DEVELOPMENT OF UNIPLEX AND MULTIPLEX PCR ASSAYS FOR THE DETECTION OF HUMAN ENTERIC PROTOZOAN PATHOGENS

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by

YousryAbdel Hameid Hawash EL-Sabbagh

Department of Infection, Immunity and Inflammation
University of Leicester

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Statement of originality

This accompanying thesis submitted for the degree of PhD entitled "Development of uniplex and multiplex PCR assays for the detection of human enteric protozoan pathogens" is based on work conducted by the author in the Department of Infection, Immunity and Inflammation mainly during the period between January 2007 and January 2010.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other University.

Signed _____ Date _____

Abstract

Cryptosporidium spp., *Giardia lamblia* and *Entamoeba histolytica* are the most frequently identified enteric protozoa in water-borne disease outbreaks. Many PCR assays, with satisfactory results in terms of sensitivities and specificities, have been developed for detection of the aforementioned three protozoa in faecal specimens but the majority of these assays have a limited usage in the clinical laboratories due to being more costly and more time-consuming than the conventional diagnostic methods.

Based on published oligonucleotide primers, three individual uniplex PCR assays were developed, properly optimised and subsequently combined into a conventional multiplex PCR format to screen for the three protozoa in the same stool specimen. The multiplex PCR assay was clinically validated with 185 control and 212 randomly selected stool samples. The assay was optimised with DNA directly retrieved from stool samples using a modified QIAamp® DNA Stool Mini Kit (Qiagen) DNA extraction protocol subsequent amplification using single-round well-controlled PCR protocol.

Like the individual PCRs, the multiplex PCR assay detected genomic DNA from control isolates matching 12, 12 and four copies of the *Cryptosporidium*, *G. lamblia* and *E. histolytica* genomes, respectively. Similarly, ~100 (oo)cysts per 200µl stool were successfully identified by the multiplex and the matching uniplex-PCR assays as detection limits. The diagnostic sensitivity, specificity, negative predictive value and positive predictive value of the multiplex and the individual PCR assays were comparable and equal to 97 %, 100 %, 95 % and 100 %, respectively. Furthermore, by nominating three nested PCRs as '*gold standards*', the multiplex PCR demonstrated specificity and sensitivity exceeding that achieved by the combined copro-antigen immunoassay adopted for *Cryptosporidium*/*Giardia* diagnosis at the Clinical Microbiology laboratory, Leicester Royal Infirmary, University Hospital of Leicester.

In conclusion, the newly developed multiplex PCR was demonstrated to be a simple, cost-effective, adequately sensitive and highly specific assay. An assay with this broad-spectrum format has a great potential to be adopted as a routine test in diagnostic laboratories especially those in resource-poor countries where parasitic protozoal infections are endemic.

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Abbreviations

ά	alpha
ß	beta
$CaCl_2$	Calcium chloride
EDTA	Ethylenediaminetetraacetic acid
EAC	External Amplification Control
EIA	Enzyme Immuno-Assay
λ	lambda
µl	microliter (s)
µM	micromolar
amp	ampicillin
BSA	bovine serum albumin
bp	base pair(s)
DTT	Dithiothreitol
dH_2O	distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
F	Forward (primer)
g	grams or gravitational acceleration
GC	Guanine-cytosine
h	hours
HCl	Hydrochloric acid
IAC	Internal Amplification Control
IPCR	Inverse PCR
Kb	kilobase pair(s)
kDa	Kilo-Dalton
KOH	Potassium hydroxide
l	liter(s)
LF	Lateral Flow
LB	Luria Bertani Medium

M	Molar
$MgCl_2$	Magnesium chloride
MCS	Multiple cloning site
min	minute(s)
ml	milliliter(s)
mM	millimolar
$MgSO_4$	Magnesium sulfate
NaCl	Sodium chloride
Na OH	Sodium hydroxide
nt	nucleotide
°C	degrees Celsius
OD_{600}	optical density at 600 nm
o/n	overnight
(oo)cysts	Oocysts or cysts
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
R	Reverse (primer)
rpm	Revolutions per minute
RT	Room temperature
RNase	Ribonuclease
PVP	Polyvinylpolypyrrolidone
SAF	Sodium acetate acetic acid formalin
SDS	Sodium dodecyl sulphate
sec	Second(s)
SOC	Super Optimal broth with Catabolite repression
TAE	Tris- acetate EDTA buffer
TE	Tris EDTA
Tm	Melting temperature
U	units
UV	Ultraviolet
v/v	volume per volume
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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1 Introduction

Enteric protozoa are uni-cellular microscopic parasites inhabiting the gastrointestinal tract of numerous vertebrate hosts. Of these protozoa, *Cryptosporidium* spp., *Giardia lamblia* and *Entamoeba histolytica* are considered responsible for the majority of human infections (Kosek *et al.*, 2001; Ortega and Adam, 1997 and Haque *et al.*, 2003). Human infections caused by *Cryptosporidium* spp., and *Giardia lamblia* are termed cryptosporidiosis and *giardia*sis respectively. Although the greatest burden of both diseases occurs in developing countries, *Cryptosporidium* has been recognized as a major cause of many waterborne and food-borne outbreaks of gastroenteritis in developