had been contaminated with one or more parasitic species. Protozoa were the predominant parasites, with microsporidia and *Cryptosporidium* spp. being more prevalent (Hassan *et al*., 2011). This study pointed to the transmission of parasites which may occur indirectly via inanimate objects in the surrounding environment. On the other hand, a report of food outbreak from South Carolina in summer camp showed that eating ham from a sandwich bar that included camp-grown raw produce and sharing a cabin with an ill person were significantly associated with illness. *Cryptosporidium* isolates from stool specimens of livestock and humans at the camp were of the *C. parvum* subtype, IIaA17G2R1, indicating that zoonotic transmission had occurred (Collier *et al*., 2011). A study showed that filth flies can be carrier for infection with *C. parvum* oocysts from unsanitary sites, deposit them on visited surfaces, and therefore may be involved in human or animal cryptosporidiosis (Graczyk *et al*., 2000). Most commonly, zoonotic transmission occurs as a consequence of cryptosporidiosis in neonatal calves and lambs, which can excrete up to 10^9 oocysts/g faeces (the environmentally resistant and infective form of the parasite). Once in the environment, infectious oocysts can contaminate water sources and food (Smith and Nichols, 2010).

Studies in slums in Peru and Brazil have shown that children infected with *C. hominis* have higher oocyst shedding intensity and longer duration diarrhea than those infected with *C. parvum* and other genotypes (Xiao *et al*., 2001; Bushen *et al*., 2007).

A. Zoonotic transmission

Farm animals are commonly infected with three other *Cryptosporidium* spp. in addition to *C. parvum*, including *C. andersoni*, *C. bovis*, and *C. ryanae*. The most frequent species and genotypes of *Cryptosporidium* among animals is shown in Table 2.12. Studies in the United States showed occurrence of *Cryptosporidium* spp. was related to the age of animals. In dairy cattle, *C. parvum* was mostly found in pre-weaned calves, *C. bovis* and *C. ryanae* in weaned calves and *C. andersoni* in young and adult cattle while only pre-weaned calves were major source of *C. parvum* (Fayer *et al*., 2006b, Fayer *et al*., 2007, Santin *et al*., 2008). Pre-weaned lambs in Australia were more infected by *C. parvum* than older animals (Yang *et al*., 2009). Few studies have genotyped *Cryptosporidium* from goats. In one study in Belgium, *C. parvum* was the only species found in 11 goat kids (Geurden *et al*., 2008b). *Cryptosporidium hominis* was the only species identified in three goats, five humans, and six cattle samples in South Korea (Park *et al*., 2006).

Many of the common bovine IIa subtypes in North America, Europe and Australia are also dominant *C. parvum* subtypes in humans in these areas (Alves *et al*., 2006, Jex *et al*., 2008, Jex *et al*., 2007, Waldron *et al*., 2009, Zintl *et al*., 2009, Feltus *et al*., 2006). For example, the predominant *C. parvum* subtype in calves in Portugal, Slovenia and the Netherlands, IIaA15G2R1, is also the major *C. parvum* subtype in humans in these countries (Alves *et al*., 2006, Soba and Logar, 2008, Wielinga *et al*.,

2008). Likewise, humans and animals (calves) found to be infected with the same subtypes IIa with dominance of IIaA18G3R1, that have been reported in the Republic of Ireland and Northern Ireland (Thompson *et al*., 2007, Zintl *et al*., 2009).

Another less common bovine *C. parvum* subtype family, IId, is found to be responsible for some zoonotic infections in Europe. In Portugal, IId subtype was recovered from the 4 cases of HIV positive persons while two of these subtypes were reported previously in calves and lambs in the same area (Alves *et al*., 2006). These subtypes have been reported in humans in Netherlands, Ireland and Australia (Wielinga *et al*., 2008, Waldron *et al*., 2009, Zintl *et al*., 2009). Another *C. parvum* subtype family in calves in Europe, IIl, has also been found occasionally in humans in Slovenia (Soba and Logar, 2008). All these were further indicators of differences in the role of zoonotic transmission of *C. parvum* among geographic areas.

Table 2.12
Species and genotypes of *Cryptosporidium* found in cattle and locations where they have been detected. Adapted from Santín and Trout (2008).

Cryptosporidium species/genotypes	Prevalence	Geographical location
C. parvum	Frequent	Worldwide
C. bovis	Frequent	Worldwide
C. andersoni	Frequent	Worldwide
C. ryanae	Frequent	Worldwide
C. hominis	Rare	Scotland, India, Korea
C. suis	Rare (2 calves)	USA and Zambia
C. suis-like	Rare (3 cattle)	Denmark
Pig genotype II	Rare (1 cow)	Denmark
C. felis	Rare (1 cow)	Poland
C. canis	Experimental infection only	USA

B. Water transmission

Cryptosporidium oocysts are frequent contaminants of water, with contributions from infected human and non-human hosts, livestock and agricultural practices, and infected feral and transport hosts (Smith *et al*., 1995, Smith and Grimason, 2003). Water is an important transmission route, with at least 165 waterborne outbreaks of cryptosporidiosis documented (Insulander *et al*., 2005, Karanis *et al*., 2007). A major outbreak of cryptosporidiosis affecting >400 000 persons occurred in Milwaukee, WI, USA (Mac Kenzie *et al*., 1994). This outbreak brought attention of water contamination by *Cryptosporidium* because of the high infection rate and association with drinking treated water. This outbreak highlighted the significance of drinking contaminated water as a major risk factor for contracting cryptosporidiosis in the USA.

Oocysts occur at low densities in water (Smith and Rose, 1998, Smith *et al*., 1995, Smith and Grimason, 2003). Oocysts are resistant to numerous disinfectants normally used in water treatment, and water is an important component of food production from crop irrigation, harvesting, sorting, to storage and distribution. In the food industry, potable water, uncontaminated with infectious oocysts, is needed for the preparation of ready-to-eat food and for the dilution of beverages. Oocysts can enter the food chain from agricultural practices such as muck-spreading and slurry spraying of oocyst-contaminated, animal-derived faecal material onto land used for cultivation and from animals pasturing near crops intended for human consumption. The use of contaminated, untreated sewage (and waste stabilization pond) effluents and untreated water for crop irrigation can also contaminate crops. Runoff from, and percolation through, contaminated pasture and soils can contaminate adjacent water bodies, and

oocysts transported into rivers and marine estuaries can lead to the contamination of shellfish, which are frequently eaten raw or lightly cooked (Smith and Nichols, 2010).

Genotyping methods for discriminating pathogens isolated from water borne outbreaks can help epidemiologists to trace the source of contamination. They also provide information about the public health significance of *Cryptosporidium* oocyst in watersheds (Thompson, 2000). In UK and USA, water sources are treated and the amount of oocysts in processed water should not exceed one oocyst in 10 *l* (Lloyd and Drury, 2002).

The oocysts are infectious immediately upon being excreted in faeces. The infectious dose is low; therefore ingestion of as few as 10–30 oocysts can cause infection in healthy persons (DuPont *et al*., 1995, Okhuysen *et al*., 1999). A single bowel movement from an infected person can contain 10^8–10^9 oocysts. Infected persons can excrete oocysts for up to 50 days after cessation of diarrhea (Jokipii and Jokipii, 1986). These parameters beside that the *Cryptosporidium* documented high resistance to chlorine disinfection (Korich *et al*., 1990, Shields *et al*., 2008b) make *Cryptosporidium* commonly transmitted via water (e.g., swimming pools, drinking water systems), where chlorine is the major public health barrier to pathogen transmission.

2.2.6.3 Risk factors

Infections with *Cryptosporidium* are either detected in sporadic cases or in outbreaks. In recent years, case-control studies performed in the US, UK, and Australia have used multivariate analysis to identify risk factors associated with sporadic cryptosporidiosis (Roy *et al*., 2004, Robertson *et al*., 2002, Hunter *et al*., 2004). Those studies were found

to share two results which is contact with person who has diarrhea and contact with animals especially calves (Roy *et al*., 2004, Hunter and Thompson, 2005b). The US and UK studies also found travel abroad as a risk factor (Roy *et al*., 2004, Hunter and Thompson, 2005b). In addition, swimming in fresh water and in a chlorinated swimming pool were risk factors in US and Australia. Both the UK and Australian studies identified a dose dependent risk in drinking unboiled drinking water and this finding was also reported in a regional study in the UK (Goh *et al*., 2004). In a massive case control study in UK where over 3000 stool samples were tested, found a significant association with clinical symptoms and they reported high prevalence of *C. hominis* in urban area with higher socioeconomic background while in contrast the *C. parvum* found in area with poor water treatment. This area used manure applied on the land and highly contaminated with *Cryptosporidium* oocysts (Lake *et al*., 2007a).

Contact with animals was found to be a significant risk for cryptosporidiosis in several studies. In 2001, there was a massive outbreak in England. About a million animals were slaughtered and heavy restrictions were placed on access to the country side. During the period of restriction, the number of cryptosporidiosis were reduced to 82% cases which indicated that there was association between cattle contact and human disease in the UK (Hunter *et al*., 2003).

The improvement in drinking water treatment also play a role in protecting and decreasing number of cryptosporidiosis cases caused by *C. parvum* from ruminants (Lake *et al*., 2007b, Sopwith *et al*., 2005). In the US, longitudinal study on human found that 46/49 cases were of the zoonotic *C. parvum* and the cervine type. Nine different subtypes of *C. parvum* were found, all of which had been previously found in cattle in the surrounding region (Feltus *et al*., 2006). This finding is supported by

surveillance data which shows that Wisconsin has a relatively high incidence of cryptosporidiosis but few cases are associated with outbreaks. Zoonotic transmission from cattle appears to be an important transmission route in Wisconsin (Feltus *et al*., 2006).

Since *Cryptosporidium* cannot multiply in food, foodborne cryptosporidiosis resulted from contamination of food by faecal product either through carrier worker or contamination of water and biosolids during preparation of food. Other factors include unhygienic food production, storage, processing or preparation processes. Several studies have found *Cryptosporidium* oocysts on products at markets in Costa Rica, Norway, and Peru (Robertson and Gjerde, 2001, Ortega and Adam, 1997, Monge and Arias, 1996). Vegetables have also been found to be contaminated with *Cryptosporidium* by water or through handling by ill workers, beside the wild animals faeces (Thurston-Enriquez *et al*., 2002, Chaidez *et al*., 2005). Multiple studies reported that the consumption of raw vegetables, particularly carrots, is a protective factor (Roy *et al*., 2004, Hunter *et al*., 2004, Robertson *et al*., 2002). The rationale of this observation was that the high fiber diet and the ingestion of small amount of *Cryptosporidium* oocyst increase the immunity against the invasion of this parasite in the small intestine. A study in Peru showed that *C. hominis* was associated with diarrhea, nausea, vomiting, general malaise, and increased oocyst shedding intensity and duration. In contrast, *C. parvum*, *C. meleagridis*, *C. canis*, and *C. felis* were associated with diarrhea only (Cama *et al*., 2008).

2.2.7 Genotyping

Different types of molecular diagnostic tools have been used in the differentiation of *Cryptosporidium* species/genotypes. For genotyping analysis, several genetic loci have been targeted such as ssU rRNA (Leng *et al*., 1996, Xiao *et al*., 1999a), internal transcribed spacer (ITS1) (Carraway *et al*., 1996), oocyst wall protein (COWP) (Spano *et al*., 1997), polythreonine repeat (polyT) (Carraway *et al*., 1996), dihydropholate reductase (DHFR) (Gibbons *et al*., 1998), thrombospondin- related adhesive protein 1 (TRAP-C1), thrombospondin-related adhesive protein 2 (TRAP-C2) (Spano *et al*., 1998, Sulaiman *et al*., 1998) and 70 KDa heat shock protein (HSP70) (Gobet and Toze, 2001).

Small subunit ssu rRNA based tools are now generally used in genotyping *Cryptosporidium* in humans, animals and water samples. The multi-copy nature and presence of semi-conserved and hyper-variable regions of ssu rRNA gene make it suitable for many researchers. The decrease in using other genes such as COWP gene which is based on the oocyst protein is due to its narrow specificity. The ssu rRNA method amplifies DNA of *C. parvum*, *C. hominis*, *C. meleagridis*, and species/genotypes closely related to *C. parvum* (Gómez-Couso *et al*., 2007, Meamar *et al*., 2007, Wolska-Kusnierz *et al*., 2007, Bajer *et al*., 2008, Duranti *et al*., 2009, Giangaspero *et al*., 2009).

The most popular gene used in subgenotyping is the 60 kDa glycoprotein (*gp60*, also called gp40/15). The *gp60* gene is similar to a microsatellite sequence by having tandem repeats of the serine-coding trinucleotide TCA, TCG or TCT at the 50 (gp40) end of the gene. Besides the trinucleotide repeat, there is also difference in non –repeat regions, which categorizes *C. parvum* and *C. hominis* into several subtype families.

Within each subtype family, subtypes differ from each other mostly in the number of trinucleotide repeats (TCA, TCG or TCT microsatellite).

The name of *gp60* subtypes starts with the subtype family designation (Ia, Ib, Id, Ie, If, etc. for *C. hominis*, and IIa, IIb, IIc, IId, etc. for *C. parvum*) followed by the number of TCA (represented by the letter A), TCG (represented by the letter G), or TCT (represented by the letter T) repeats (Sulaiman *et al*., 2005). Major *gp60* subtype families and representative sequences are shown in Table 2.13.

Thus, the name IbA10G2 indicates that parasite belongs to *C. hominis* subtype family Ib and has 10 copies of the TCA repeat and two copies of the TCG repeat in the trinucleotide repeat region of the *gp60* gene. In the *C. parvum* IIa subtype family, a few subtypes also have two copies of the ACATCA sequence right after the trinucleotide repeats, which are represented by ‘‘R2” (R1 for most subtypes). Additionally, within the *C. hominis* subtype family Ia, subtypes are further identified by the copy number of a 15-bp repetitive sequence (represented by the letter R) 5 AA/GGACGGTGGTAAGG-3 (the last copy is 13-bp: AAA/GACGGTGAAGG) that is located shortly downstream of the trinucleotide repeats. Therefore, the name IaA28R4 indicates that parasite belongs to *C. hominis* subtype family Ia, and has 28 copies of the TCA repeat in the trinucleotide region and four copies of the 13–15 bp repeat. In the case of *C. parvum* subtype family IIc, all subtypes have five copies of TCA and three copies of TCG repeats: IIcA5G3. They differ from each other in the nucleotide sequence of the 30 region of the gene. The original *gp60* sequence for *C. parvum* subtype family IIc (GenBank Accession No. AF164491) was assigned as IIcA5G3a. Subtypes that diverge from this sequence are assigned subsequent alphabetical extensions. For example subtype IIcA5G3b has a trinucleotide deletion (ACA) shortly after the trinucleotide repeats and 31 nucleotide substitutions whereas subtype IIcA5G3c has 33 nucleotide

substitutions. It should be kept in mind that *gp60* and other subtyping tools (including the multilocus typing and multilocus sequence typing tools below) do not amplify DNA of *C. felis, C. canis,* and other species distant from *C. parvum* and *C. hominis*.

The *gp60* gene is widely used because it is the single most polymorphic marker identified so far in the *Cryptosporidium* genome (Gatei *et al*., 2006a-b, Leoni *et al*., 2007, Wielinga *et al*., 2008). Other subtyping targets, such as double stranded RNA, internal transcribed spacer-2 and traditional microsatellites and minisatellites are generally considered non-functional while *gp60* is located on the surface of apical region of invasive stages of the parasite, and is one of the dominant targets for neutralizing antibody responses in humans (O'Connor *et al*., 2007). Thus, it is possible to link biologic characteristics of the parasites and clinical presentations with the subtype family identity. Subtypes families of *C. parvum* found in animals and humans such as IIa and IId indicate the possibility of zoonotic transmission. In Spain, it is found to be of high prevalence in calves and IId mostly isolated from goats and lambs (Quilez *et al*., 2008a, Quilez *et al*., 2008b). The subtype IIc is only found in human (Xiao and Feng, 2008, Alves *et al*., 2003). There are also significant differences in clinical presentations and virulence among some common *C. hominis* subtype families in cryptosporidiosis endemic areas (Cama *et al*., 2007, Cama *et al*., 2008).

The multilocus analysis increases the subtyping resolution and there are two types of technique used for that. The recent whole genome sequencing of *C. parvum* and *C. hominis* has allowed the identification of microsatellite and minisatellite sequences in *C. parvum* and *C. hominis* genomes and other highly polymorphic targets in *C. parvum* and *C. hominis*. There are two types of techniques used in the subtyping, multilocus typing (MLT), which allows the use of many targets in the MLT techniques economically. The variations in microsatellites and minisatellites are assessed on the

basis of length variations using polyacrylamide gel electrophoresis or the GeneScan technology (Ngouanesavanh *et al*., 2006, Tanriverdi *et al*., 2006, Tanriverdi and Widmer, 2006, Leoni *et al*., 2007, Morrison *et al*., 2008). The second type of typing techniques, multilocus sequence typing (MLST), relies on the detection of genetic heterogeneity by DNA sequencing of the amplified PCR products (Cama *et al*., 2006b, Gatei *et al*., 2006a-a, Gatei *et al*., 2006, Gatei *et al*., 2008). It allows the detection of length polymorphism and nucleotide substitution in microsatellite and minisatellite markers and the inclusion of other markers with only single nucleotide polymorphisms (SNP).

The PCR technique used in genotyping and subtyping has problem in detecting small amount of oocyst genome in addition to failure in detecting the different genotypes in the same sample. All narrowly specific genotyping and subtyping tools detect only *C. hominis*, *C. parvum* and species or genotypes related to them. It results in the failure to detect many concurrent infections with mixed *Cryptosporidium* species/genotypes, whereas this mixed of *Cryptosporidium* species/genotypes leads to the failure in detecting other divergent species/genotypes and the inability to subtype these parasites. Another problem faced when utilizing PCR tools is that the technique chosen will affect the results greatly. As an example of the effect of the technique was on a study on HIV patients in Peru, *C. canis* and *C. felis* have been detected using ssu rRNA-based PCR-RFLP genotyping tools but after reanalysis the specimen using different PCR tool which was COWP- and dihydrofolate reductase-based PCR-RFLP genotyping tools, it failed to detect *C. canis* and *C. felis* and identified *C. hominis*, *C. parvum* or *C. meleagridis*. So there was a difference in the genotype identified and also on the number of positive cases. Therefore, the type of molecular diagnostic tools used

and their order of usage would greatly affect the results of molecular epidemiologic investigations of cryptosporidiosis transmission (Cama *et al*., 2006a).

PCR tools also do not provide information of infectivity or viability of the oocyst. Therefore, using other techniques like Reverse-Transcriptase PCR (RT-PCR) or adding indirect methods could help to overcome this problem. The Reverse-Transcriptase PCR relies on the integrity of mRNA which helps in detecting the viability of the oocyst by targeting the heat shock protein (hsp) 70 gene. Moreover, with the recent introduction of real-time PCR, it allows the continuous monitoring of amplicon formation throughout the reaction. Quantitative aspect of the infection could be studied with exquisite sensitivity. This will, for example, allows (1) the detection of carrier states, (2) determination of the number of oocysts present in a sample, (3) quantitative aspects of gene expression during the various phases of the infection (Caccio, 2004).

Table 2.13

Major *gp60* subtype families and representative sequences. Modified from Xiao *et al*, (2010).

Species	Subtype family	Dominant trinucleotide repeat	Other repeat (R)
C. hominis	Ia	TCA	AA/GGACGGTGGTAAGG
		TCA, TCG, TCT	
	Ib	TCA, TCG	
		TCA, TCG, TCT	
	Id	TCA, TCG	
		TCA	
	Ie		
	If		
	Ig		
C. parvum	IIa	TCA, TCG	TCA, TCG ACA
	IIb	TCA	
	IIc	TCA, TCG	
	IId	TCA, TCG	
	IIe	TCA	
	IIf	TCA	
	IIg	TCA, TCG	
	IIh	TCA	
	IIi	TCA	
	IIk	TCA	

2.2.8 Diagnosis

The microscopic identification of oocyst after staining by modified acid fast stain which is easy to perform and low in cost, it is the routine technique to screen *Cryptosporidium* (Arrowood, 1997). Using fluorescent stain (auramine-rhodamine) enhances the sensitivity of microscopic method but sometimes it may give false positive result (Arrowood, 1997). The tissue sample of biopsy from intestine of patient is a useful histological investigation for confirmation of the diagnosis on post-mortem using haematoxylin and eosin stains.

Anti-cryptosporidial IgM, IgG, and IgA can be detected by the enzyme-linked immunoabsorbent assay (ELISA) or by the antibody immunofluorescence assay (IFA), but unfortunately neither of these assays can provide a direct diagnosis of cryptosporidiosis. Although ELISA showed sensitivity ranging from 66% to 100%, false positive result have been reported (Garcia *et al*., 2003, Arrowood, 1997).

Rapid immunochromatographic cartridge can be used on preserved specimens. It is easy and quick to perform. Low sensitivity is the problem where not all positive cases can be detected either in repeated samples, while asymptomatic host does not mean the absence of the parasite (Garcia *et al*., 2003, Johnston *et al*., 2003). In this case and when the clinical suspicion is high, the oocyst negative stool samples should be subjected to antigen and/or PCR-based detection, as sufficient *Cryptosporidium* antigen or DNA from asexual life cycle forms should be present in faeces. PCR and RFLP and/or sequencing can be used to determine some or all *Cryptosporidium* species/genotypes or subtypes. The typing and subtyping systems can be used for veterinary, human and for environmental samples, to avoid any confusion arising from using different systems during the investigation of disease outbreaks with both veterinary and public health implications. However the sensitivity of subtyping systems

will need to be increased so that they can be used for clinical and environmental samples containing small numbers (<10) of oocysts. Specimens for primary diagnosis should be collected during acute infection, and should be processed as soon as possible, ideally, within 24 hours. Transportation to the laboratory should be in accordance with the International Air Transport Association regulations.

There are no international standards for the preparation of purified oocysts, antisera, antigens, monoclonal antibodies or hybridomas, although a variety of purified oocysts and coproantigen detection kits using monoclonal antibodies are available commercially.

It must be noticed that the sensitivity of PCR is affected by inhibitors present in the faeces. Stool samples containing *Cryptosporidium* oocysts can be stored at 4°C in either 2.5% potassium dichromate or 10% formalin for reference purposes. Similarly, oocyst-positive faecal smears, air dried and fixed in absolute methanol, can be prepared in advance from previous positive samples for use as positive controls. Where bronchio-pulmonary involvement is suspected, similar tests can be performed on bronchial and pleural exudates or lavages. Oocyst positive stool samples or partially purified oocysts stored in 2.5% potassium dichromate and intended for nucleic acid amplification by PCR should be washed in deionised water to remove its residual prior to DNA extraction. A series of three washes each followed by centrifugation (3000 g for 10 minutes), removal of the supernatant and resuspension of the pellet in deionised water should minimise PCR inhibition. It should be noted that inhibitory factors can still be present even after long-term (>6 months) storage (Oie, 2008).

2.2.9 Treatment

There is no commonly advised specific treatment for cryptosporidiosis, and recovery usually depends on the health of the immune system. Most healthy people recover within two weeks without medical attention. If patients have a compromised immune system, the illness may be prolonged leading to significant malnutrition and wasting. The goal of treatment is to alleviate symptoms and improve the immune response. The management of dehydration is by supplementation of nutrient and fluid (Carneiro-Filho *et al*., 2003, Kotier *et al*., 1990). *Cryptosporidium* treatment options include: anti-motility agents. These medications slow down the movements of the intestines and increase fluid absorption to relieve diarrhea and restore normal stools (anti-motility drugs include loperamide and its derivatives (Imodium A-D, others), anti-parasitic drugs include (nitazoxanide, paromomycin, macrolide, spiramycin, azithromycine and refaxmin). Medications such as nitazoxanide (Alinia) can help alleviate diarrhea by attacking the metabolic processes of *Cryptosporidium*. Broad-spectrum anti parasitic drug has been approved for treating cryptosporidiosis and giardiasis in the USA (White and Clinton, 2004, Huang *et al*., 2004). Azithromycin (Zithromax) may be given along with one of these medications in people with compromised immune systems. Azithromycin acts by binding the 50S ribosomal subunit of susceptible microorganisms, thus interfering with microbial protein synthesis (Giacometti *et al*., 1999).

The use of highly active antiretroviral therapy (HAART) in people with AIDS has dramatically reduced the prevalence of cryptosporidiosis and the length and severity of its clinical course. This effect is attributed to the recovery of host immunity, as demonstrated in other cases of cryptosporidiosis associated with immunodeficiencies such as primary immunodeficiencies, organ transplantation, cancer, diabetes and

malnutrition for which antiretroviral therapy is not indicated. A prime example is the extremely high prevalence of cryptosporidiosis in the setting of X-linked immunodeficiency with hyper-immunoglobulin M, a rare form of primary immunodeficiency disease, whereby T lymphocytes cannot induce B cells to undergo immunoglobulin class-switching from immunoglobulin M (IgM) to IgG, IgA and IgE. In this specific case, the key mediator of the immune response to *Cryptosporidium* is the production of interferon-gamma. Some studies using protease inhibitors such as ritonavir, saquinavir and indinavir claim a drastic reduction of *C. parvum* infection both *in vivo* and *in vitro* (Hommer *et al*., 2003, Mele *et al*., 2003)

Whether or not aspartyl proteases could have some important functions is not known, as there are no reports of its presence in *C. parvum*. Despite the reduction of opportunistic infection in patients with AIDS under HAART, opportunistic infections continue to be the most important cause of death in HIV-infected individuals. While HAART should increase patients' CD4+ cells above risk thresholds, concomitant targeting of the opportunistic infection remains important in order to prevent ongoing morbidity.

CHAPTER III

METHODOLOGY

3.1 STUDY AREA AND STUDY POPULATION

The study was conducted among out patients from Al Jomhury hospital, Al-Kuwait hospital and Alzahrawy Clinical Center, which are situated in the middle of Sana'a, the capital city of Yemen. Figure 3.1 shows map of Yemen indicating the main governorates. The priority in choosing these sites was due to the high number of patients who come to these hospitals and the clinical center. Faecal samples were collected from patients referred to the parasitological lab in the above mentioned hospitals and clinical center for stool examination. A total of 503 samples were collected from December 2008 to March 2009.

Yemen is one of the least developed countries in the world, ranking 148 out of the 174 countries covered by the United Nation's Development Program's 2003 Global Human Development Report. The population growth rate in Yemen is the highest in the world exceeding 3 % per year. In the next two decades, its population is expected to double to over 40 million (Boucek., 2009). The government is unable to provide adequate educational or other public services for the rapidly expanding population growth (Boucek., 2009). The World Bank currently reports that about 73% of Yemen's population lives in rural areas, and agriculture provides 58% of all employment but accounts for only 15% of the Gross Domestic depending Product (GDP).

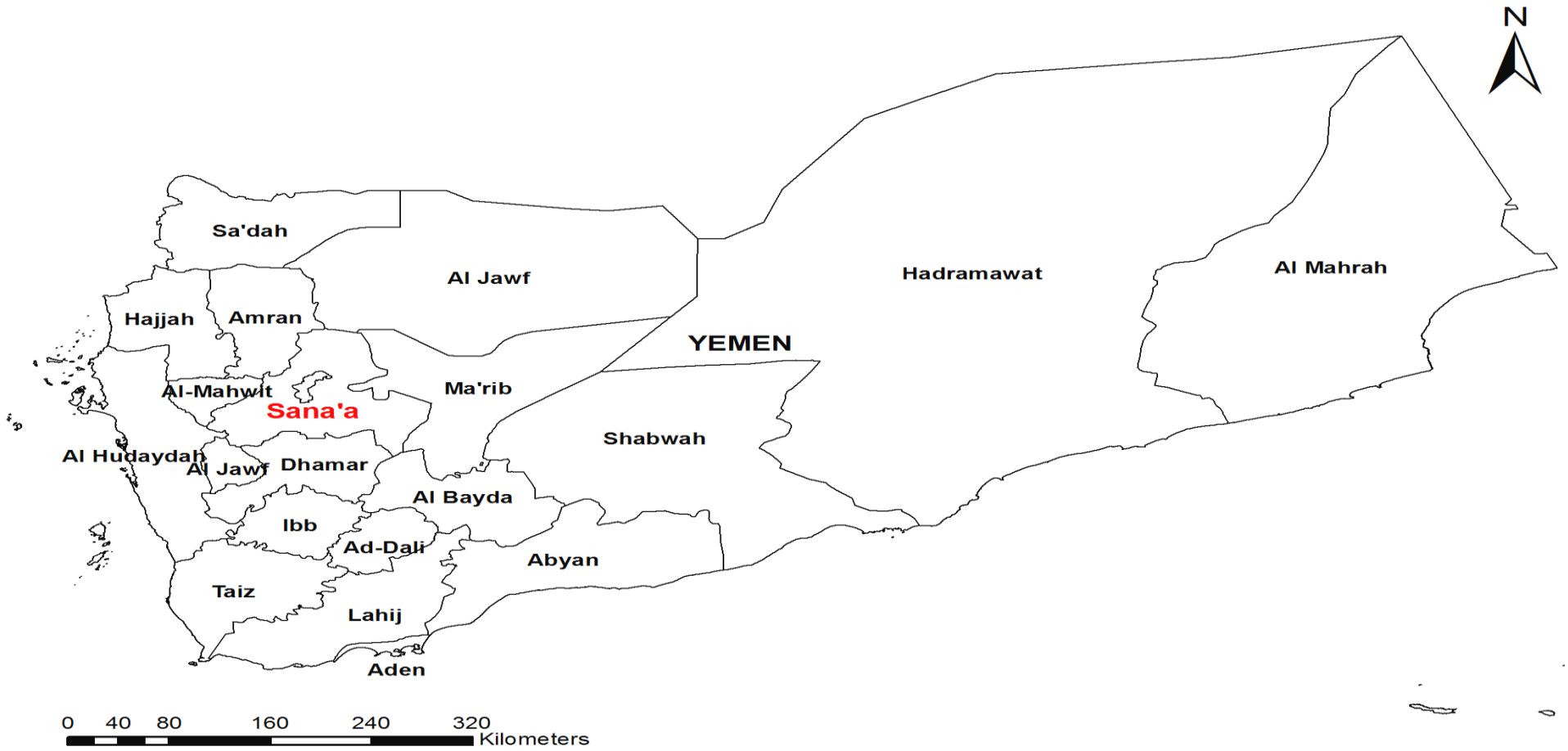


Figure 3.1 Map of Yemen indicating the location of major cities and governorates.

3.2 ETHICAL CLEARANCE

The study protocol was approved by the research and ethical committee of Faculty of Medicine, University of Malaya, Malaysia (MEC RF. No: 782.9). Permission was obtained from the hospital authorities before the commencement of the study. Individuals voluntarily participated after a clear explanation of the research objectives. If the subject was a child, informed consent was obtained from their parents or guardians.

3.3 STUDY DESIGN

This is a cross sectional study.

3.4 SAMPLE SIZE

The sample size needed for this study was 246 individuals. The expected sample size was calculated according to the following parameters - expected prevalence of *Giardia* at 20% (Azazy and Raja'a, 2003), confidence level at 95% and absolute precision at 0.05 (Lwanga and Lemeshow, 1991). However, 503 samples were collected during the 4 months study period from December 2008 to March 2009.

3.5 DEFINITION OF VARIABLES

Age group : ≤ 12 years (children)

≥ 12 years (adults)

Gender : Male or Female

Education level : Considered educated if he/she has primary school education or higher.

Educated mother/ father : Mother and/or father are considered educated if they had primary school education or higher.

Non-educated mother/father : Mother and/or father are considered non- educated if they do not have primary school education.

Household income : Total income of the family members that share the same house. It is classified into two levels: ≤20000 Yemeni Ryal (≤100USD) and ≥20000 Yemeni Ryal (≥100USD) according to the minimum wage in Yemen.

Household members : Number of family members living in the same house.

Gastroenteritis : Diarrhea, vomiting, nausea, fever and abdominal pain. Diarrhea was defined as loose stool for three consecutive days.

Rearing activity : Rearing animals was defined as the traditional rearing which depends on sending animals to mountains and valleys for grazing and caring of animals in shelters within the vicinity of the

farmer's house.

Grazing activity : Grass collection activity from open fields for animal feed was defined by the practice of collecting grass from mountains and valleys and bring it back to the shelters as animal feed

3.6 SAMPLE COLLECTION

3.6.1 Stool collection

Stool samples were collected in 50 ml screw-capped containers. The name and the number of respondent were written on the container for proper identification purposes. On the same day of collection, approximately 10 mg of stool was put into a 15 ml centrifuge tube containing 8 ml of normal saline and was processed according to the formal ether concentration technique. The rest of the sample was put in 2.5% potassium dichromate and kept in the Parasitology Department at the Al Jomhury hospital, Yemen at - 4 ºC prior to transfer to the Department of Parasitology, University of Malaya, Malaysia for molecular analysis. Once the samples reached Malaysia, they were kept in - 80 ºC freezer.

3.6.2 Structured questionnaire

Data were collected from every patient using pre-tested questionnaire (Appendix A). The questionnaire included socio-demographic information such as age, gender,

residence, education level, occupation, monthly income and behavioral habits (i.e., washing hands before eating, washing hands after defaecation, eating with hands, washing fruits and vegetables and taking bath at least twice a week), health condition which included symptoms and history of symptoms (e.g., diarrhea, nausea, vomiting and abdominal pain). Environmental conditions such as water supply, provision of sanitation system and existence of animal in households were also included. Every patient was personally interviewed by expert interviewers from the staff of the hospitals where the samples were collected. In the case of children, their parents or guardians were interviewed.

3.7 PROCESSING OF STOOL SAMPLES

3.7.1 Formal ether concentration technique

Approximately 10-15mg of stool was transferred into a clean container before being processed using the formal ether concentration technique (Appendix B3). A smear from the stool sediment was made on a glass slide and covered with a cover slip. It was then examined under the microscope using 100x magnifications to detect ova, cyst and trophozoite. For confirmation, the 400x magnification was used to observe internal structures. In bright field microscopy, *G. duodenalis* cyst appears ovoid to ellipsoidal in shape and measures 11-14µm (range: 8-19 µm). Immature and mature cysts have 2 and 4 nuclei, respectively. Axonemes and median bodies are visible. The trophozoites appear as pear-shaped organisms, measuring 12-15 µm (range: 10-20 µm). Trophozoites are characterized by two anterior positioned nuclei on each side of the paired and longitudinally oriented axonemes. Repeated examination once more was

done for confirmation. The smears were also stained with iodine to clearly observe the cyst and trophozoite component for more confirmation.

3.7.2 Ziehl-Neelsen staining technique

Cryptosporidium oocysts were detected using modified Ziehl-Neelsen staining technique (Appendix C). Smears were prepared from fresh stool, air dried and fixed with alcohol. Fixed smears were stained with carbol fuchsin for 10 minutes, decolorized using acid alcohol for 10-15 sec and stained with malachite green for 3 minutes. Stained smear was examined under 1000 x magnification using light microscope. *Cryptosporidium* oocysts stained red or pink and can be differentiated from green stained yeasts, and the size ranged from 4-6 µm as shown in Figure C1 and C2 (Appendix C).

3.8 MOLECULAR ANALYSIS

3.8.1 DNA extraction

About 15 mg of the stool sample was mixed with about 10 ml of distilled water. Then the stool was sieved using 2 layers of gauze, centrifuged for 10 minutes at 3000 rpm. The sediment was used for DNA isolation. The Power Soil® DNA Isolation Kit (cat# 12855-50) was used for DNA isolation according to the manufacturers manual (Appendix D). DNA was purified and isolated using the PowerLyzer™ with a Bead Tube suitable for high powered bead beating of soil to break down the cyst and oocyst walls. The isolated DNA was finally eluted in 50 µl of the elution buffer instead of 100 µl to concentrate the isolated DNA. The DNA was stored in -20 ºC until use.

3.8.2 DNA amplification

3.8.2.1 *Giardia* protocols

3.8.2.1.1 Genotyping of *Giardia* based on 16S rRNA gene

Nested PCR assay was used to amplify a partial region of the 16s rRNA gene according to Read *et al*.(2002) with minor modifications (Mahdy *et al*., 2009) (Appendix E1.2). The primary PCR primer pair RH11 (5'-CAT CCG GTC GAT CCTGCC-3'') and RH4 (5'-AGT CGA ACC CTG ATTCTC CGC CAGG-3') (Hopkins *et al*., 1997) were used to amplify an approximately 292 bp of the primary PCR product. The secondary PCR was carried out using primer pair Giar-F (5'-GAC GCTCTC CCC AAG GAC-3') and Giar-R (5'-CTGCGT CAC GCT GCTC-3') (Read *et al*., 2002). Primary and secondary PCRs were performed in a total of 25 µl reaction mixture containing 12.5 pmol of each primer (Research Biolab, Singapore), 2U Taq polymerase (New England Biolabs, cat. no.M0267L, Ipswich, USA), 2X PCR ThermoPol buffer (New England Biolabs, cat. no.M0267L, Ipswich, USA), 200 µM dNTPs (Fermentas, cat. no. #R0192, Ontario, Canada), 2 mM $MgCl_2$ (Fermentas, cat. no. #R0971, Ontario, Canada). 5% dimethyl sulfoxide (DMSO) (Sigma, cat. no. 673439, USA) and 400 mg/ml bovine serum albumin (BSA) (New England biolabs, cat.no. #B14, Ipswich, USA). Two µl of DNA template was used in both primary and secondary PCRs. Samples were incubated in MyCycler thermal cycler (Bio-Rad, cat. no. 170-9705, Hercules, USA) under the following conditions: denaturing step at 95 ºC for 2 min, followed by 35 cycles of denaturing for 20 s at 96 ºC, annealing for 20 s at 59 ºC and extension for 20 s at 72 ºC, followed by a final extension at 72 ºC for 7 min.

3.8.2.1.2 Subgenotyping of *G. duodenalis* based on *b*-giardin gene

A semi-nested PCR was used in the amplification of the partial *b*-giardin gene (Appendix E1.3). In the primary PCR, a 753-bp fragment was amplified using primers G7 (5'- AAG CCC GAC GAC CTC ACC CGC AGT GC-3') and G759 (5'-G AGG CCG CCC TGG ATC TTC GAG ACG AC -3') (Caccio *et al*., 2002a), whereas in the secondary PCR, a 390-bp fragment was amplified using primers G376 (5'CAT AAC GAC GCC ATC GCG GCT CTC AGG AA-3') and G759 (Caccio *et al*., 2002a). The PCR mixture consisted of 1X ThermoPol PCR buffer, 1.5 mM $MgCl_2$ (Fermentas, Ontario, Canada), 200 µM of dNTP (Fermentas), 0.2 µM of each primer, 2 U of Taq polymerase (New England Biolabs, Ipswich, USA), 400 mg/ml of non-acetylated bovine serum albumin (New England Biolabs, Ipswich, USA) and 2 µl of purified DNA in a final volume of 50 µl. The PCR cycling conditions were as follows: after an initial denaturation step of 5 min at 95 ºC, 40 cycles of amplification were run, each consisting of 30 sec at 95 ºC, 30 sec at 65 ºC and 60 sec at 72 ºC, followed by a final extension of 7 min at 72 ºC.

3.8.2.2 *Cryptosporidium* protocols

3.8.2.2.1 Species identification of *Cryptosporidium* based on 18S rRNA

A partial polymorphic region of 18S rRNA gene was amplified according to Nichols *et al.* (2003) (Appendix E1.4). In the primary reaction, a 655 to 667bp fragment was amplified depending on the species of *Cryptosporidium* or *C. parvum* genotype, using a 27-mer forward primer (N-DIAGF; 5′-CAA TTG GAG GGC AAG TCT GGT GCC AGC-3′) and a 26-mer reverse primer (N-DIAGR2;5′-CCT TCC TAT GTC TGG ACC

TGG TGA GT-3′). The secondary PCR amplified a 435 bp fragment using the forward primer (CPB-DIAGF; 5′-AAG CTC GTA GTT GGA TTT CTG-3′) and the reverse primer (CPB-DIAGR; 5′-TAA GGT GCT GAA GGA GTA AGG-3′) that were previously developed by Johnson *et al*. (Johnson *et al*., 1995).

The primary reaction was run in a total volume of 50 µl in 0.2 ml thin walled tubes consisting of premixed reagents containing 200 µM of each of the four deoxynucleoside triphosphates (dNTP) (Fermentas, cat .no. #R0192, Ontario, Canada), 0.2 µM of each primer CPB-DIAGF2/R2 (Research, Singapore), 400 mg/ml BSA (New England Biolabs, cat.no. #B14, Ipswich, USA), 3.5 mM $MgCl_2$ (Fermentas, cat. no.#R0971, Ontario, Canada) and 2.5 U Taq Polymerase (New England Biolabs, cat. No. M0267L, Ips, USA). Two µl of DNA template was used in the primary PCR whereas 5 µl of the first PCR product was used as template in the secondary PCR. Reagents concentrations in the secondary PCR were similar to the primary PCR as mentioned above. The cycling conditions were as follows: hot start at 95 ºC for 5 min followed by 35 cycles of denaturing at 94 ºC for 30 sec, annealing at 60 ºC for 1 min and extension at 72 ºC for 30 sec whereas the final extension was at 72 ºC for 10 min. The secondary PCR had similar cycling conditions except that the annealing temperature in the secondary PCR was 60 ºC instead of 68 ºC.

3.8.2.2.2 Subtyping of *Cryptosporidium* based on *gp60* gene

Two step nested-PCR assays (Mallon *et al*., 2003, Strong *et al*., 2000) were used to generate amplicons for subgenotyping by sequencing a fragment of the *gp60* gene (Appendix E1.5). In the first round of PCR, the *gp60* gene (~980–1000 bp) was amplified from DNA using a 21-mer forward primer (gp15-ATG; 5′-ATG AGA TTG

TCG CTC ATT ATC-3′) and a 21-mer reverse primer (gp15-STOP; 5′-TTA CAA CAC GAA TAA GGC TGC-3′). Subsequently, a 450-bp fragment was amplified in the secondary reaction using primers gp15-15A (5′-GCC GTT CCA CTC AGA GGA AC -3′) and gp15-15E (5′-CCA CAT TAC AAA TGA AGT GCC GC -3′) (Appendix F2.5). The primary and the secondary reactions were prepared in a total volume of 50 µl in 0.2 ml tubes consisting of premixed reagents containing 200 µM of each of the four deoxynucleoside triphosphates (dNTP) (Fermantas, cat. No. #R0192, Ontario, Canada), 4 µM of each of primers gp15-ATG/ gp15-STOP and gp15-15A/ gp15-15E (Bio-Basic, Canada), 3 mM $MgCl_2$ (Fermentas, cat.no. # R0971, Ontario, Canada), 2.5 U Taq polymerase (New England Biolabs, cat. No. M0267L, Ipswich, USA), and 1× ThermoPol PCR buffer (New England Biolabs, cat. No. M0267L, Ipswich, USA). Two µl of DNA template was used in the primary PCR whereas 5 µl of the first PCR product was used as template in the secondary PCR. The cycling conditions were as follows; hot start at 94°C for 5 min, followed by 40 cycles of denaturing for 30 sec at 94°C, annealing for 45 sec at 55°C and extension for 1 min at 72°C, followed by a final extension at 72°C for 10 min. The secondary PCR had a 35 cycle conditions with annealing and extension times for 30 sec for each one instead of 45 sec and 1 min, respectively.

3.8.3 PCR product analysis

i. Preparation of agarose gels

Agarose solution (2%) was prepared by adding 2 g of agarose to 250 ml conical flask containing 100 ml of 1X TBA buffer. The agarose was melted thoroughly by heating in a microwave oven at high power for 1-2 min and left to cool to approximately 50 ºC.

Subsequently, 2 µl of SYBR® Safe DNA gel stain (catalog# S33102 Invitrogen) was added and mixed. The agarose solution was poured into a gel mould fitted with appropriate comb for moulding the agarose gel wells. The gel was left at room temperature to solidify and transferred to the gel tank containing (1X) TAE buffer (catalog# 129237).

ii. Sample preparation for gel electrophoresis

An aliquot of 3 µl of 6X gel loading buffer (fermantas, cat. no. #SM0241, Ontario, Canada) was mixed by repeated pipetting with 10 µl of each amplification reaction product on small sheet of clean parafilm (PARAFILM® USA). The individual mixture was then transferred carefully with a pipette into the respective well set in the agarose gel. As a reference for molecular size, 5 µl of 100-basepair DNA ladder (Fermentas, cat. no. #SM 024, Ontario, Canada) was added to the well designated as reference.

iii. Gel electrophoresis

The electrophrosis was run at 100V and visualization of Sybr safe stained DNA was performed using the ultraviolet light illumination of gels using UV transilluminator and viewed in the monitor. Gels were photographed by using a camera fixed to the transilluminator. Figure F (Appendix F) showed gel electrophoresis of PCR products of 18S rRNA gene generated from *Cryptosporidium* positive faecal specimen on 2% agarose gel.

3.9 DNA PURIFICATION AND SEQUENCING

The PCR product was analysed on electrophoresis gel and the band was excised using clean scalpel blade and transferred to a clean microcentrifuge tube. The DNA was purified using QIAquick Gel Extraction Kit (QIA gen, cat. no. 28704, Germany) according to manufacturer's instructions (Appedix F). The gel purification step was done for the product that has low DNA concentration. For sample with high DNA concentration, the DNA was purified directly from the second PCR product using QIAquick DNA isolation kit according to manufacturer's instructions (Appendix G). The volume of elution buffer added to the column was 30 µl instead of 50 µl to increase the concentration of the eluted DNA. DNA was sequenced in both directions using the secondary PCR primers in an automated DNA sequencer (3130x1 Genetic Analyzer (Applied Biosystems, Foster City, USA) and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

3.10 PHYLOGENETIC ANALYSIS

3.10.1. *Giardia duodenalis*

DNA sequences were edited and the consensus sequences were created using the BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The consensus sequences were compared with those deposited in the GenBank database using The Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1997). Phylogenetic analysis for the partial sequences of 16S rRNA and *b*-giardin genes was carried out using the MEGA4 program (www.megasoftware.net). Sequences of *G. muris* (GenBank

accession no. AF113895) and *G. ardeae* (GenBank accession no. Z17210) were used as outgroups for 16S rRNA and sequence of *G. muris* (GenBank accession no. AY258618) was used as an outgroup for *b*-giardin. Neighbour Joining analyses was performed with distances calculated with the Kimura 2-parameter (Kimura, 1980). To evaluate the support for inferred topologies, bootstrapping was carried out using 1,000 replicates (Felsenstein, 1985).

3.10.2 *Cryptosporidium*

DNA sequences were edited and the consensus sequences were created using BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The consensus sequences were compared with those deposited in the GenBank database using The Basic Local Alignment search tool (BLAST) (Altschul *et al.*, 1997). Phylogenetic analysis for the partial sequences of 18S rRNA and *gp60* genes was carried out by Bayesian Inference using Monte Carlo Markov Chain (MCMC) in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). The General Time Reversible (GTR) model of evolution was used for nucleotide substitution (Rodriguez *et al*., 1990) with gamma distribution rate. Phylogenetic trees were viewed using TreeView software (http://darwin.zoology.gla.ac.uk/~ rpage/treeviewx/download.html). Reference sequences from previous published studies were selected for the two genes and used in the analysis (Figure 4.5, 4.6 and 4.7).

3.11 STATISTICAL ANALYSIS

Data were analysed using SPSS programme for windows version 11.5 (SPSS Inc., Chicago, IL, USA). The associations between dependent and independent variables

were tested using chi-square test. The risk was estimated with odds-ratio and the significant was tested using 95% confidence interval and *P* value. Multivariate analysis using logistic regression was applied to confirm the significant risk factors. The significant was defined as $P<0.05$.

3.12 QUALITY CONTROL

To minimize the cross-contamination during pre-PCR, DNA extraction and template addition to reaction tubes and post-PCR manipulation lab work were performed in three separate laboratories area by using designated micropipettes and tips. Micropipettes were new and tested for calibration. Laboratory coat and gloves that have been worn before starting the laboratory were changed when necessary and the working areas were always wiped with 70% alcohol. The positive control was used for every gene taken from previous study while the negative control was distilled water.

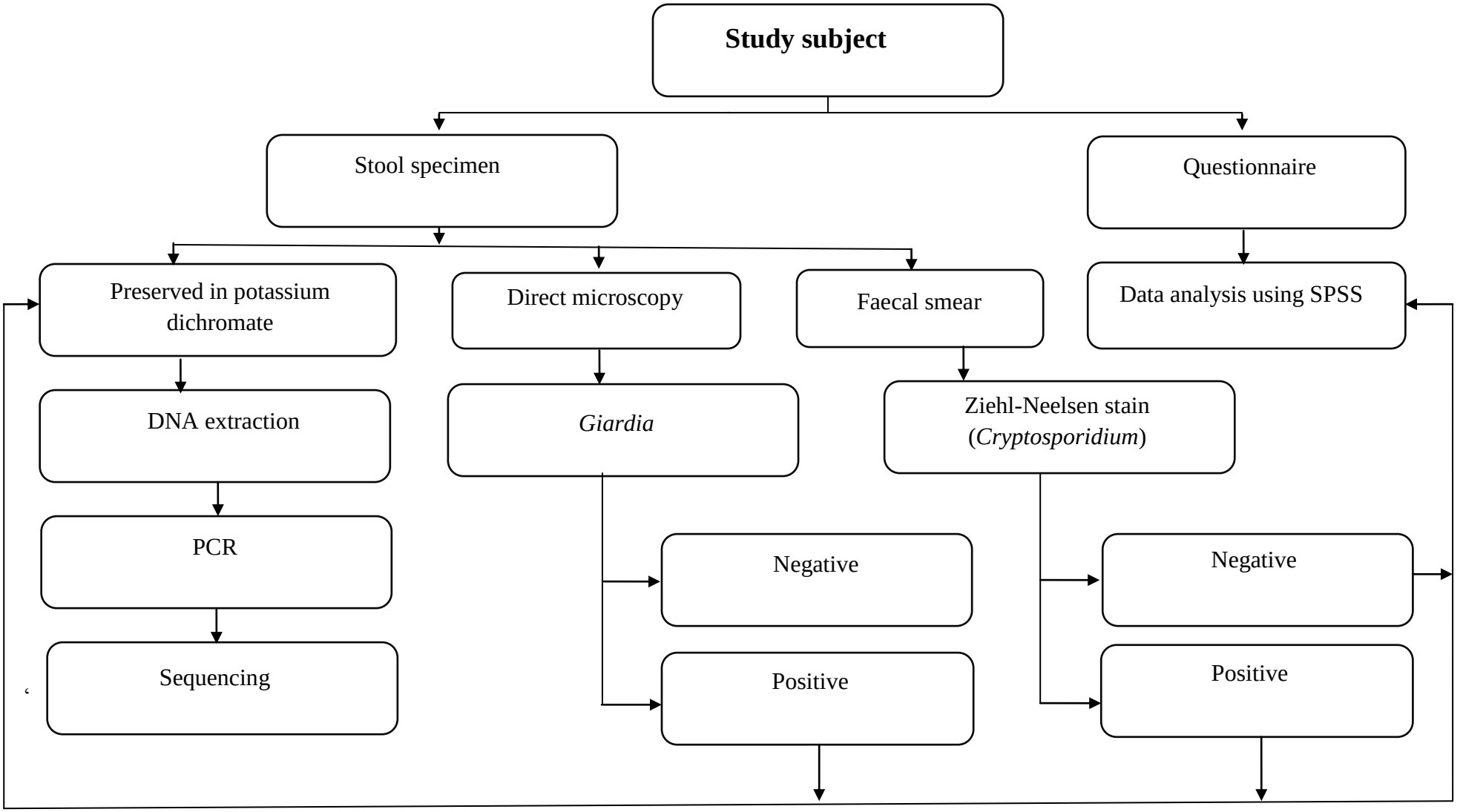


Figure 3.2: Schematic diagram of the processing of stool specimen

CHAPTER IV

RESULTS

4.1 EPIDEMIOLOGY OF INTESTINAL PARASITES AMONG PATIENTS SEEKING HEALTHCARE IN SANA'A CITY, YEMEN

4.1.1 Characteristics of the patients who participated

A total of 503 stool samples was collected from patients attending two hospitals and a health center, in Sana'a City, and were referred to a parasitological laboratory for faecal examination. Of these patients, 219 were males and 284 were females. The age range of participants was between 1 to 80 years old. The characteristics of the studied population were as follows: 71.4% were adults, 75.5% patients came from outside Sana'a (towns around Yemen) while only 24.6% came from Sana'a, the capital city of Yemen, 43.5% were male and 56.5% were female patients. There were 80% of those sampled came from household income of >100 US dollars. Family size more than 5 represented 69.4% of the samples. There were 68% patients who were not working and they were mostly women and children. The level of education among the studied population is 60.6%, whereas, 29.7% kept animals in their house. Only 32.1% patients have diarrhea. The majority of the patients did not drink treated water (Table 4.1).

4.1.2 The prevalence of parasitic infections among the study subjects

The overall prevalence of parasitic infection was 40.3%. Patients with multiple infections were registered at 11.7%, with 30.9% being protozoan infections. The prevalence of each parasite is indicated in Table 4.2. *Giardia duodenalis* showed the highest infection rate (17.7%) followed by *Entamoeba histolytica/dispar* (17.1%) and *Cryptosporidium* (1%). Other intestinal parasites detected included *Ascaris lumbricoides* (2.4%), *Hymenolepis nana* (1.4%), *Enterobius vermicularis* (0.4%) and *Schistosoma mansoni* (0.3%).

Table 4.1 The characteristics of the patients who participated in the present study.

Variables		**N**	**%**
Age (years)	> 12	359	71.4
	≤ 12	144	28.6
Address	Urban	366	73.3
	Rural	133	26.7
Gender	Male	218	43.5
	Female	283	56.5
Income (Yemeni Ryal)	> 20000	405	80
	≤ 20000	98	19.5
Family size	< 5	154	30.6
	≥ 5	349	69.4
Education	Educated	303	60.6
	Not educated	197	39.4
Occupation	Working	158	32.0
	Not working	335	68.0
Sewage disposal	Common drainage	429	85.8
	Others	71	14.2
Existence of animal	No	352	70.3
	Yes	149	29.7
Washing hands before eating	Yes	211	42.7
	No	283	57.3
Eating raw vegetables	No	95	19.5
	Yes	391	80.5
Eating fresh fruits	No	257	52.3
	Yes	234	47.7
Washing fruits and vegetables	Yes	391	79.1
	No	103	20.9
Drinking water	Treated	189	38.1
	Not treated	307	61.9
Bathing twice weekly	Yes	375	75.2
	No	124	24.8
Washing hands after defecation	Yes	225	46.4
	No	260	53.6
Watering plants	No	441	88.0
	Yes	60	12.0
Grazing activity	No	429	85.6
	Yes	72	14.4
Rearing activity	No	424	84.8
	Yes	76	15.2
Diarrhea	No	334	62.8
	Yes	166	31.2
Vomiting	No	404	80.6
	Yes	97	19.4

Table 4.1 continued

Variables		N	%	
Anorexia	No	332	62.4	
	Yes	169	31.8	
Fever	No	335	66.6	
	Yes	165	33.1	
Mother education	Educated	111	23.0	
	Non educated	371	77.0	
Father occupation	Others	330	69.5	
	Farmer	145	30.5	
Mother occupation	Others	436	90.5	
	Farmer	46	9.50	
Living site	**Governorates**			
	Sana’a	121	24.6	
	Sana'a governorate	65	13.2	
	Taiz	60	12.2	
	Haja	21	4.30	75.5
	Thamar	55	11.2	
	Ibb	81	16.5	
	Rima	21	4.3	
	Other governorates	68	13.8	

N: Number of patients

Table 4.2 Prevalence of intestinal parasitic infections according to species (N=503) among patients seeking healthcare in Sana'a city, Yemen.

Parasite	**No Infected**	**%**
Protozoa		
Giardia duodenalis	89	**17.7**
Entamoeba histolytica/dispar	86	**17.1**
Cryptosporidium	5	1
Helminthes		
Ascaris lumbricoides	12	2.4
Schistosoma mansoni	2	0.3
Hymenolepis nana	7	1.4
Enterobius vermicularis	2	0.4
Total	**203**	**40.3**

4.1.3 Factors associated with intestinal protozoan infections

Univariate analysis identified six factors associated with intestinal protozoan infections (Table 4.3) which include contact with animals (OR = 1.75, 95% CI 1.17-2.62), not washing fruits and vegetables before eating (OR = 1.66, 95% CI 1.060-2.601), drinking untreated water (OR = 1.50, 95% CI 1.01-2.25), taking bath less than twice a week (OR = 1.82, 95% CI 1.19-2.78), watering plants using untreated water (OR = 1.85, 95% CI 1.07-3.21) and working mother (farmers) (OR=2.26, 95% CI 1.22-4.17). Those living in the rural areas (OR = 1.52, 95% CI 0.99-2.305) and do not practice hand washing (OR = 1.47, 95% CI 0.99-2.17) had higher infection rates. Multivariate analysis using forward stepwise logistic regression confirmed contact with animals (OR = 1.75, 95% CI 1.17-2.62) and taking bath less than twice a week (OR = 1.82, 95% CI 1.19-2.78) as significant risk factors of intestinal protozoan infections.

4.1.4 Factors associated with *Giardia duodenalis* infection

Univariate analysis was also carried out based on single infection with *G. duodenalis* (Table 4.4). It was found that drinking untreated water was a significant predictor of giardiasis (OR=1.73. 95% CI = 1.05-2.86). Unexpectedly, people with income ≤ 20000 Yemeni Ryal appeared as protective factor (OR=0.4, 95% CI = 0.24-0.95) (Table 4.4). Logistic regression analysis confirmed that drinking untreated water is a significant risk factor of *G. duodenalis* infections in Yemen (OR = 2.09, 95% CI = 1.22 – 3.61).

Table 4.3 Factors associated with protozoan infections among patients seeking health care in Sana'a City.

Variables		**Infected %**	**OR (95%CI)**	***p* value**
Age (years)	>12	29.6	1	0.33
	≤12	34	1.23(0.81-1.85)	
Address	Urban	28.4	1	0.05
	Rural	37.6	1.52(0.99-2.31)	
Gender	Male	29.4	1	0.50
	Female	32.2	1.14(0.78-1.67)	
Income (Yemeni Ryal)	> 20000	31.4	1	0.63
	≤ 20000	28.9	0.89(0.55-1.45)	
Family size	< 5	27.9	1	0.34
	≥ 5	32.2	1.23(0.81-1.86)	
Education	Educated	33.3	1	0.13
	Not educated	26.9	0.74(0.49-1.09)	
Occupation	Working	27. 8	1	0.29
	Not working	32.5	0.80(0.53-1.213)	
Sewage disposal	Common drainage	29.6	1	0.15
	Others	38.0	1.459(0.87-2.46)	
Existence of animal	No	27.3	1	**<0.05***
	Yes	39.6	1.75(1.17-2.617)	
Washing hands before eating	Yes	26.5	1	0.055
	No	34.6	1.47(0.99-2.169)	
Eating raw vegetables	No	31.6	1	0.94
	Yes	31.2	0.98(0.61-1.59)	
Eating fresh fruits	No	28.7	1	0.29
	Yes	33	0.82(0.56-1.19)	
Washing fruits and vegetables	Yes	29.0	1	**<0.05**
	No	40.4	1.66(1.06-2.601)	
Drinking water	Treated	25.9	1	**<0.05**
	Not treated	34.5	1.50(1.01-2.25)	
Bathing two times weekly	Yes	27.7	1	**<0.05***
	No	41.1	1.82(1.19-2.78)	
Washing hands after defecation	Yes	28. 6	1	0.23
	No	33.7	1.27(0.86-1.873)	
Watering plants	No	29.7	1	**<0.05**
	Yes	43.3	1.185(1.07-3.20)	
Diarrhea	No	29.6	1	0.35
	Yes	33.72	1.28(0.81-1.80)	
Father occupation	Others	35.2	1	0.19
	Farmer	29.1	1.32 (0.87-2.00)	
Mother occupation	Others	28.9	1	**<0.05**
	Farmer	47.8	2.26(1.22-4.17)	

**Confirmed by logistic regression*

Table 4.4 Factors associated with *G. duodenalis* infections among patients seeking health care in Sana'a City.

Variables		**Infected %**	**OR (95%CI)**	**p value**
Age (years)	>12	16.8	1	0.37
	≤12	20.1	1.25(0.77-2.05)	
Address	Urban	16.1	1	0.14
	Rural	21.8	1.45(0.88-2.39)	
Gender	Male	15.6	1	0.27
	Female	19.4	1.31(0.82-2.09)	
Income (Yemeni Ryal)	> 20000	19.5	1	**<0.05**
	≤ 20000	10.3	0.47(0.24-0.95)	
Family size	< 5	17.8	1	0.94
	≥ 5	17.5	1.02(0.62-1.68)	
Education	Educated	19.1	1	2.26
	Not educated	15.2	0.76(0.47-1.23)	
Occupation	Working	18.8	1	0.33
	Not working	15.2	0.77(0.46-1.29)	
Sewage disposal	common drainage	17.1	1	0.40
	Others	21.1	1.31(0.70-2.44)	
Existence of animal	No	17.7	1	0.70
	Yes	18.0	1.10(0.67-1.81`1	
Washing hands before eating	Yes	18.0	1	0.92
	No	17.7	0.98(0.61-1.56)	
Eating raw vegetables	No	15.8	1	0.59
	Yes	18.2	1.18(0.64-2.17)	
Eating fresh fruits	No	16.7	1	0.52
	Yes	18.9	0.86(0.54-1.36)	
Washing fruits and vegetables	Yes	17.4	1	0.51
	No	20.2	0.86(0.54-1.37)	
Drinking water	Treated	13.2	1	**<0.05***
	Not treated	20.8	1.73(1.05-2.86	
Bathing two times weekly	Yes	16.0	1	0.06
	No	20.8	1.60(0.97-2.64)	
Washing hands after defecation	Yes	16.1	1	0.20
	No	20.3	1.33(0.83-2.12)	
Watering plants	No	17.0	1	0.23
	Yes	23.3	1.49(0.78-2.84)	
Diarrhea	No	15.9	1	0.11
	Yes	21.7	1.47(0.92-2.35)	
Father occupation	Others	19.3	1	0.49
	Farmer	16.7	1.19(0.72-1.98)	
Mother occupation	Others	16.3	1	0.10
	Farmer	26.5	1.78(0.88-3.61)	

*Confirmed by logistic regression

4.1.5 Factors associated with *Entamoeba histolytica/dispar* infection

Univariate analysis indicated that *E. histolytica/dispar* infection was significantly associated with contact with animals (OR=2.75, 95% CI = 1.71-4.43), watering activity (OR=2.13, 95% CI = 1.15-3.95), taking bath less than twice a week (OR=1.82, 95% CI = 1.10-2.99), not washing hands before eating (OR=2.19, 95% CI = 1.31-3.64), living in rural areas (OR=1.72, 95% CI = 1.05- 2.82) and the absence of common drainage (OR=1.97, 95% CI = 1.09-3.54) (Table 4.5). Logistic regression confirmed that not washing hands before eating (OR=1.98, 95% CI = 1.15-3.41) and contact with animals as significant predictors of *Entamoeba* infections (OR=3.09, 95% CI = 1.88-5.06).

Table 4.5 Factors associated with *E. histolytica/dispar* infection among patients seeking health care in Sana'a City.

Variables		**Infected %**	**OR (95%CI)**	***p* value**
Age (years)	>12	15.5	1	0.54
	≤12	18.8	1.17(0.71-1.93)	
Address	Urban	15.0	1	**<0.05**
	Rural	23.3	1.72(1.05-2.82)	
Gender	Male	17.9	1	0.71
	Female	16.6	0.91(0.57-1.45)	
Income (Yemeni Ryal)	>20000	17.5	1	0.91
	≤20000	17.0	1.04(0.58-1.86)	
Family size	<5	19.3	1	0.06
	≥5	12.3	1.69(0.98-2.93)	
Education	Educated	16.3	1	0.26
	Not educated	17.8	1.07(0.67-1.71)	
Occupation	Working	17.9	1	0.33
	Not working	16.5	0.90(0.55-1.50)	
Sewage disposal	Common drainage	15.6	1	**<0.05**
	Others	26.8	1.97(1.09-3.54)	
Existence of animal	No	12.5	1	**<0.05***
	Yes	28.2	2.75(1.71-4.43)	
Washing hands before eating	Yes	11.4	1	**<0.05***
	No	21.9	2.19(1.31-3.64)	
Eating raw vegetables	Yes	16.9	1	0.47
	No	20.0	0.81(0.46-1.43)	
Eating fresh fruits	No	16.7	1	0.52
	Yes	18.9	0.86(0.54-1.37)	
Washing fruits and vegetables	Yes	15.6	1	**<0.05**
	No	24.0	1.71(1.01-2.89)	
Drinking water	Treated	13.2	1	0.06
	Not treated	19.9	1.63(0.98-2.69)	
Bathing two times weekly	Yes	14.9	1	**<0.05**
	No	24.2	1.82(1.10-2.99)	
Washing hands after defecation	Yes	15.2	1	0.29
	No	18.8	1.29(0.80-2.09)	
Watering plants	No	15.6	1	**<0.05**
	Yes	28.3	2.13(1.15-3.95)	
Diarrhea	No	15.7	1	0.52
	Yes	18	0.85(0.51-1.40)	
Father occupation	Others	15.2	1	**<0.05**
	Farmer	22.8	2.33(1.18-4.59)	
Mother occupation	Others	15.8	1	**<0.05**
	Farmer	30.4	1.97(1.10-3.55)	

*Confirmed by logistic regression

4.2 MOLECULAR CHARACTERIZATION OF *GIARDIA DUODENALIS*

4.2.1 Identification of *G. duodenalis* assemblages

PCR analysis of the 89 microscopy-positive samples targeting ssu rRNA and/or *b*-giardin produced the expected targeted amplicons in 65 samples, which were successfully sequenced. Sequence comparison with *G. duodenalis* sequences available in the GenBank database revealed that 66 % (43 of 65) were assemblage A and 34 % (22 of 65) were assemblage B based on ssu rRNA and/or *b*-giardin genes. Molecular analysis of the ssu rRNA gene identified assemblages A and B in 32 and 9 of the 41 positive samples, respectively.

Based on *b*-giardin, assemblages A and B were identified in 18 and 16 of the 34 positive samples, respectively. Discrepancies were noted in four samples where they were identified as assemblage A using ssu rRNA and assemblage B using *b*-giardin.

4.2.2 Factors associated with *G. duodenalis* assemblages

Statistical analysis showed no significant difference in the distribution of assemblages A and B by age, gender, area of residence, type of water and family size. However, the occurrence of assemblage A, the predominant assemblage, showed a significant association with animal contact ($p < 0.05$) and grass collection activity for animal feed ($p < 0.05$). More patients were infected with assemblage A than B and presented with abdominal pain and diarrhea. However, the difference was not statistically significant.

Patients infected with assemblage A showed a significant association with vomiting (p <0.05). The socioeconomic factors assessed and their association with assemblages A and B are listed in Table 4.6.

Table 4.6 Factors assessed for the association with *G. duodenalis* assemblages A and B infections among patients seeking health care in Sana'a City.

Variables		A n (%)	B n (%)	χ^2	***p* value**
Demographic					
Age (years)	>12	28 (67)	14 (33)	0.21	0.64
	≤12	14 (61)	9 (39)		
Address	Urban	27 (60)	18 (40)	1.07	0.29
	Rural	14 (74)	5 (26)		
Gender	Male	12 (52)	11 (48)	2.41	0.12
	Female	30 (71)	12 (29)		
Household members	< 5	11 (55)	9 (45)	1.17	0.28
	≥ 5	31 (69)	14 (31)		
Personal hygiene					
Washing hands after defecation	Yes	12 (55)	10 (46)	1.32	0.25
	No	29 (69)	13 (31)		
Washing hands before eating	Yes	16 (70)	7 (30)	0.58	0.45
	No	24 (60)	16 (40)		
Washing fruits and vegetables	Yes	32 (68)	15 (32)	1.24	0.27
	No	9 (53)	8 (47)		
Drinking water	Treated	9 (50)	9 (50)		
	Not treated	33 (70)	14 (30)	2.33	0.13
Rearing animals	No	31 (59)	22 (42)	-	0.027*
	Yes	11 (92)	1 (8.0)		
Grass collection activity from open fields for animal feed	No	32 (59)	22 (41)	-	0.043*
	Yes	10 (91)	1 (09)		
Clinical symptoms					
Abdominal pain	No	13 (72)	5 (28)	0.63	0.43
	Yes	29 (62)	18 (38)		
Diarrhea	No	22 (59)	15 (41)	1.00	0.318
	Yes	20 (71)	8 (29)		
Watery stool	No	28 (58)	20 (42)	-	0.085*
	Yes	13 (81)	3 (19)		
Vomiting	No	27 (56)	21 (44)	-	0.016*
	Yes	15 (88)	2 (12)		

n: number of patients

*The association was examined using Fisher Exact test

The level of significance was defined as $p < 0.05$

4.2.3 Phylogenetic analysis based on 16S rRNA and genetic polymorphism in *b*-giardin gene

Phylogenetic analysis based on 16S rRNA was conducted using 9 representative sequences from this study and 2 sequences from GenBank database representing assemblages A and B (GenBank accession numbers HQ616613 and HQ616612, respectively) and the phylogenetic tree is shown in Figure 4.1 .

Phylogenetic analysis of the 32 *b*-giardin sequences together with 11 references from the GenBank representing subtypes A1, A2, A3, B1, B2, B3 and assemblages C, D, E and F (GenBank accession numbers X85958 and AY258617, AY072723, AY072724, AY072725, AY072726, AY072727, AY545646, AY545648, AY072729 and AY647264, respectively) produced two main clusters. Isolates belonging to Assemblage A were grouped in two sub-clusters for subtypes A2 and A3. Isolates belonging to assemblage B were placed in one cluster with the reference sequences of subtypes B1, B2 and B3 (bootstrap 90%) (Figure 4.2). Alignment analysis showed that A2 cluster differs from A3 cluster in two substitutions in this partial sequence of *b*-giardin. Assemblage B group appeared to be polymorphic. The isolate 48 was 99% similar to subtype B1 (difference in one substitution). Isolates 7, 43, 72, 79, 262, 303, 305, 330, 353, 409 and 455, which were represented by two sequence types 7 and 330, were identical to subtypes B2 and B3. Isolates 126, 371 and 476 showed substitutions that did not seem to belong to a known subtype (Figure 4.3).

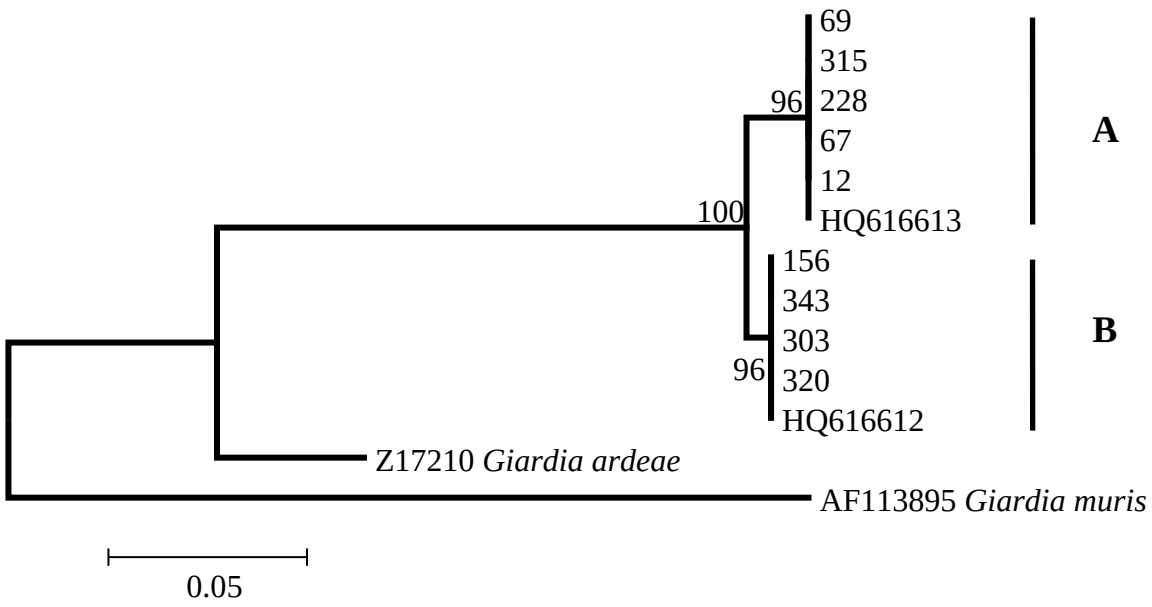


Figure 4.1 Neighbor-joining (NJ) tree displaying the relationships of 16S rRNA sequences representing *G. duodenalis* isolates. Bootstrap probabilities (% based on 1000 replicates) of more than 90% are indicated. Sample numbers (69 , 315, 228, 67, 12, 156, 343, 303, 320). *G. muris* and *G. ardeae* were used as outgroups. Accession numbers represent reference sequences from the GenBank.

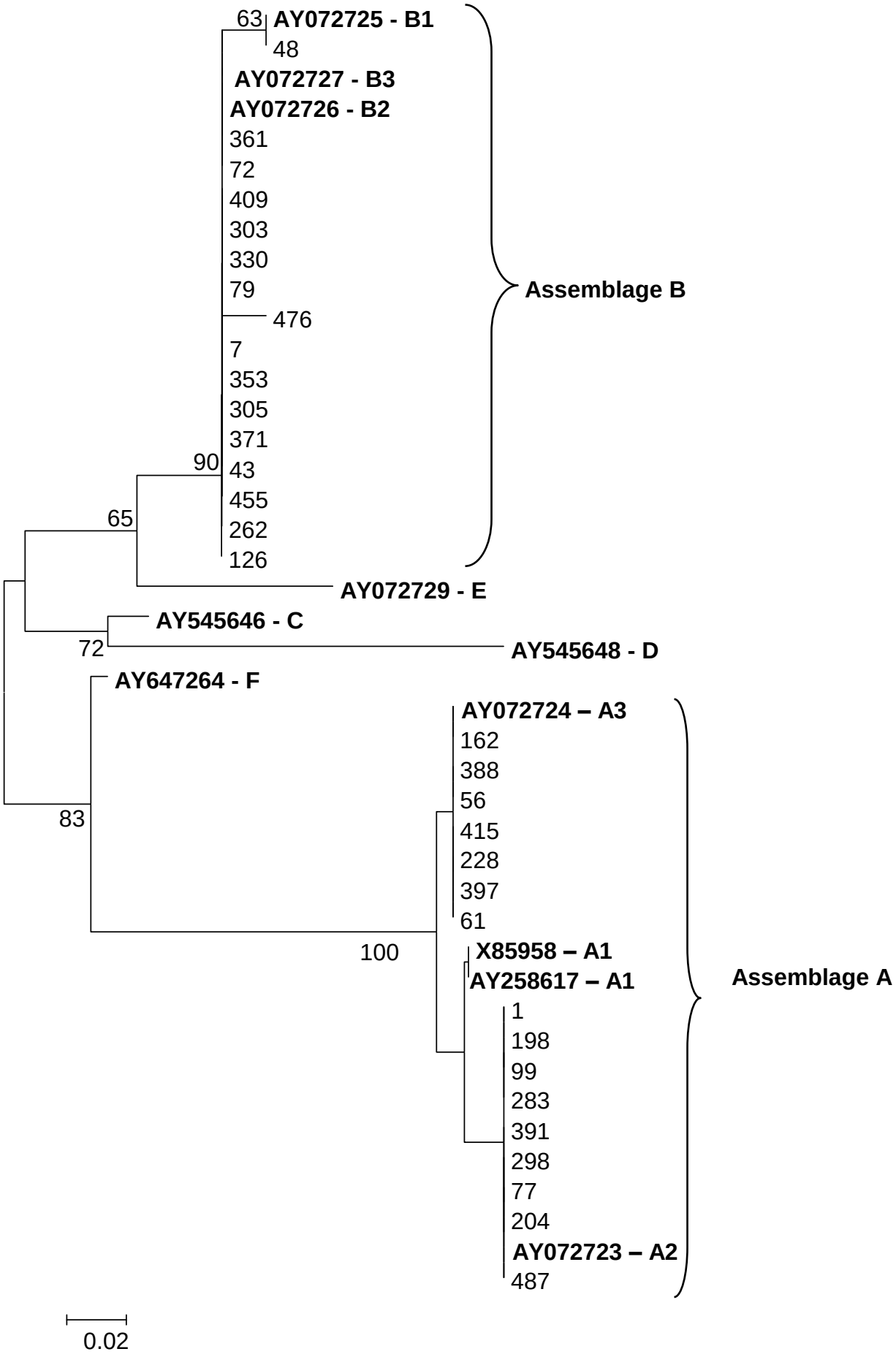
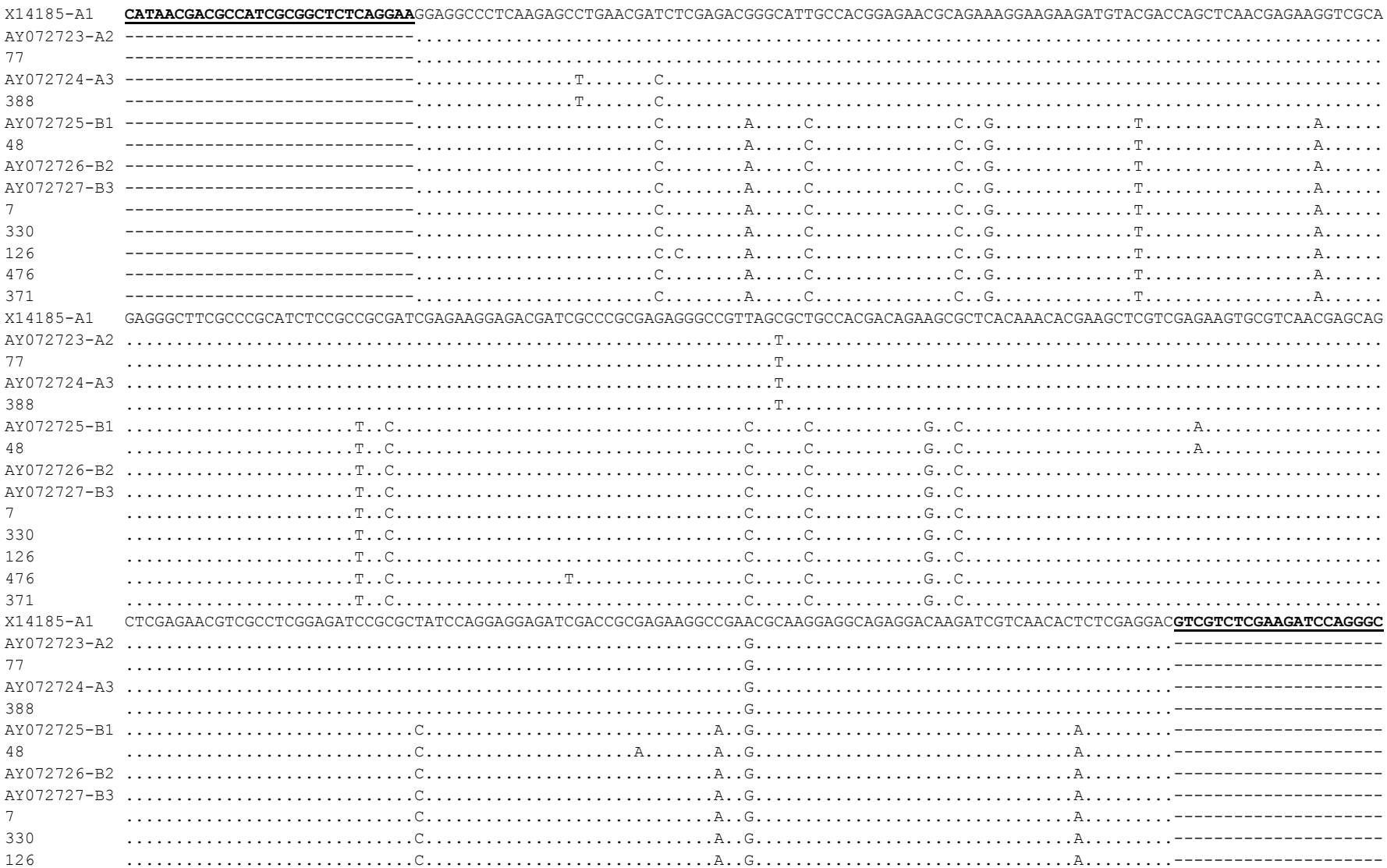

63
AY072725 - B1
48
AY072727 - B3
AY072726 - B2
361
72
409
303
330
79
476
7
353
305
371
90
43
455
65
262
126
Assemblage B
AY072729 - E
AY545646 - C
72
AY545648 - D
AY647264 - F
AY072724 – A3
162
388
56
415
228
397
61
83
100
X85958 – A1
AY258617 – A1
1
198
99
283
391
298
77
204
AY072723 – A2
487
Assemblage A
0.02

Figure 4.2 Neighbor-joining (NJ) tree displaying the relationships of *b-giardin* sequences of *G. duodenalis*. Bootstrap values (% based on 1,000 replicates) of more than 60% are indicated. Sample numbers (361, 72, 409, 303,79, 476, 7, 353, 305, 371, 43, 455, 262, 126, 162, 388, 56, 415, 228, 397, 61, 1, 198, 99, 283, 391, 298,77, 204, 487). Accession numbers represent reference sequences from the GenBank.


X14185-A1 CATAACGACGCCATCGCGGCTCTCAGGAAGGAGGCCCTCAAGAGCCTGAACGATCTCGAGACGGGCATTGCCACGGAGAACGCAGAAAGGAAGAAGATGTACGACCAGCTCAACGAGAAGGTCGCA
AY072723-A2
77
AY072724-A3
388
AY072725-B1
48
AY072726-B2
AY072727-B3
7
330
126
476
371
X14185-A1 GAGGGCTTCGCCCGCATCTCCGCCGCGATCGAGAAGGAGACGATCGCCCGCGAGAGGGCCGTTAGCGCTGCCACGACAGAAGCGCTCACAAACACGAAGCTCGTCGAGAAGTGCGTCAACGAGCAG
AY072723-A2
77
AY072724-A3
388
AY072725-B1
48
AY072726-B2
AY072727-B3
7
330
126
476
371
X14185-A1 CTCGAGAACGTCGCCTCGGAGATCCGCGCTATCCAGGAGGAGATCGACCGCGAGAAGGCCGAACGCAAGGAGGCAGAGGACAAGATCGTCAACACTCTCGAGGACGTCGTCTCGAAGATCCAGGGC
AY072723-A2
77
AY072724-A3
388
AY072725-B1
48
AY072726-B2
AY072727-B3
7
330
126

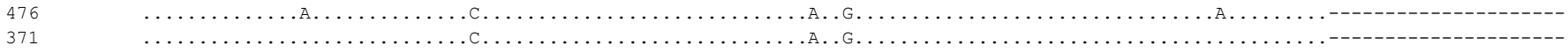


Figure 4.3 Multiple alignment using Bioedit program shows nucleotide changes at the *b*-giardin gene that distinguish between assemblage A and B and subtypes within assemblage A and B. Dots indicate nucleotide identity to the sequence in the first line (X85958). Primers used in the secondary PCR are underlined. Sequences with accession numbers are references from the GenBank. Sequences coded using Arabic numbers are representative sequences from this study.

4.3 MOLECULAR CHARACTERIZATION OF *CRYPTOSPORIDIUM*

4.3.1 Prevalence and distribution of *Cryptosporidium* based on PCR detection

Microscopic examination of *Cryptosporidium* identified only five positive samples. Given that molecular technique such as PCR is more sensitive in the detection of *Cryptosporidium* compared to microscopy, a total of 335 faecal specimens were screened for *Cryptosporidium* based on PCR. Of those examined, 42.2% were males and 57.6% were females. The age of patients ranged from 1 to 80 years with a median of 22 years and interquartile range of 79.9. Molecular detection using 18S rRNA-based nested PCR identified *Cryptosporidium* in 9% of the patients (33 of 335). Females had higher infection rate of *Cryptosporidium* than males (10.4% *vs* 9.3%). Higher prevalence of cryptosporidiosis was seen in patients who did not have a bath for at least two times a week (14%) and who drank untreated water (16%). The prevalence of *Cryptosporidium* was slightly higher in patients with a family size greater than five, adults, and individuals who reared animals. However, these differences were not statistically significant (Table 4.7).

Table 4.7 Prevalence and distribution of *Cryptosporidium* among patients seeking health care in Sana'a City.

Variables		N	Infected %	OR (95%CI)	*p* value
Demographic					
Age (years)	>12	24	10.3	1	0.68
	≤12	9	8.80	0.84 (0.38-1.88)	
Address	Urban	25	10.3	1	0.70
	Rural	8	8.90	0.85 (0.37-1.95)	
Gender	Male	14	9.30	1	0.74
	Female	19	10.4	1.13 (0.55-2.35)	
Household members	<5	9	9.60	1	0.92
	≥5	24	10.0	1.05 (0.47-2.34)	
Personal hygiene					
Washing hands after defecation	Yes	15	9.90	1	0.85
	No	16	9.30	0.93 (0.44-1.95)	
Washing hands before eating	Yes	16	11.7	1	0.41
	No	17	8.90	0.74 (0.36-1.52)	
Sewage disposal	common drainage	28	9.70	1	0.77
	Others	5	11.1	1.16 (0.42-3.18)	
Washing fruits and vegetables	Yes	25	9.90	1	0.87
	No	8	10.5	1.07 (0.46-2.49)	
Drinking water	Treated	11	8.7	1	0.57
	Not treated	22	10.7	1.25 (0.58-2.67)	
Bathing two times weekly	Yes	21	8.50	1	0.13
	No	12	14.1	1.78 (0.83-3.79)	
Rearing animals	No	23	9.70	1	0.84
	Yes	10	10.4	1.09 (0.49-2.38)	
Clinical symptoms					
Diarrhea	No	20	8.90	1	0.37
	Yes	13	12.0	1.40 (0.67-2.94)	
Clinical symptoms*	No	3	7.5	1	0.59
	Yes	30	10.2	1.40 (0.41-4.8)	

N: number of patients.

*Clinical symptoms includes: fever, vomiting, abdominal pain and nausea.

4.3.2 Identification of *Cryptosporidium* species

Out of these 335 samples, 33 were successfully sequenced and compared to *Cryptosporidium* 18S rRNA sequences deposited in the GenBank database using BLAST. Approximately 96% sequences were identified as *Cryptosporidium parvum* while only one sequence was identified as *Cryptosporidium hominis* (4%). Eight representative sequences from this study and 22 sequences representing different *Cryptosporidium* species obtained from GenBank were used in the phylogenetic analysis by Bayesian Inference. Details of reference sequences used for phylogenetic analysis on *Cryptosporidium* species, host and the country of origin are mentioned in Table 4.8. The phylogram placed seven isolates (i.e. 306, 343, 368, 379, 414, 444 and 463) in one cluster with representative sequences of *C. parvum* (pp = 0.96). Isolate 343 was grouped with representative sequences of *C. hominis* (pp = 0.89) (Figure 4.4).

4.3.3 Subtyping of *C. parvum* and *C. hominis* based on *gp60* gene

Phylogenetic analysis based on selected partial sequences of *gp60* (n = 7) representing *C. parvum* from this study together with 24 reference sequences representing the different *C. parvum* subtype families (Table 4.9), identified that IIa subtype family (IIaA15G2R1) is the prominent allelic family (pp = 0.87) (Figure 4.5). The phylogenetic analysis of the *gp60* sequence representing *C. hominis* with 26 reference sequences from GenBank (Table 4.10) inferred the *C. hominis* sequence as Ie genotype (subgenotype IeA11G3T3R1) (Figure 4.6).

Table 4.8 *Cryptosporidium* species used in the phylogenetic analysis.

Species	Source of origin	Countries	Genbank accession number	References
C. parvum	HIV patient	Malaysia	HQ450685	(Lim *et al*., 2011)
	Grey Langur	Sri Lanka	EF446678	(Ekanayake *et al*., 2007)
	Squirrel	USA	AF297509	(Ekanayake *et al*., 2007)
	Water rats	Japan	AB271070	(Kimura *et al*., 2007)
	Purple faces langur	Sri Lanka	EF446679	(Ekanayake *et al*., 2007)
	Cattle	Australia	AF108864	(Morgan *et al*., 1999)
	Cattle	Brazil	JN120853	(Olson *et al*., 2004)
	Human	Yemen	JX032678 JX032684	The present study
C. hominis	Human	Australia	AF108865	(Morgan *et al*., 1999)
	Human	UK	GQ983352	(Bouzid *et al*., 2010)
	Human	Chile	DQ286403	(Mercado *et al*., 2007)
	Human	UK	FJ031236	(Davies *et al*., 2009)
	Human	Slovenia	AJ849464	(Šoba *et al*., 2006b)
	Human	Yemen	X032685	The present study
C. suis	Pig	USA	AF115377	(Xiao *et al*., 1999b)
C. felis	Cat	Australia	AF108862	(Morgan *et al*., 1999)
C. bovis	Cattle	US	AY741305	(Fayer *et al*., 2006b)
C. xiaoi	Drinking water	Scotland	HM015881	(Nichols *et al*., 2010)
C. ryanai	Cattle	China	FJ463193	(Liu *et al*., 2009)
C, baileyi	Water	USA	L19068	(Johnson *et al*., 1995)
C. andersoni	Cattle	USA	AF093496	(Xiao *et al*., 1999a)
C. muris	Mouse	Japan	AB089284	(Satoh *et al*., 2003)
C. serpentis	Corn snake	USA	AF151376	(Kimbell III *et al*., 1999)
C. galli	Gouldian finch	Australia	AF316624	(Morgan *et al*., 2001)
C. fragile	Amphibian pet	Malaysia	EU162751	(Morgan *et al*., 2001)

Table 4.9 *Cryptosporidium parvum* subtypes used in the phylogenetic analysis.

Subtype family	Subtype	Source of origin	Countries	Genbank accession number	References
IIa	IIaA13G2R1	Cattle	Canada	DQ192502	(Trotz-Williams *et al*., 2006)
IIa	IIaA18G3R1	Human	Australia	EU164808	(Jex *et al*., 2007)
IIa	IIaA23G3R1	Human	Australia	EU164811	(Jex *et al*., 2007)
IIa	IIaA22G3R1	Human	Australia	EU164810	(Jex *et al*., 2007)
IIa	IIaA16G1R1	Cattle	Canada	DQ192504	(Trotz-Williams *et al*., 2006)
IIa	IIaA15G2R1	Human	Yemen	JX032686-JX032692	Present study
IIg	IIgA23	Human	Uganda	AY873780	(Akiyoshi *et al*., 2006)
Ilb	IIb12	Human	Global	AF402285	(Peng *et al*., 2001)
IId	IIdA17G1	Human	Portugal	DQ280495	(Alves *et al*., 2006)
IId	IIdA22G2R1	Cattle	Portugal	AY166806	(Alves *et al*., 2003)
IId	IIdA21G1	Sheep	Portugal	DQ280497	(Alves *et al*., 2006)
IId	IIdA20G1c	Human	Kuwait	AY738189	(Sulaiman *et al*., 2005)
IId	IIdA20G1b	Human	Kuwait	AY738185	(Sulaiman *et al*., 2005)
IId	IIdA19G1	Human	Portugal	DQ280496	(Alves *et al*., 2006)
IId	IIdA18G1b	Cattle	Belgrade	AB242226	(Misic and Abe, 2007)
IIj	IIjA15G4	Cattle	Ireland	DQ648547	(Thompson *et al*., 2007)
IIe	IIeA7G1	Human	Malawi	AY382675	(Peng *et al*., 2003)
IIf	IIfA6	Human	Kuwait	AY738188	(Sulaiman *et al*., 2005)
IIi	IIiA29	Human	Uganda	AY873782	(Akiyoshi *et al*., 2006)
IIc	IIcA5G3k	Human	Japan	AB237133	(Abe *et al*., 2006)
IIc	IIcA5G3a	Human	Global	AF403177	(Peng *et al*., 2001)
IIh	IIhA21G8	Human	Uganda	AY873781	(Akiyoshi *et al*., 2006)

Table 4.10 *Cryptosporidium hominis* subtypes used in the phylogenetic analysis

Subtype family	Subtypes	Source of origin	Countries	Genbank accession number	References
Ie	IeA12G3T3	Wastewater	Milwaukee	AY262032	(Zhou *et al*., 2003)
Ie	IeA11G3T3	Wastewater	Milwaukee	AY262033	(Zhou *et al*., 2003)
Ie	IeA11G3T3	Human	Japan	AB237138	(Abe *et al*., 2006)
Ie	IeA11G3T3	Human	Malawi	AY382668	(Peng *et al*., 2003)
Ie	IeA11G3T3	Human	Bangladesh	AY700389	(Hira *et al*., 2011)
Ie	IeA11G3T3	Human	Bangladesh	AY700399	(Hira *et al*., 2011)
Ie	IeA11G3T3	Human	Ontario	DQ192509	(Trotz-Williams *et al*., 2006)
Ie	IeA11G3T3	Human	UK	GU214354	(Pangasa *et al*., 2010)
Ie	IeA11G3T3R1	Human	Yemen	JX032693	The present study
If	IfA12G1	HIV children	South Africa	AF440629	(Leav *et al*. 2002)
If	IfA19G1	HIV children	South Africa	AF440638	(Leav *et al*., 2002)
If	IfA12G1	HIV children	South Africa	AF440639	(Leav *et al*., 2002)
Ib	IbA10G2R2	Human	Kuwait	AY738187	(Sulaiman *et al*., 2005)
lb	IbA9G3	Human	Kuwait	AY738196	(Sulaiman *et al*., 2005)
Ib	IbA20G2	Human	-	AF403175	(Peng *et al*., 2001)
Ib	Ib2 A18G1R4	Human	Australia	EU164807	(Jex *et al*., 2007)
Ia	IaA19R3	Human	-	AF403172	(Peng *et al*., 2001)
Ia	IaA21G1R1	Human	-	AF403171	(Peng *et al*., 2001)
Ia	IaA15R3	Human	Japan	AB237130	(Abe *et al*., 2006)
Ia	IaA15G1R1	Human	Italy	AF403162	(Sulaiman *et al*., 2001)
Ia	IaA17	Wastewater	Milwaukee	AY262027	(Zhou *et al*., 2003)
Id	IdA15	Human	Malawi	AY382669	(Peng *et al*., 2003)
Id	IdA19	Human	Canada	DQ192512	(Trotz-Williams *et al*., 2006)
Id	IdA21	HIV children	South Africa	AF440624	(Leav *et al*., 2002)
Id	IdA17G1	Human	Global	AF403165	(Peng *et al*., 2001)
Id	IdA15G1	HIV children	South Africa	AF440625	(Leav *et al*., 2002)

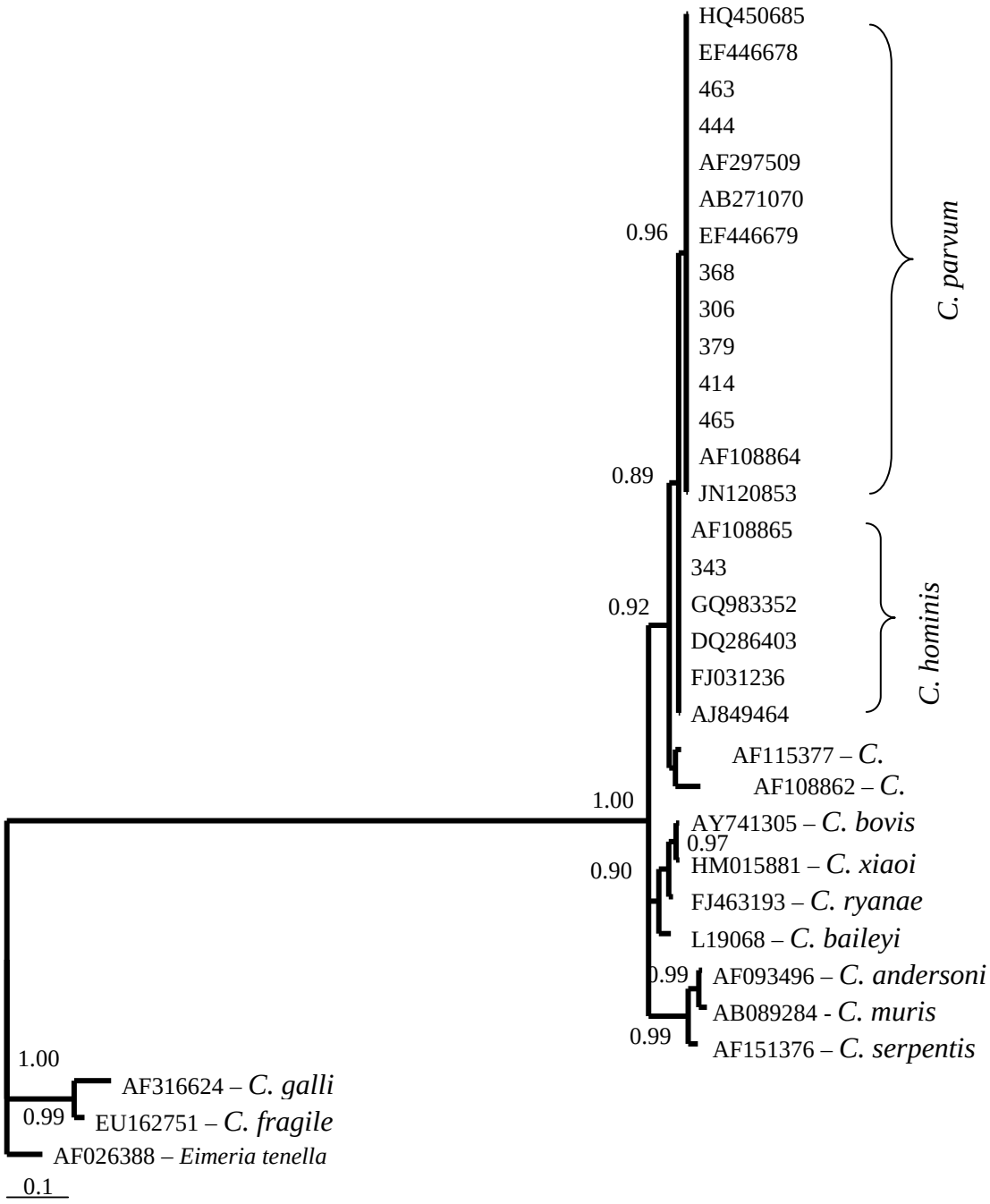


Figure 4.4: Phylogenetic analysis of partial 18S rRNA sequence data, representing *C. hominis* using Bayesian Inference. Sequences from the present study were the isolates (306,343,368,379,414,444,463 and 465). Details about the references used are available in Table 4.8. Posterior probabilities above 0.80 are indicated at major nodes. *Eimeria tenella* (GenBank, Accession no AF026388) was used as outgroup.

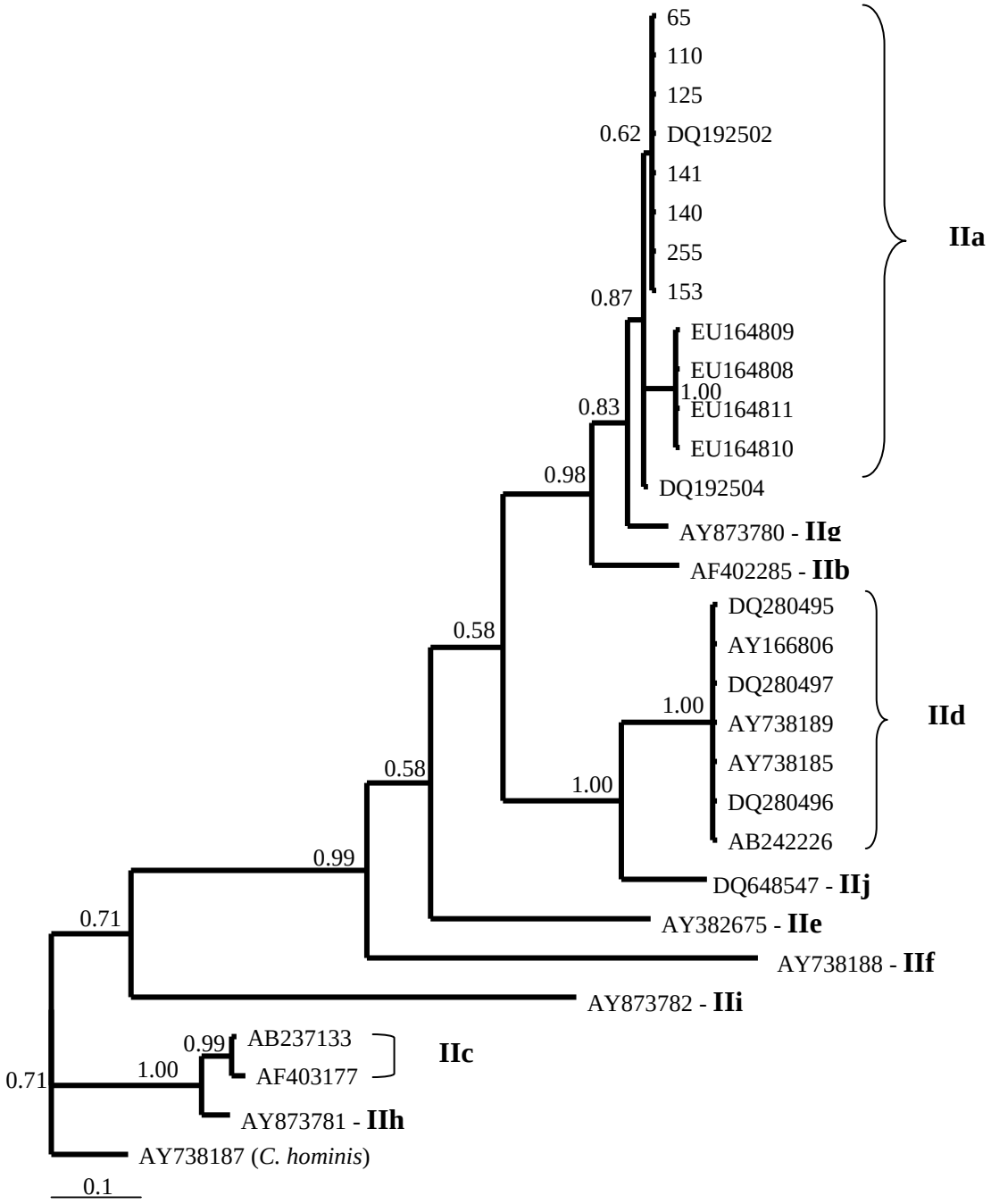


Figure 4.5 Phylogenetic analysis of partial *gp60* sequence data, representing *C. parvum* using Bayesian Inference. Sequences from the present study are isolates (65, 110, 125, 140, 141, 153, 255). Details about the references used are available in Table 4.9. Posterior probabilities above 0.50 are indicated at major nodes. *C. hominis* (GeneBank, Accession no AY738187) was used as outgroup.

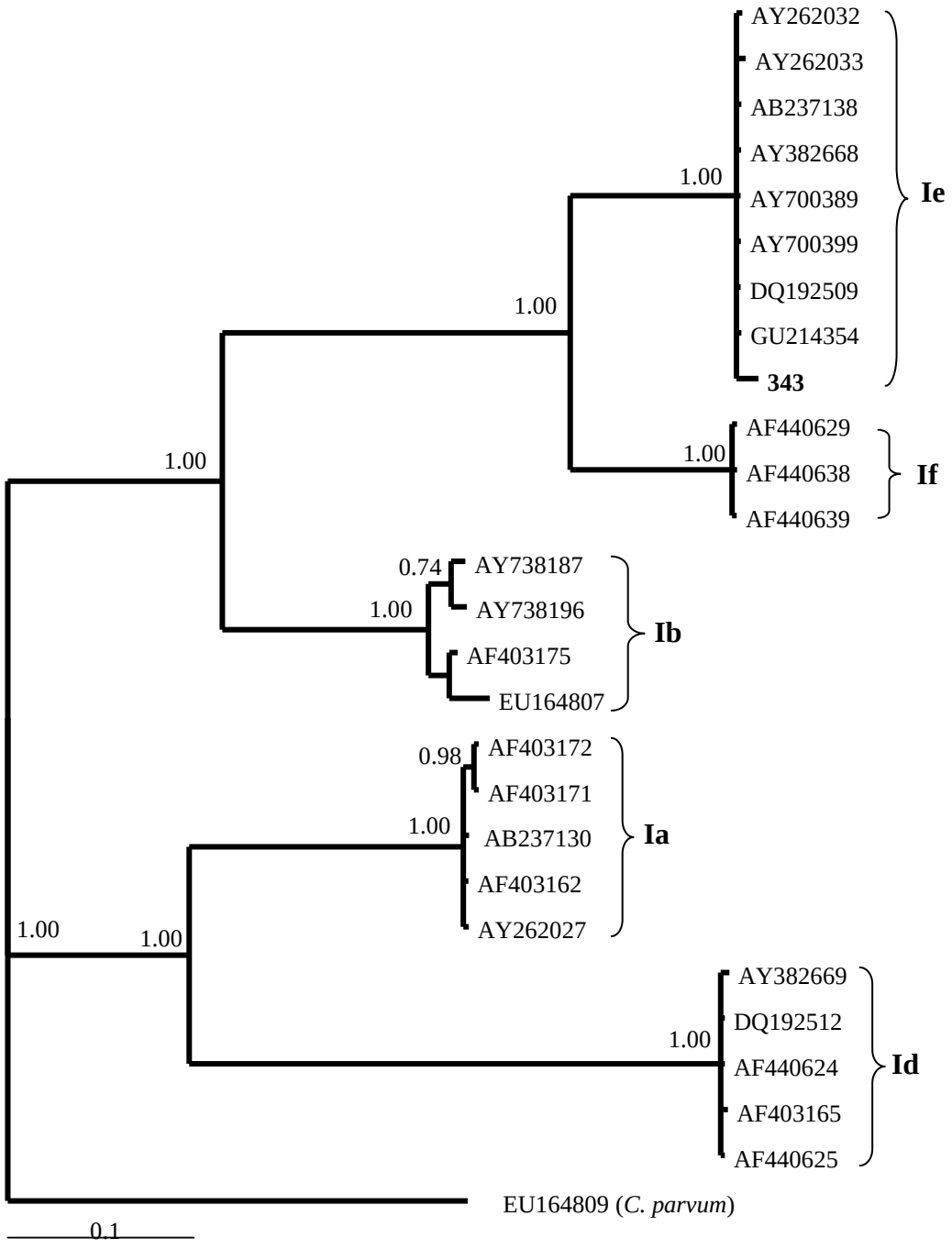


Figure 4.6 Phylogenetic analysis of partial *gp60* sequence data, representing *C. hominis* using Bayesian Inference. Sequence from the present study is isolate 343. Details about the references used are available in Table 4.10. Posterior probabilities above 0.70 are indicated at major nodes. *C. parvum* (GeneBank, Accession no EU164809) was used as outgroup.

CHAPTER V

DISCUSSION

5.1 EPIDEMIOLOGY OF INTESTINAL PARASITES

5.1.1 Prevalence of intestinal protozoa

The current findings indicated that the prevalence of intestinal protozoan infections was 38.9% based on a single stool examination. This infection rate was lower compared to a previous study carried out in Yemen on 37,000 outpatients in which the prevalence of intestinal protozoa was 53% (Hazza *et al*., 1983). In comparison with other Middle Eastern countries, the infection rate of intestinal protozoa in this study is higher than that which have been reported in Saudi Arabia among patients seeking health care (27.8% - 32.2%) (Akhter *et al*., 1994, Al-Shammari *et al*., 2001), Iran (19.9%) (Saab *et al*., 2004, Sayyari *et al*., 2005) and Oman (18%) (Prakash, 2008), and lower compared to the prevalence in Pakistan (52%) (Mehraj *et al*., 2008). The most dominant protozoa in this study were *G. duodenalis* and *E. histolytica/dispar*, which were rated at 17.7% and 17.1%, respectively. This finding is comparable to the previous studies carried out in Yemen (Raja A *et al*., 2000, Azazy and Raja'a, 2003), except studies among restaurant workers (Al-shibani. *et al*., 2009a, Baswaid and Al-Haddad, 2008). These differences could be attributed to the differences in the study subjects and study areas. Besides that, different diagnostic methods used from one study to another should also be considered as a possible reason behind the disparity in the infection rates (Ebrahim *et al*., 1997, Baker *et al*., 1987).

5.1.2 Factors associated with intestinal protozoan infections

This study showed a significant association between low personal hygiene practices and behavior with intestinal protozoan infections. The result of univariate analysis showed that existence of animals, drinking untreated water, not washing fruits and vegetables, not taking bath at least twice a week and watering activity as significant predictors of protozoan infections. Logistic regression analysis indicated that those who did not take their bath at least twice a week were at 2-fold higher risk of getting infections with intestinal protozoa. The current water crisis might contribute in some ways to the low hygienic practices in Yemen.

Yemen depends totally on ground water, which is dropping by 20 – 65 feet a year as reported by the World Bank (World Bank, 2008). Furthermore, the Carnegie Endowment for International Peace noted that 19 of Yemen's 21 main aquifers were not being replenished because of lower rainfall (Boucek and Peace, 2009). The impact of water quantity on the health status has been well documented since plentiful and accessible supplies of water do encourage better hygiene.

Two review articles which covered 84 studies in 28 countries have concluded that quantity of water available has more impact on endemic diarrhea cases in developing countries than water purity (Esrey *et al*., 1989, Esrey *et al*., 1985) . Another study carried out in Nicaragua, found that children from homes with insufficient water supply had 34% higher infection rate of diarrhea (Gorter *et al*., 1998). Water availability may also affect the frequency of hand washing as it has been stated that a mother needs 20 liters of water to wash her hands after using the latrine, changing a nappy, before preparing food, eating, giving food to the infant and handling of cooking or drinking utensils (Curtis *et al*., 2000).

In the present study, logistic regression analysis also showed that those people who came in contact with animals were at significant risk in acquiring protozoan infections. This association implicated animals as a significant source of protozoan infection in Yemen. However, this postulation should be confirmed by further studies which incorporate molecular tools. It is common for the rural communities in Yemen to keep animals such as cattle, goats and donkeys in the ground floor of the same house. Evidence of zoonotic transmission of some intestinal protozoa, especially *Giardia* and *Cryptosporidium* have been provided by several studies via molecular data analysis (Thompson and Lymbery, 2009, Xiao and Fayer, 2008).

5.1.3 Factors associated with *G. duodenalis* infection

Prevalence of *Giardia* has been reported to be high in Yemen and other Middle East countries. In Oman, the latest report among children was 10.4% (Patel and Khandekar, 2006), Saudi Arabia reported a prevalence of 37.7% (Al Shammari *et al*., 2001) whilst Syria documented a prevalence of 14% among children (Almerie *et al*., 2008). In Iraq, the prevalence was high (i.e., 31%-48%) among children (Al-Saeed and Issa, 2006) and in Palestine, a rate of 11.6% was recorded (Hussein *et al*., 2009). Other Arabic countries such as Egypt reported a prevalence of 31% among patients attending clinical hospital (Helmy *et al*., 2009b), whilst 9.7% was recorded in Sudan among food handlers (Babiker *et al*., 2009) and in Algeria, there were 34.2% of children in the Sahrawi region were infected (Lalle *et al*., 2009).

In this study, *Giardia* infections were shown to be significantly associated with drinking untreated water. In Yemen, ground water is the main source of drinking water. Given that most of the homes are without proper sanitary system, the possibility of

faecal contamination is high via ground seepage (Schmidt *et al*., 2008). Furthermore, it was noted that people in rural areas are dependent on dams besides wells as drinking water resources. Dam water is a collection of rainwater, which is exposed to contamination especially during the rainy season due to soil runoff contaminated with parasite oo(cysts) and ova from animals and humans faeces.

Previously, usage of well water has been identified as significant predictors of *E. histolytica* and *Giardia* infections in Saudi Arabia. Comparatively, those who use desalinated water have the lowest degree of exposure to the risk of infections (Omar *et al*., 1995). In a study in the village of Ethiopia, there was more cases of *Giardia* detected among children using unprotected water sources (Tigabu *et al*., 2011). In rural area of Pakistan, the most prevalent parasite detected was *Giardia* (i.e., 50%) (Siddiqui *et al*., 2002). Usage of untreated water was also a significant risk for polymicrobial and protozoan source of diarrheal infections among children in rural areas of Jordan (Nimri *et al*., 2004).

As with Yemen, Jordan also suffers from shortage of clean water sources. Study on low quality of treated wastewater in Jordan valley found that 73% of subjects studied were infected by *Giardia*, suggesting that the low quality of wastewater treatment reused in the agriculture sector may be a source of infection (Al-Alawi, 2007). In Iraq, a study on the risk factors of giardiasis among children showed that living in rural area and drinking raw or municipal water were significantly associated with *Giardia* infection (khudair Hussein, 2010). Even in Southeastern Brazil, it has been discovered that the risk factors of giardiasis among children in day care center found drinking unfiltered and unboiled water (OR 2.12, CI 1.26–3.69, $p<0.001$) and washing hands only with water (OR 2.14, CI 1.19–4.04, $p < 0.001$) were related risk factors (Santos *et al*., 2012).

Contamination of drinking water with *Giardia* cyst during transporting and storing of drinking water is highly possible in Yemen as rural people use containers to transfer water from dams or wells to their houses where water is stored to be used for drinking and cooking. Faecal contamination of drinking water between the source and the point of use is a well known fact (Wright *et al*., 2004) and improving household water management has been promoted as a low cost health intervention to combat waterborne infections (Mintz *et al*., 2001). Furthermore, drilling water wells in areas which are not far from the sewage disposal areas increases the possibility of water contamination with human faeces.

A recent study in Poland highlighted the treatment of water as an effective way for the removal of parasite from drinking water. In that study, *Giardia* was found to be 61% in surface water, 6% in raw water and in 19% in treated water. It also highlighted the frequent occurrence of parasites in surface waters in Poland (Bajer *et al*., 2012). In addition, a study in a village in Brazil on the natural spring water highlighted that contamination of water by soil is constant for a long duration of time. This survey concluded that in order to control and prevent parasite infections, the inclusion of consistent public health interventions with measures that include the protection of springs, the installation of minimum health infrastructure, and primary education of the population are necessary (Branco *et al*., 2012).

5.1.4 Factors associated with *E. histolytica/dispar* infection

As for *E. histolytica/dispar* infection, the present findings showed that those who do not practice proper hand washing before eating was at two fold higher risk of acquiring *E. histolytica/dispar* infection. The major role of contaminated hands in the faecal-oral

transmission of diseases has been well documented in developing countries and washing hands before eating or after defecation has been considered as a secondary barrier. In Indonesia, it has been reported that people who never or sometimes wash hands had a four times higher risk of getting severe diarrhea (Gasem *et al*., 2001). In Nepal, the practice of hand washing had a strong correlation with the prevalence of parasitic infection (Gyawali *et al*., 2010). Another case-control study in the same country indicated that people who never used soap for washing hands were at 30 times higher risk of acquiring typhoid (Velema *et al*., 1997). In addition, the practice of not washing hands has been reported to be significantly associated with diarrhea in Malaysia (Knight *et al*., 1992) and Myanmar (Han and Hlaing, 1989). Intervention trials and case-control studies conducted in Bangladesh, have also indicated that the practice of not washing hands was a significant risk for diarrhea (Khan, 1982, Shaid *et al*., 1996, Hoque *et al*., 1999).

In addition, animal contact was also identified as a significant factor associated with *Entamoeba* infection. Although it is still unclear whether *Entamoeba* infection is zoonotic or not, this parasite has been isolated from animals. In Ethiopia, a study on baboon and Cercopithecus (old world monkey) found that the prevalence of *E. histolytica* was 24.4% (Legesse and Erko, 2004). Another study, carried out in Ethiopia, found that cockroaches serve as carriers of human intestinal parasites (Kinfu and Erko, 2008). In Uganda, *E. histolytica* and *Giardia* have been detected in monkeys (Gillespie *et al*., 2005). In the Philippines, *E. histolytica* and *E. dispar* were detected among captive macaques in a primate facility. In the same study, using PCR, they found that 23 *E. histolytica* isolates were identical to human *E. histolytica* (Rivera *et al*., 2010) highlighting a possibility of zoonotic transmission.

5.2 MOLECULAR CHARACTERIZATION OF *G. DUODENALIS*

5.2.1 Prevalence and distribution of *G. duodenalis* assemblages

This is the first study in Yemen that has genotyped *G. duodenalis* isolated from humans. The two loci targeted were 16S rRNA which enabled the identification of assemblages and *b*-giardin which is a polymorphic locus that has the power to discriminate between subtypes. The present study identified assemblages A and B with the predominance of assemblage A from human samples in Yemen. The global incidence of assemblages A and B is different from country to country (Feng and Xiao, 2011).

In Middle East countries, giardiasis registered high prevalence but very limited molecular work has been carried out (Al-Mohammed, 2011, Al-Shammari *et al*., 2001, Helmy *et al*., 2009b, Lalle *et al*., 2009, Saab *et al*., 2004). A study conducted in Saudi Arabia, a neighbouring country of Yemen, showed that assemblage A was more prevalent than B (57% vs 37.5%) (Al-Mohammed, 2011). In Ethiopia, out of 59 positive isolates examined, 31 (52%) were typed as assemblage A and 13 (22%) as assemblage B; while the remaining were mixed infections. It was observed that there was a strong correlation between the presence of symptoms and infection with assemblage B was observed among humans in Ethiopia (Gelanew *et al*., 2007a). In Egypt, a community based study reported that prevalence of *Giardia* assemblage B was 80% compared to 5% for assemblage A (Foronda *et al*., 2008). A recent study in Egypt identified assemblage B in 13 of 15 samples positive for *G. duodenalis* (Soliman *et al*., 2011). However, a hospitalized based study conducted in Egypt indicated a higher infection rate of assemblage A than assemblage B (75.6% vs 19.5%) (Helmy *et al*., 2009b). In both Saudi Arabia and Egypt, assemblage B was symptomatic. The predominance of

assemblage A may not be surprising in an agricultural community such as the rural areas of Yemen where humans live in close contact with animals.

5.2.2 Subtyping of *G. duodenalis* assemblages

Subtyping analysis identified that all assemblage A isolates belonged to subtypes A2 and A3. The subtyping of assemblage B isolates was however challenging. It should be noted that subtypes A2 and A3 belong to sub-assemblage AII which is more commonly found in humans than animals (Feng and Xiao, 2011). Thus, it would seem that anthroponotic transmission of giardiasis is probably more common in Yemen. Human to human transmission of *Giardia* infection in this country could be aggravated by the water crises which has a direct effect on the health status since less water affects hygiene levels. The present study showed that factors indicating low personal hygiene were significant predictors of protozoan infection in Yemen (Alyousefi *et al*., 2011). This result is similar to a study in Nablus Palestine where assemblage A2 was the dominant subassemblage in clinical faecal samples (Hussein *et al*., 2009) .

However, the finding of the present study has also indicated that the potential of zoonotic transmission of giardiasis. The study showed that people who have close contact with animals are more prone to assemblage A infection compared to assemblage B, which implicates animals as a possible source of *Giardia* cysts. The zoonotic transmission of giardiasis has received support from a few studies conducted in endemic areas where the same genotype was isolated from humans and animals living in the same locality (Traub *et al*., 2004, Hopkins *et al*., 1997). Animals have also been considered as sources of *Giardia* cysts contaminating water resources and causing

human infections in other parts of the world (Isaac-Renton *et al*., 1994, Isaac-Renton *et al*., 1993, Robertson *et al*., 2006, Fernandes *et al*., 2011, Eligio-Garcia *et al*., 2008).

Furthermore, Yemeni farmers have close contact with animals. In rural and sub-urban areas of Yemen, livestock represents 50% of the farmers' income and 75% of the farmers keep at least a cow to provide milk for the household (United States Agency for International Development, 2008). Sheep and goats represent large proportion of livestock owned by farmers and are usually kept in small groups inside or next to the farmer's house rather than on farms. Farmers would collect grass by hands from open fields in the mountains and valleys and bring them back to feed their animals.

Veterinary services are not commonly available, thus these animals may harbor parasitic infections without being detected. In addition, animals imported to Yemen from Africa are also not being screened for parasitic infections (United States Agency for International Development, 2008). Livestock have been considered as a potential reservoir host of giardiasis (Castro-Hermida *et al*., 2007, Geurden *et al*., 2008a, van der Giessen *et al*., 2006b, Sprong *et al*., 2009). To date, there is only one study on giardiasis in animals in Yemen which indicated that crows are carrier for *Giardia* and other protozoa (Al-Sallami, 1991). Another study which has implicated animals but not related to parasite is the rift valley fever caused by importing virus infected animals from Ethiopia. This outbreak killed 97 people in Yemen and 1797 cattle. The outbreak spread to Saudi Arabia causing the death of 88 people (ProMED, 2000, Thabet *et al*., 2006). Unfortunately, the current study did not include animals in the survey which could support the assessment of the occurrence of zoonotic transmission.

5.2.3 Factors associated with *G. duodenalis* assemblages

The current study showed no significant difference in signs and symptoms between assemblages A and B. The link between *Giardia* assemblages and clinical manifestations is still not conclusive (Sahagun *et al*., 2008). This finding is similar to the findings reported in Portugal (Ceu Souza and Poiares da Silva, 2004), and in patients > 5 years old in Spain (Sahagun *et al*., 2008) and children in Brazil (Kohli *et al*., 2008b). Some studies have reported significant association between assemblage A and diarrhea but assemblage B cause no symptom (Haque *et al*., 2005a, Sahagun *et al*., 2008). In contrast, studies in Malaysia, Ethiopia and Netherlands showed that assemblage B patients were symptomatic (Homan and Mank, 2001a, Mahdy *et al*., 2009). The severity of disease is determined by the interplay between the virulence of the parasite and the developmental, nutritional and immunological status of the host. The discrepancies in the clinical manifestations of *Giardia* assemblages in different reports could be due to the variation in the virulence of the different genotypes, host factors or the combination of both (Thompson *et al*., 2000).

5.3MOLECULAR CHARACTERIZATION OF *CRYPTOSPORIDIUM*

5.3.1 Prevalence and distribution of *Cryptosporidium* species

The prevalence of *Cryptosporidium* detected via microscopy in this study was only 1%. Various reports have shown PCR detection is more sensitive in detecting the DNA of *Cryptosporidium* compared to conventional microscopy. In a comparison study between microscopy fluorescence and PCR, PCR sensitivity was shown to be 97% (Bialek *et al*., 2002) and a recent study demonstrated the lack of sensitivity of microscopic

identification for *Cryptosporidium* (Stark *et al*., 2011). An earlier report showed microscopy exhibited 83.7% sensitivity compared to PCR, which was more sensitive and easier to interpret (Morgan *et al*., 1998). Of 28 patients with primary immunodeficiencies 25, cases of *Cryptosporidium* were detected by PCR but not microscopy (Mclauchlin *et al*., 2003). Therefore, in order to increase the sensitivity of detection, PCR was employed in this present study.

The current work documented the first report of *Cryptosporidium* species and genotypes/subtypes in Yemen. Results showed that all isolates were *C. parvum* except one isolate which was identified as *C. hominis*. These findings are consistent with previous reports in other Middle Eastern countries (Xiao, 2010, Nazemalhosseini-Mojarad *et al*., 2011, Al-Brikan *et al*., 2008, Sulaiman *et al*., 2005, Hijjawi *et al*., 2010, Tamer *et al*., 2007). In Saudi Arabia, a neighbouring country of Yemen, out of 31 human faecal samples examined, Al-Brikan and his colleagues (2008) detected *C. parvum* and *C. hominis* in 15 and 13 samples, respectively. Another study In Saudi Arabia found *C. parvum* in 43 of 53 samples examined (Nazemalhosseini-Mojarad *et al*., 2012). Beside that a large water-borne outbreak involving over 800 inhabitants of Bani Hassan infected by *Cryptosporidium* in Jordan apparently associated with contaminated drinking water (Mohammed and Nicholas, 2007).

Similar results have been reported from Kuwait where *C. parvum* was the predominant species causing cryptosporidiosis among children (Sulaiman *et al*., 2005, Iqbal *et al*., 2011). A molecular epidemiological study conducted in Jordan which included 44 *Cryptosporidium* positive specimens, identified *C. parvum* and *C. hominis* in 22 and 20 specimens respectively, while *C. meleagridis* and *C. canis* was detected each in two cases (Hijjawi *et al*., 2010). In Iran, collective data generated from four molecular epidemiological studies showed predominance of *C. parvum*

(Nazemalhosseini-Mojarad *et al*., 2012). In contrast, two molecular epidemiological studies reported the presence of *C. parvum* and *C. hominis* in Egypt with predominance of *C. hominis* (Abd El Kader *et al*., 2011). In general, it would seem that the distribution of *Cryptosporidium* species in Middle Eastern countries including Yemen is not concurrent with other developing countries where *C. hominis* is the most common species (Xiao, 2010). In developed countries, both *C. parvum* and *C. hominis* are present with *C. parvum* being more prevalent (Xiao, 2010).

The factors that may contribute to the variation of the burden of *C. parvum* and *C. hominis* among human include age and seasonality (Wielinga *et al*., 2008, Zintl *et al*., 2009). In the current study, most of the infected patients were adults which are in agreement with previous studies conducted in the UK and Netherlands where *C. parvum* appeared to be adult biased whilst *C. hominis* was most common among children and infants (Wielinga *et al*., 2008, Chalmers *et al*., 2009, Chalmers *et al*., 2008). However, most of the studies carried out in Middle Eastern countries showed a predominance of *C. parvum* among children suggesting that there is no preferential infection with *C. parvum* in this region (Al-Brikan *et al*., 2008, Sulaiman *et al*., 2005, Iqbal *et al*., 2011, Hijjawi *et al*., 2010).

5.3.2 Subtyping of *C. parvum* and *C. hominis*

An understanding of the geographic distribution of *Cryptosporidium* species is not a conclusive indication of a difference in the dynamic of transmission. Although *C. parvum* has been known as zoonotic species, 9 subtype families of *C. parvum* (IIb, IIc, IIe – IIl) are not zoonotic (Xiao, 2010). The *C. parvum* subtype families, such as IIa and

IId, are found in both humans and ruminants responsible for zoonotic cryptosporidiosis (Xiao, 2010).

In this study we have applied *gp60* subtyping and analysis which showed high degree of sequence polymorphism in *C. parvum* and *C. hominis* and this allows the identification of subtype families which could link the parasite and its mode of transmission. In this study, all *C. parvum* subtypes belonged to IIa subtype family (IIaA15G2R1). The subtype IIaA15G2R1 is the major bovine subtype causing *C. parvum* infection among cattle in developed and developing countries (Abe *et al*., 2006, Alves *et al*., 2003, Broglia *et al*., 2008, Cohen *et al*., 2006, Feng *et al*., 2007, Sulaiman *et al*., 2005, Thompson *et al*., 2007, Trotz-Williams *et al*., 2006, Wielinga *et al*., 2008, Wu *et al*., 2003). This bovine subtype has also been found in humans in USA (Santin *et al*., 2008), the UK (Chalmers *et al*., 2009), Canada (Budu-Amoako *et al*., 2012), Japan (Abe *et al*., 2006), Australia (Ng *et al*., 2008), Netherlands (Wielinga *et al*., 2008), Slovenia (Soba and Logar, 2008), Portugal (Alves *et al*., 2006) and Ireland (Thompson *et al*., 2007). However, it has been rarely reported among human in developing countries where IIc is the most common subtype of *C. parvum* (Xiao, 2010) whereas in other developing countries such as Malawi and Kenya, IIe (anthroponotic *C. parvum* subtype family) is seen in humans in addition to IIc (Peng *et al*., 2003, Ajjampur *et al*., 2007).

In Middle Eastern countries, the bovine *C. parvum* subtype family IIa, is less common among human. It has been reported in 7 of 22 *C. parvum* infected patients from Iran (Nazemalhosseini Mojarad *et al*., 2010), 3 of 13 patients from Jordan (Hijjawi *et al*., 2010), and 1 of 37 patients from Saudi Arabia (Nazemalhosseini-Mojarad *et al*., 2012). In contrast, *C. parvum* subtype IId is the dominant subtype in this region (Nazemalhosseini-Mojarad *et al*., 2012). In Kuwait, both bovine *C. parvum* subtypes,

IIa and IId have been found (Sulaiman *et al*., 2005, Iqbal *et al*., 2011). The bovine IId *C. parvum* subtype family has never been detected in humans or animals in USA and Canada (Xiao, 2010) but it has been found in a number of sporadic cases in Europe and Australia (Alves *et al*., 2006, Wielinga *et al*., 2008, Zintl *et al*., 2009). In the current study in Yemen, IId subtype was not found, however the IIa subtype family is the sole parasite detected; suggesting that molecular epidemiology of *Cryptosporidium* in Yemen may be different from other Middle Eastern countries.

The predominance of the zoonotic subtype families of *C. parvum* IIa and IId, in the Middle Eastern regions suggests animal-to-human transmission as the most common transmission route of *Cryptosporidium*. However, the postulation has been contradicted by findings from two studies conducted in Kuwait (Sulaiman *et al*., 2005, Iqbal *et al*., 2011) where there were no animals. Instead urbanization characterizes the society and desalinated sea water is the source of drinking water. It must be noted that the existence of animal is a key factor to elucidate the possibility of the zoonotic transmission. Therefore, authors assumed that the source of infection in Kuwait was from human.

Recent report from Northern New South Wales found that animals and humans living in farms shared the same subgenotype (IIaA18G3R1) of *Cryptosporidium* (Yin *et al*., 2012). The predominance of *C. parvum* have been reported among farm workers in Zambia (Siwila *et al*., 2007). Elsewhere, the zoonotic *C. parvum* was responsible for about 85% of the *Cryptosporidium* infections in pre-weaned calves (Santin *et al.*, 2004). In the rural areas in Australia, it was found that all the four subtypes (IIa-A17G2R1, IIa-A18G3R1, IIa-A19G3R1 and IIaA20G3R1) found in human were also found in cattle suggesting that zoonotic transmission may be an important contributor to sporadic human cases (Ng *et al.,* 2008). Recent published data from Canada reported that farm animals and wildlife animals were important contributors of *Cryptosporidium* oocysts in

the watershed in urban and rural areas (Van Dyke *et al.*, 2012). The presence of the same zoonotic *C. parvum* subtypes (IIaA16G2RI, IIaA15G2RI) in cattle and human isolates implied that transmission was largely zoonotic and cattle might be a source of sporadic human infections on Prince Edward Island, Canada (Budu-Amoako *et al*., 2012). In a survey in Madagascar, results highlighted suggest varying risks of zoonotic transmission across the human-animal interface. One infected human with *C. suis*, suggests a potential risk for human-pig transmission in Ankialo, whereas, large numbers of infected cattle in Ambodiaviavy suggest a potential risk for human-cattle transmission (Bodager, 2012). PCR-RFLP methods, and Ssp1 and Vsp1 restriction enzymes, sequence determination and analysis of the results revealed that *C. parvum* in Tehran rats matched 99 to 100 % with *C. parvum* isolates in human and cattle (Bahrami *et al*., 2012).

In Yemen, the situation is different from other Middle Eastern countries. In rural and sub-urban areas of Yemen, animal husbandry is the mainstay of life. The factors enhancing the spread of *Cryptosporidium* are the same as *Giardia* that is as mentioned before; i.e., animals usually kept inside or next to the farmer's house and not in farms. However, the structure of the community in Yemen is also quite different from other Middle East countries. Urbanization level is lower and contact with animal is higher. Besides, local veterinary services are not commonly available and thus these animals may harbor parasitic infections without being detected. In addition, animals imported to Yemen from Africa are also not being screened for parasitic infections (United States Agency for International Development, 2008). These circumstances provide a suitable environment for zoonotic transmission of zoonotic parasites. The predominance of the bovine IIa subtype family among humans in the Yemeni community is an evidence for the possible zoonotic transmission of *Cryptosporidium*. However, this should be

supported by genotyping/subtyping study among humans and animals in the same locality.

5.4 LIMITATIONS OF THE PRESENT STUDY

The present study did not include water samples and there is no previous study focusing on contamination of water sources by these parasites in Yemen. Animal samples were required for analysis to know the spread of these parasites among livestock and other animals in Yemen, and further study should include animals to investigate the zoonotic potential.

CHAPTER VI

CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

1- The present study showed high prevalence of intestinal protozoan infections with *G. duodenalis* and *E. histolytica/dispar* being the most predominant protozoa among patients seeking health care in Yemen. The microscopic results for *Giardia* and *Cryptosporidium* are 17.7% and 1% respectively.

2- Multivariate analysis using forward stepwise logistic regression based on intestinal protozoan infections showed that contact with animals (OR = 1.748, 95% CI = 1.168–2.617) and taking bath less than twice a week (OR = 1.820, 95% CI = 1.192–2.779) were significant risk factors of protozoan. Logistic regression showed that drinking untreated water is a significant risk factor of *Giardia* infections in Yemen (OR = 2.09, 95% CI = 1.22 – 3.61)

. 3- *Giardia duodenalis* assemblages A and B are prevalent with the predominance of assemblage A (i.e., 66%). Subtyping analysis showed that assemblage A isolates belong to subtype A2 and A3, suggesting anthroponotic transmission of giardiasis in Yemen.

4- *Cryptosporidium* genotyping analysis showed that 96% were identified as *C. parvum* whilst one case was caused by *C. hominis*. All *C. parvum* isolates subtype belonged to the IIaA15G2R1 subtype.

6.2 RECOMMENDATIONS

1- Genotyping *G. duodenalis* and *Cryptosporidium* from human, animals and water resources are highly recommended to understand the actual dynamics of transmission of these protozoa in Yemen

2- *Cryptosporidium* should be included in the routine faecal examination and physicians should have high suspicion of this parasite.

3- Government services should coincide with an effective system for tracking infectious diseases, especially in remote areas.

4- Health authorities must take into consideration the development of health awareness among the community through active encouragement of individuals in adopting hygienic behaviors via audio, visual and curriculum programs.

5- Introduction of health education about the risk of the zoonotic diseases is crucial in Yemen especially among farmers.

6- Imported animals should be examined for pathogenic infection before entering the country through the provision of efficient health control measures.

7- Veterinary services must be available and accessible for farmers. Veterinary centers are essential to help animal breeders train and rehabilitate animals.

8- Radical solution to water scarcity is also an important requirement to combat the proliferation of these waterborne infections in Yemen. Future studies should target humans and animals living in the same community via the application of subtyping technology which has high discrepancy power.

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APPENDIX A

QUESTIONNAIRE (ENGLISH)

I. Subject NO._____

A. Personal information:

Name: _____

Address: Name of place: _____

House no.: _____

Marital status: [1] Single [2] Married

Ethnic group: _____

Age: _____

Gender [1] Male [2] Female

Monthly income: _____

No. of family members: _____

Educational level: [1] No formal education [2] Primary

[3] Secondary [4] Tertiary [5] University

Occupation: [1] Self-sufficient farmer [2] Government employees

[3] Odd-jobs [4] Professional [5] Factory

B. Source of water and environmental sanitation

1- Source of water for drinking and cooking water	☐ 1	Well	☐ 2	River
	☐ 3	Government pipe water	☐ 4	Rain water
2- Source of water for bathing	☐ 1	Well	☐ 2	River
	☐ 2	Government pipe water	☐ 3	Rain water
3- Source of water for washing	☐ 1	Well	☐ 2	River
	☐ 3	Government pipe water	☐ 4	Rain water
4- Existence of poor flush-toilet	☐	Yes	☐	No
5- Existence of bathroom	☐	Yes	☐	No
6- Sewage disposal	☐ 1	Bucket	☐ 2	Socking pits
	☐ 3	Common drainage	☐ 4	To the river
7- Animals	☐	Pets	☐	Livestock
	☐	Others (specify): _____		

C. Personal hygiene, habits and sanitation

1- Do you eat with hands?	☐	Yes	☐	No	☐ Sometimes

2- Do you wash your hands before eating? ☐ Yes ☐ No ☐ Sometimes

3- Do you eat raw vegetables? ☐ Yes ☐ No ☐ Sometimes

4- Do you eat fresh fruit? ☐ Yes ☐ No ☐ Sometimes

5- Do you wash fruit and vegetables? ☐ 1 Always ☐ 2 Usually

☐ 3 Sometimes ☐ 4 Never

6- Do you always drink boiled water? ☐ Yes ☐ No ☐ Sometimes

7- Do you bath at least once a day? ☐ Yes ☐ No ☐ Sometimes

8- Do you change your clothes at least once a day? ☐ Yes ☐ No ☐ Sometimes

9- Do you wear shoes when you go outside? ☐ Yes ☐ No ☐ Sometimes

10-Do you wash your hands after playing with soil? ☐ Yes ☐ No ☐ Sometimes

11- Do you clean yourself after defaecation? ☐ Yes ☐ No ☐ Sometimes

12- Do you wash your hands after defaecation? ☐ Yes ☐ No ☐ Sometimes

13- Where do you defaecate? ☐ 1 River ☐ 2 Pit latrine ☐ 3 Pour flush toilet

		4	Bush latrine	5	Outdoor	6	Others
14- Activities of children	i) Running barefooted		Yes		No		Sometimes
	ii) Playing with soil		Yes		No		Sometimes
	iii) Geophagia		Yes		No		Sometimes
15- Activities of adult	i) Forest activities		Yes		No		Sometimes
	ii) River activities		Yes		No		Sometimes
	iii)Rearing livestock		Yes		No		Sometimes

D. Health status

1- Any diarrhoea complain		Yes		No
2- How many times in a day?	/day			
3- How long has the diarrhea persisted?	/days			
4- Is the stool watery?		Yes		No

5- Is the stool bloody? ☐ Yes ☐ No

6- Are there any symptoms?

☐ 1 Vomiting ☐ 2 Anorexia

☐ 3 Abdominal pain ☐ 4 Mild fever

☐ 5 Other symptoms? (specify): _____

Yes = 1, No = 2 Sometimes = 3

II) Father

Name: _____

Age: _____

Monthly income: _____

Educational level:

☐ 1 No formal education ☐ 2 Primary

☐ 3 Secondary ☐ 4 Tertiary ☐ 5 University

Occupation:

☐ 1 Self-sufficient farmer ☐ 2 Government employees

☐ 3 Odd-jobs ☐ 4 Professional ☐ 5 Factory

III) <u>Mother</u>

Name: _____

Age: _____

Monthly income: _____

Educational level:

1	No formal education	2	Primary		
3	Secondary	4	Tertiary	5	University

Occupation:

1	Self-sufficient farmer	2	Government employees		
3	Odd-jobs	4	Professional	5	Factory

APPENDIX B

PREPERATION OF STOCK SOLUTIONS AND FORMAL ETHER TECHNIQUE

B1. Preparation of 2.5% potassium dichromate (1L)

1. Dissolve 25 g of Potassium dichromate in 500 ml of distilled water
2. Top up with distilled water to 1 litre

B2. Preservation of stool

1. Collect the stool in 60 ml screw-capped container
2. Cover the stool with 2.5% potassium dichromate or in 1:1 dilution
3. Keep the stool at 4 c until use

B3.Formal Ether technique (Nithiuthai S, 1997)

1. Two to five grams of stool were used for each test.

2-The stool was poured onto a double layer of wetted gauze taking special care to include any blood or mucus.

3-Ten milliliters of a preparation of normal saline were mixed.

4-The mixture was left for 1 hour before centrifugation for 5 minutes at 2,000 rpm.

5- The supernatant was poured off and mixed with the reagent in step 2 again.

6-Repeat centrifugation, at the same rate and for the same time, was conducted.

7-The supernatant was poured off and 10ml of 0.1% formalin were added.

8-The mixture was left for 10 minutes.

9-Three milliliters of ether were added; the tube was closed and mixing took place for 30 seconds.

10-The upper separated supernatant was poured off; the sediment was used for slide preparation

B4 Preparation of fecal sample before extraction

1. Small part of faecal specimen was mixed with 10 ml of distilled water and sieved using cotton**.**
2. The suspended faeces were centrifuge at 1500 xg for 10 min and the supernatant was discarded to the waste.
3. Repeat Step one and two for 3 times to remove the preservative

APPENDIX C

ZIEHL-NEELSEN STAIN

C1 ZIEHL-NEELSEN STAINING TECHNIQUE

A. Materials and equipments

- Carbol fuchsin
- Malachite green
- Acid alcohol (3ml concentrated hydrochloric acid + 97 ml of 95% ethyl alcohol)
- Methanol
- Microscopic slides
- Forceps
- Staining rakes

B. Procedures

1. A thin smear of faecal specimen was made and left to air dry.
2. The smear was fixed with methanol.
3. The smear was stained with Carbol fuchsin for 10 min.
4. The smear was differentiated with acid alcohol for 15 sec or until the colour ceased to flood out.
5. The smear was counterstained with Malachite green fro 3 min and washed with tape water.
6. The stained smear was air dried and examined using the magnification 1000X.

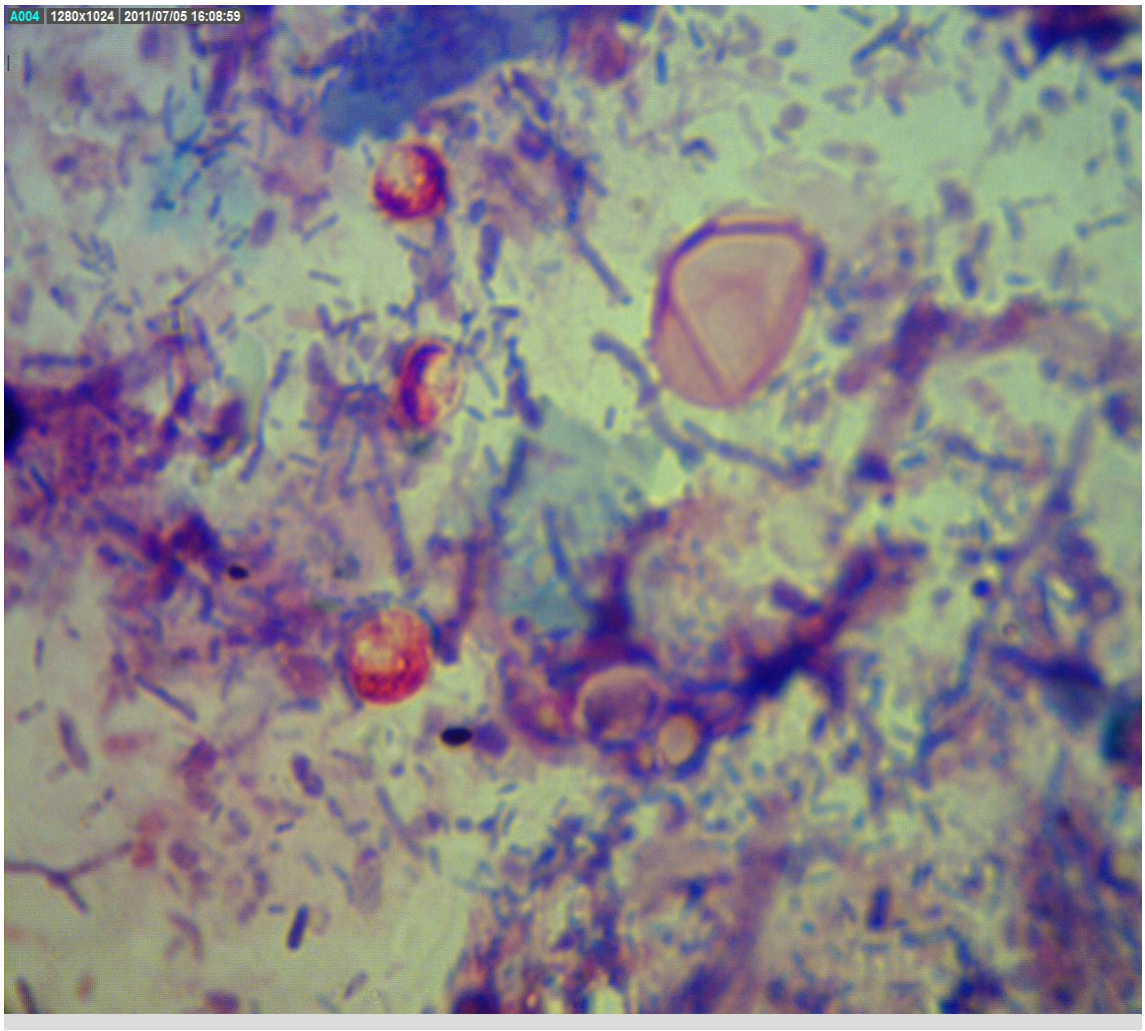

Figure C1

Cryptosporidium oocyst. Sample number 343

APPENDIX D

MANUFACTURE INSTRUCTIONS DNA ISOLATION

Experienced User Protocol
Please wear gloves at all times

1. To the **PowerBead Tubes** provided, 0.25 grams of soil sample.
2. Gently vortex to mix.
3. **Check Solution C1.** If **Solution C1** is precipitated, heat solution to 60°C until dissolved before use.
4. Add 60 µl of **Solution C1** and invert several times or vortex briefly.
5. Secure **PowerBead Tubes** horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.
   Note: If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.
6. Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds at room temperature. **CAUTION:** Be sure not to exceed 10,000 x *g* or tubes may break.
7. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).
   Note: Expect between 400 to 500 µl of supernatant. Supernatant may still contain some soil particles.
8. Add 250 µl of **Solution C2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.
10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean **2 ml Collection Tube** (provided).
11. Add 200 µl of **Solution C3** and vortex briefly. Incubate at 4°C for 5 minutes.
12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.
13. Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean **2 ml Collection Tube** (provided).
14. Shake to mix Solution C4 before use. Add 1200 µl of **Solution C4** to the supernatant and vortex for 5 seconds.
15. Load approximately 675 µl onto a **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature. Load the remaining supernatant onto the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature.
    Note: A total of three loads for each sample processed are required.
16. Add 500 µl of **Solution C5** and centrifuge at room temperature for 30 seconds at 10,000 x *g*.
17. Discard the flow through.
18. Centrifuge again at room temperature for 1 minute at 10,000 x *g*.
19. Carefully place spin filter in a clean **2 ml Collection Tube** (provided). Avoid splashing any **Solution C5** onto the **Spin Filter**.
20. Add 100 µl of **Solution C6** to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10).
21. Centrifuge at room temperature for 30 seconds at 10,000 x *g*.
22. Discard the **Spin Filter**. The DNA in the tube is now ready for any downstream application. No further steps are required.

We recommend storing DNA frozen (-20° to -80°C). **Solution C6** contains no EDTA. To concentrate the DNA see the Hints & Troubleshooting Guide.

APPENDIX E

DNA AMPLIFICATION PROTOCOLS

E1 PCR protocols

E1.1 Preparation of chemicals for PCR.

2.1.1 dNTP (1.25mM)

- Add 175 µl of dH2O to sterile 1.5 microcentrifuge tube.
- Add 25 µl of 10 mM dNTP to the tube.
- Label the tube and store at – 20 ^{o}C until use.

2.1.2 Primers (4 µM)

- Add 192 µl of dH2O to sterile 1.5 microcentrifuge tube.
- Add 8 µl of each 100 µM primer to the tube.
- Label the tube and store at – 20 ^{o}C until use.

E1.2 Giardia 16S rRNA PCR protocols (read *et al*.2002) with minor modifications (Mahdy *et al*., 2009)

A. Equipment

- Micropipettes and sterile corresponding tips
- Gloves
- Ice
- PCR tubes- 0.2ml thermal tubes
- Microtubes holder tray (P10,P20, P200. P1000)
- Thermocycler

B. Reagents

- PCR buffer (10X)
- dNTP (1.25mM)
- Magnesium chloride (25mM)
- Bovine Serum Albumin (10mg/ml)
- Dimethyl sulfoxide (DMSO) (Sigma, cat. no. 673439, USA
- RH 11 (4 µM)
- RH 4 (4 µM)
- Giar-F (4 µM)
- Giar-R (4 µM)
- dH_2O

C. Procedure

1. Pre-sterilise pipttes, microtube holder tray, gloves, PCR thermal tubes and the distilled water using UV for 20 min.
2. Wear PCR lab coat and gloves.
3. Label each thermal tube lid with the appropriate sample number.
4. Label two 1.5 ml microcentrifuge tubes for preparing the master mix.
5. Prepare the primary PCR master mix as following:

Reagent	1X	Master Mix
PCR buffer (10X)	5 µl	
dNTP (1.25mM)	4µl	
Magnesium chloride (25mM)	2µl	
Bovine Serum Albumin (10mg/ml)	1 µl	
DMSO	2 µl	
RH11 (4 µM)	3.125 µl	
RH4 (4 µM)	3.125µl	
dH2O	3 µl	
Taq polymerase (5U/ µl)	0.5 µl	
Total	23µl	

6. The number of samples being assayed will dictate the volume of the master mix prepared. Enough master mix is prepared to be sufficient for each sample plus two samples.

7. Turn on the PCR to warm.

8. Vortex the master mix.

9. Add 23 µl to each tube including positive and negative controls tubes.

10. In another bench using another micropipette add 2 µl of the extracted DNA to the appropriate tube.

11. Add 2 µl of the positive control to the positive control tube at the end to avoid contamination.

12. Vortex PCR tubes lightly

13. Run the primary PCR under the following condition

	Temperature / Time	Cycle
Denaturation	95 ºC / 2 min	1 cycle
Denaturation Annealing Extension	96 ºC / 20 sec 59 ºC / 20 sec 72 ºC / 20 sec	35 cycles
Extension	72 ºC / 7min	1 cycle

14. Store the primary PRC product at -20 ºC for long time or at 4 ºC for short time.

15. While running the primary PCR, prepare the secondary PCR master mix.

Reagent	1X	Master Mix
PCR buffer (10X)	5 µl	
dNTP (1.25mM)	4 µl	
Magnesium chloride (25mM)	2µl	
Non-acetylated BSA (10mg/ml)	1µl	
DMSO	1.25	
Gia-R (4 µM)	3.125µl	
Gia-F (4 µM)	3.125 µl	
dH2O	3 µl	
Taq polymerase (5U/ µl)	0.5 µl	
Total	23 µl	

16. Add 23µl of PCR mixture to each tube.

17. Add 2 µl of the primary PCR product using special pipette (DNA pipette) to PCR tubes including positive and negative control.

18. Mix by vortixing.

19. Run the secondary PCR under the previous cycling conditions as mentioned the primary PCR.

20. Store the secondary PRC product at -20 ºC for long time or at 4 ºC for short time.

E1.3 *b*-giardin gene according (caccio *et al* 2002) and (lalle *et al* 2005)

A Equipment

- Micropipettes and sterile corresponding tips
- Gloves
- Ice
- PCR tubes- 0.2ml thermal tubes
- Microtubes holder tray (P10,P20, P200. P1000)
- Thermocycler

B Reagents

- PCR buffer (10X)
- dNTP (1.25mM)
- Magnesium chloride (25mM)
- G7 (4 µM)
- G759 (4 µM)

- G376 (4 µM)
- Taq polymerase (5U/ µl)
- DNAse free water

C. Procedure

1. Pre-sterilise pipttes, microtube holder tray, gloves, PCR thermal tubes and the distilled water using UV for 20 min.
2. Wear PCR lab coat and gloves.
3. Label each thermal tube lid with the appropriate sample number.
4. Label two 1.5 ml microcentrifuge tubes for preparing the master mix.
5. Prepare the primary PCR master mix as following:

Reagent	1X	Master Mix
PCR buffer (10X)	5 µl	
dNTP (1.25mM)	8 µl	
Magnesium chloride (25mM)	3 µl	
Bovine Serum Albumin (10mg/ml)	2 µl	
G7 (4 µM)	2.5 µl	
G795 (4 µM)	2.5µl	
dH2O	24.5µl	
Taq polymerase (5U/ µl)	0.5 µl	
Total	48 µl	

6. The number of samples being assayed will dictate the volume of the master mix prepared. Enough master mix is prepared to be sufficient for each sample plus two samples.

7. Turn on the PCR to warm.

8. Vortex the master mix.

9. Add 48 µl to each tube including positive and negative controls tubes.

10. In another bench using another micropipette add 2 µl of the extracted DNA to the appropriate tube.

11. Add 2 µl of the positive control to the positive control tube at the end to avoid contamination.

12. Vortex PCR tubes lightly

13. Run the primary PCR under the following condition

	Temperature / Time	Cycle
Denaturation	95 ºC / 5 min	1 cycle
Denaturation Annealing Extension	94 ºC / 30 sec 65ºC / 30 sec 72 ºC / 60 sec	35 cycles
Extension	72 ºC / 7 min	1 cycle

14. Store the primary PRC product at -20 ºC for long time or at 4 ºC for short time.

15. While running the primary PCR, prepare the secondary PCR master mix.

Reagent	1X	Master Mix
PCR buffer (10X)	5 µl	
dNTP (1.25mM)	8 µl	

Magnesium chloride (25mM)	3µl	
Non-acetylated BSA (10mg/ml)	2 µl	
G376 (4 µM)	2.5µl	
G759 (4 µM)	2.5 µl	
dH2O	24.5 µl	
Taq polymerase (5U/ µl)	0.5 µl	
Total	45 µl	

16. Add 45µl of PCR mixture to each tube.

17. Add 5 µl of the primary PCR product using special pipette (DNA pipette) to PCR tubes including positive and negative control.

18. Mix by vortixing.

19. Run the secondary PCR under the previous cycling conditions as mentioned the primary PCR

20. Store the secondary PRC product at -20 ºC for long time or at 4 ºC for short time.

E1.4 DNA amplification targeting 18 SSU RNA PCR protocol (Nichols *et al*., 2003)

A Equipment

- Micropipettes and sterile corresponding tips
- Gloves
- Ice

- PCR tubes- 0.2ml thermal tubes
- Microtubes holder tray (P10,P20, P200. P1000)
- Thermocycler

B Reagents

- PCR buffer (10X)
- dNTP (1.25mM)
- Magnesium chloride (25mM)
- Bovine Serum Albumin (10mg/ml)
- CPB-DIAGF2 (4 µM)
- CPB-DIAGR2 (4 µM)
- CPB-DIAGF (4 µM)
- CPB-DIAGR (4µM)
- Taq polymerase (5U/ µl)
- DNAse free water

C. Procedure

1. Pre-sterilise pipttes, microtube holder tray, gloves, PCR thermal tubes and the distilled water using UV for 20 min.
2. Wear PCR lab coat and gloves.
3. Label each thermal tube lid with the appropriate sample number.

4. Label two 1.5 ml microcentrifuge tubes for preparing the master mix.

5. Prepare the primary PCR master mix as following:

Reagent	1X	Master Mix
PCR buffer (10X)	5 µl	
dNTP (1.25mM)	8 µl	
Magnesium chloride (25mM)	7 µl	
Bovine Serum Albumin (10mg/ml)	2 µl	
CPB-DIAGF2 (4 µM)	2.5 µl	
CPB-DIAGR2 (4 µM)	2.5µl	
dH2O	20.5µl	
Taq polymerase (5U/ µl)	0.5 µl	
Total	48 µl	

6. The number of samples being assayed will dictate the volume of the master mix prepared. Enough master mix is prepared to be sufficient for each sample plus two samples.

7. Turn on the PCR to warm.

8. Vortex the master mix.

9. Add 48 µl to each tube including positive and negative controls tubes.

10. In another bench using another micropipette add 2 µl of the extracted DNA to the appropriate tube.

11. Add 2 µl of the positive control to the positive control tube at the end to avoid contamination.

12. Vortex PCR tubes lightly

13. Run the primary PCR under the following condition

	Temperature / Time	Cycle
Denaturation	95 ºC / 5 min	1 cycle
Denaturation Annealing Extension	94 ºC / 30 sec 68 ºC / 1 min 72 ºC / 30 sec	35 cycles
Extension	72 ºC / 10 min	1 cycle

14. Store the primary PRC product at -20 ºC for long time or at 4 ºC for short time.

15. While running the primary PCR, prepare the secondary PCR master mix.

Reagent	1X	Master Mix
PCR buffer (10X)	5 µl	
dNTP (1.25mM)	8 µl	
Magnesium chloride (25mM)	7 µl	
Non-acetylated BSA (10mg/ml)	2 µl	
CPB-DIAGF (4 µM)	2.5µl	
CPB-DIAGR (4 µM)	2.5 µl	
dH2O	17.5 µl	
Taq polymerase (5U/ µl)	0.5 µl	
Total	45 µl	

16. Add 45µl of PCR mixture to each tube.

17. Add 5 µl of the primary PCR product using special pipette (DNA pipette) to PCR tubes including positive and negative control.

18. Mix by vortixing.

19. Run the secondary PCR under the previous cycling conditions as mentioned the primary PCR except the annealing temperature which should be decreased to 60 ºC instead of 68 ºC.

20 Store the secondary PRC product at -20 ºC for long time or at 4 ºC for short time.

E1.5 DNA amplification targeting *gp60* gene (Alves *et al.*, 2003)

i. Equipment

- Micropipettes and sterile corresponding tips
- Gloves
- Ice
- PCR tubes- 0.2ml thermal tubes
- Microtubes holder tray
- Thermocycler

ii. Reagents

- PCR buffer (10X)
- dNTP (1.25mM)
- Magnesium chloride (25mM)
- gp15-ATG (4 µM)
- gp15-STOP (4 µM)

- gp15-15A (4 µM)
- gp15-15E (4 µM)
- Taq polymerase (5U/ µl)
- DNAse free water

iii. Procedure

1. Pre-sterilise pipttes, microtube holder tray, gloves, PCR thermal tubes and the distilled water using UV for 20 min.
2. Wear PCR lab coat and gloves.
3. Label each thermal tube lid with the appropriate sample number.
4. Label two 1.5 ml microcentrifuge tubes for preparing the master mix.
5. Prepare the primary PCR master mix as following:

Reagent	1X	Master Mix
PCR buffer (10X)	5 µl	
dNTP (1.25mM)	5 µl	
Magnesium chloride (25mM)	6 µl	
gp15-ATG (4 µM)	6.25 µl	
gp15-STOP (4 µM)	6.25 µl	
dH2O	19 µl	
Taq polymerase (5U/ µl)	0.5 µl	
Total	48 µl	

6. The number of samples being assayed will dictate the volume of the master mix prepared. Enough master mix is prepared to be sufficient for each sample plus two samples.

7. Turn on the PCR to warm.

8. Vortex the master mix.

9. Add 48 µl to each tube including positive and negative controls tubes.

10. In another bench using another micropipette add 2 µl of the extracted DNA to the appropriate tube.

11. Add 2 µl of the positive control to the positive control tube at the end to avoid contamination.

12. Vortex PCR tubes lightly

13. Run the primary PCR under the following condition:.

	Temperature / Time	Cycle
Denaturation	95 ºC / 5 min	1 cycle
Denaturation Annealing Extension	94 ºC / 30 sec 55 ºC / 30 sec 72 ºC / 1min	40 cycles
Extension	72 ºC / 10 min	1 cycle

14. Store the primary PRC product at -20 ºC for long time or at 4 ºC for short time.

15. While running the primary PCR, prepare the secondary PCR master mix.

Reagent	1X	Master Mix
PCR buffer (10X)	5 µl	
dNTP (1.25mM)	5 µl	
Magnesium chloride (25mM)	6 µl	
gp15-15A (4 µM)	6.25 µl	
gp15-15E (4 µM)	6.25 µl	
dH2O	16 µl	
Taq polymerase (5U/ µl)	0.5 µl	
Total	45 µl	

16. Add 45µl of PCR mixture to each tube.

17. Add 5 µl of the primary PCR product using special pipette (DNA pipette) to PCR tubes including positive and negative control.

18. Mix by vortixing.

19. Run the secondary PCR under the previous cycling conditions as mentioned the primary PCR except the annealing time and extension time was 30 sec with 35 cycles.

20. Store the secondary PRC product at -20 ºC for long time or at 4 ºC for short time

APPENDIX F

PICTURE OF GEL ELECTROPHORESIS

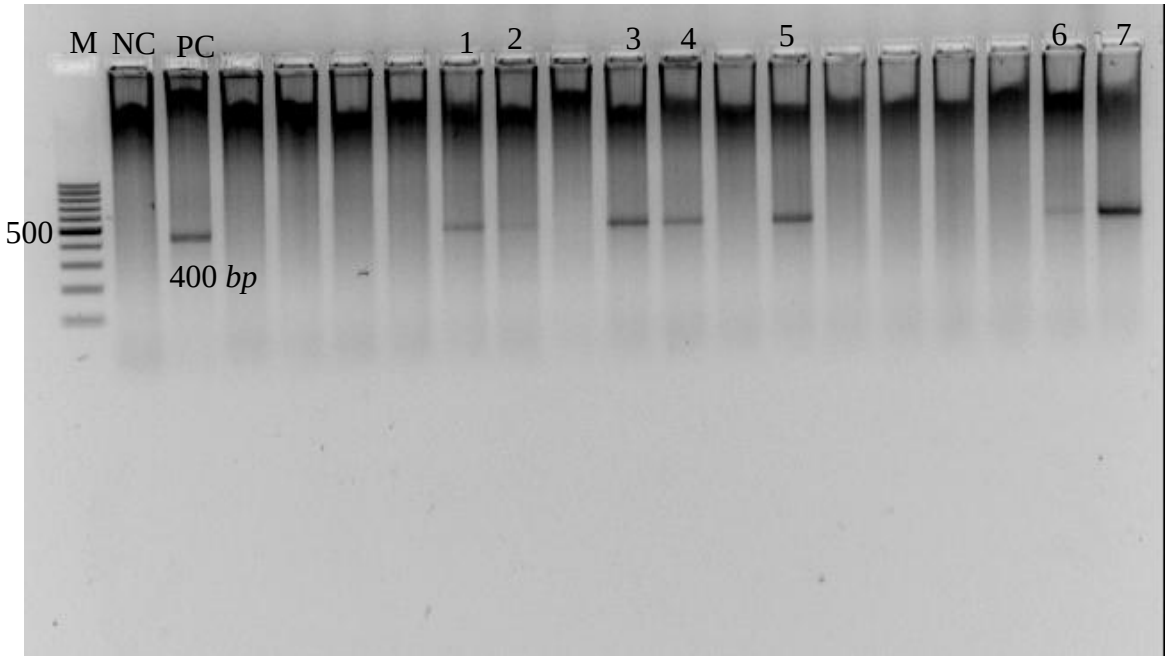

Figure F1

Representative presentation of PCR products generated from *Cryptosporidium* positive faecal specimen on 2% agarose electrophoretic gel for 18Sr RNA

M : 100-bp DNA marker, with molecular size indicated in base-pair unit for prominent band

NC: Negative control

PC : Positive control (*Cryptosporidium parvum*)

Lanes 1-7: secondary PCR product

APPENDIX G

MANUFACTURE INSTRUCTIONS DNA PURIFICATION

QIAquick PCR Purification Kit Protocol

using a microcentrifuge

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions (see page 8). For cleanup of other enzymatic reactions, follow the protocol as described for PCR samples or use the MinElute Reaction Cleanup Kit. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge.

Important points before starting

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature.
- Add 1:250 volume pH indicator I to Buffer PB (i.e., add 120 µl pH indicator I to 30 ml Buffer PB or add 600 µl pH indicator I to 150 ml Buffer PB). The yellow color of Buffer PB with pH indicator I indicates a pH of ≤7.5.
- Add pH indicator I to entire buffer contents. Do not add pH indicator I to buffer aliquots.
- If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I.

Procedure

1. **Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene.**

   For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).

2. **If pH indicator I has beein added to Buffer PB, check that the color of the mixture is yellow.**

   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

3. **Place a QIAquick spin column in a provided 2 ml collection tube.**
4. **To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.**
5. **Discard flow-through. Place the QIAquick column back into the same tube.**

   Collection tubes are re-used to reduce plastic waste.

6. **To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.**
7. **Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.**

   IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

PCR Purification Spin Protocol

8. **Place QIAquick column in a clean 1.5 ml microcentrifuge tube.**

9. **To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.**

   IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer.

   Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

10. **If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.**

    Loading dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type.

QIAquick Gel Extraction Kit Protocol

using a microcentrifuge

This protocol is designed to extract and purify DNA of 70 bp to 10 kb from standard or low-melt agarose gels in TAE or TBE buffer. Up to 400 mg agarose can be processed per spin column. This kit can also be used for DNA cleanup from enzymatic reactions (see page 8). For DNA cleanup from enzymatic reactions using this protocol, add 3 volumes of Buffer QG and 1 volume of isopropanol to the reaction, mix, and proceed with step 6 of the protocol. Alternatively, use the MinElute Reaction Cleanup Kit.

Important points before starting

- The yellow color of Buffer QG indicates a pH ≤7.5.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional table-top microcentrifuge at room temperature.

Procedure

1. **Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.**

   Minimize the size of the gel slice by removing extra agarose.

2. **Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).**

   For example, add 300 µl of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one QIAquick column.

3. **Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.**

   IMPORTANT: Solubilize agarose completely. For >2% gels, increase incubation time.

4. **After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).**

   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

   The adsorption of DNA to the QIAquick membrane is efficient only at pH ≤7.5. Buffer QG contains a pH indicator which is yellow at pH ≤7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

5. **Add 1 gel volume of isopropanol to the sample and mix.**

   For example, if the agarose gel slice is 100 mg, add 100 µl isopropanol. This step increases the yield of DNA fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb, addition of isopropanol has no effect on yield. Do not centrifuge the sample at this stage.

6. **Place a QIAquick spin column in a provided 2 ml collection tube.**
7. **To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.**

   The maximum volume of the column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.
8. **Discard flow-through and place QIAquick column back in the same collection tube.**

   Collection tubes are reused to reduce plastic waste.
9. **Recommended: Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min.**

   This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing, in vitro transcription, or microinjection.
10. **To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.**

    Note: If the DNA will be used for salt-sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 min after addition of Buffer PE, before centrifuging.
11. **Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at 17,900 x *g* (13,000 rpm).**

    IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
12. **Place QIAquick column into a clean 1.5 ml microcentrifuge tube.**
13. **To elute DNA, add 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.**

    IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl.

    Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.
14. **If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.**

    Loading dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type.

APPENDIX H

PICTURES OF RURAL AREAS IN YEMEN

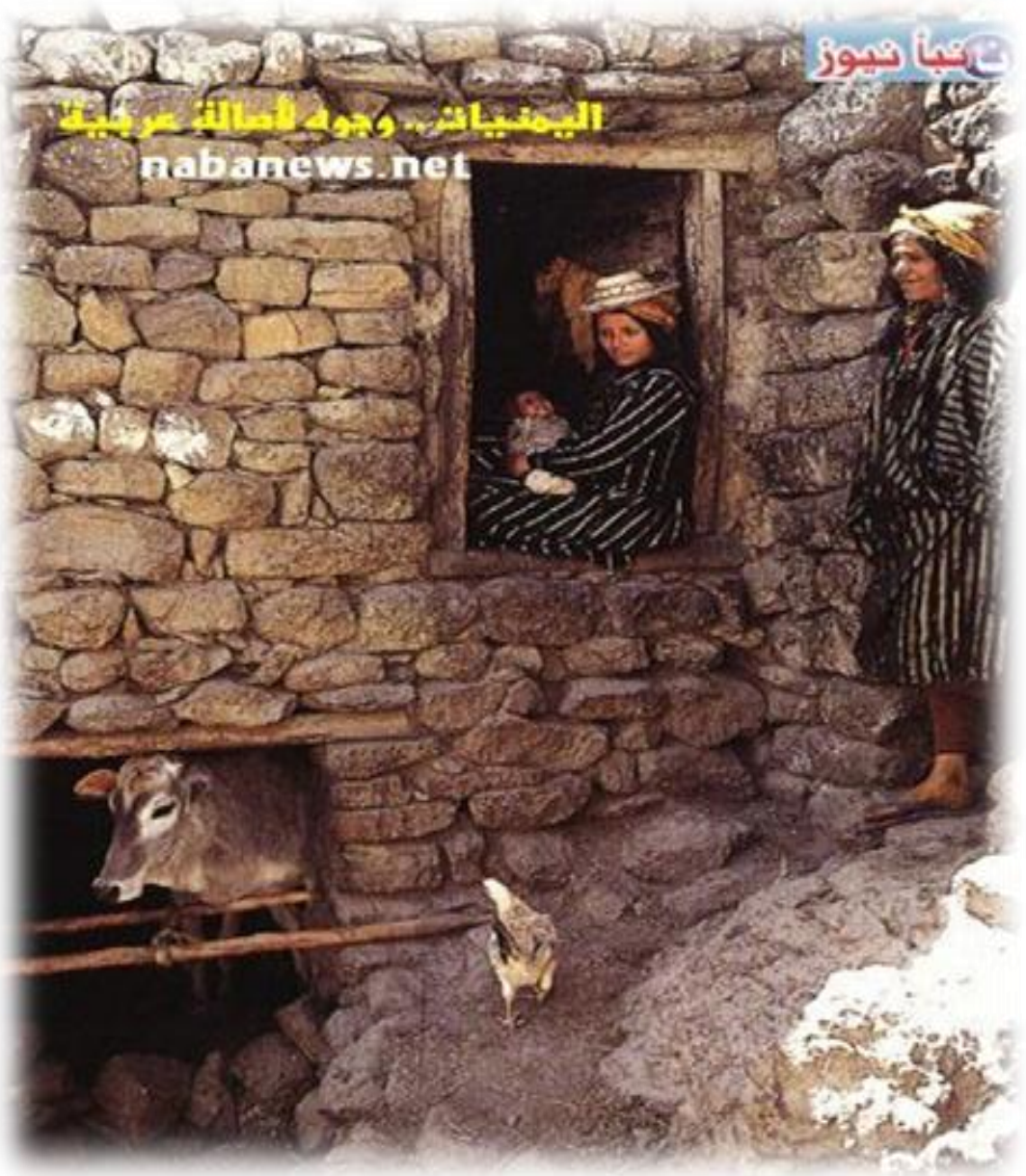

Figure H1

Showed animals living in the basement while family on the first floor (rural area in Yemen) picture from NabaNews.net

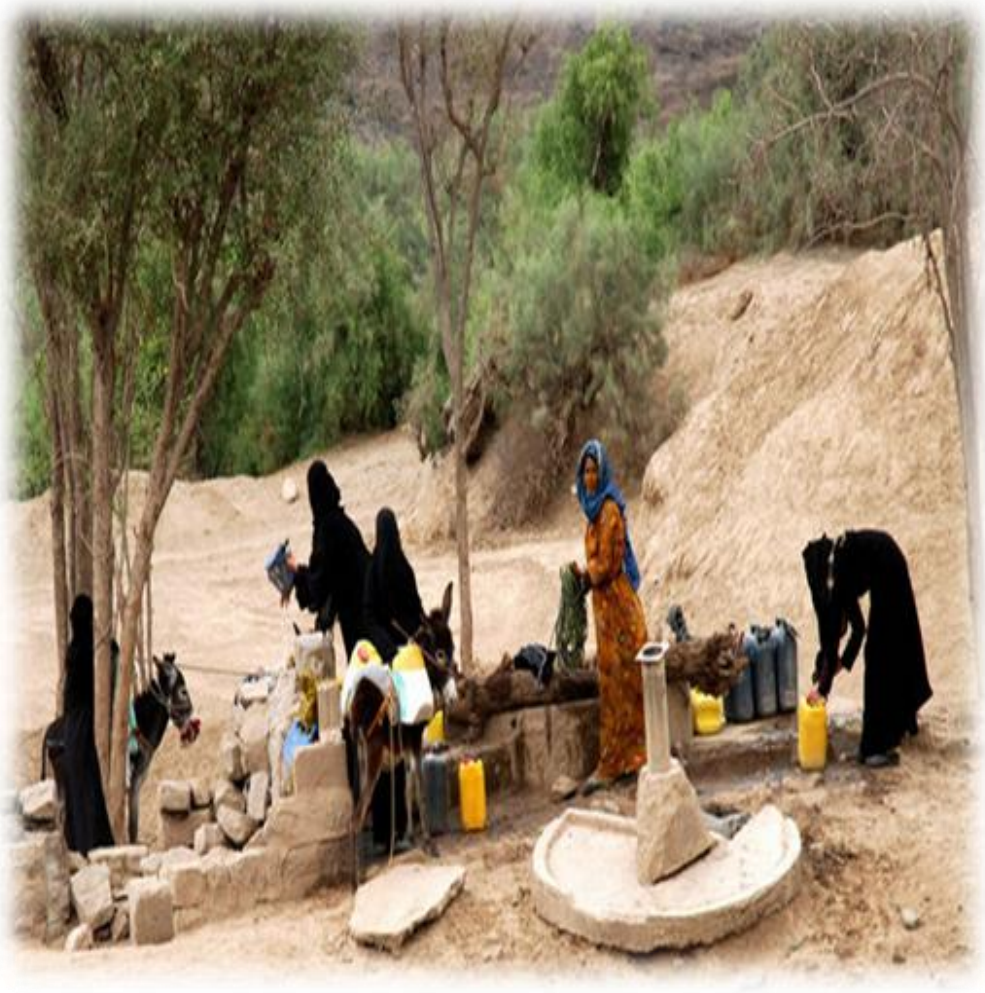

Figure H2

Showed women bring water from well for house hold need

APPENDIX I

LIST OF PUBLICATIONS

Publication in international journal

N. A **Alyousefi.**, M. A. K. Mahdy, R. Mahmud and Yvonne A. L. Lim. 2011.

Factors Associated with High Prevalence of Intestinal Protozoan Infections among Patients in Sana'a City, Yemen. PLOS ONE, vol. 6,NO. 7, P. e22044.

N . A **Alyousefi.**, M. A. K. Mahdy, L. Xiao and, R . Mahmud and Yvonne A. L. Lim. 2013.

Molecular characterization of *Giardia duodenalis* in Yemen. Experimental parasitology.

N . A **Alyousefi.**, M. A. K. Mahdy, Yvonne A. L. Lim L, Xiao and and R . Mahmud. 2013.

First molecular characterization of *Cryptosporidium* in Yemen. Parasitology, 1-6.

Presentation in international conferences

Oral presentation In 46th MSPTM "Malaysian Society of Parasitology and Tropical Medicine "Annual Seminar Student Competition" Prevalence and risk factors of protozoan infections among patients attending hospitals in Sana'a City, Yemen" on 24th-25th Mac 2010, Kuala Lumpur, Malaysia.

Poster presentation in international congress of parasitology (ICOPA) N. A. Alyousefi , M. A.K. Mahdy , R. Mahmud, Y. A.L. Lim 1 "Molecular characterization of *Giardia duodenalis* isolated from patients attending hospitals in Sana'a City, Yemen" Melbourne, Australia, from 15-20th August 2010.

Oral presentation in 16th BSGC Biological Sciences Graduate Congress "Molecular classification of *Giardia duodenalis* and the correlation with risk factors study on outpatient Sana'a city, Yemen on 12-14 Dec 2011, Singapore.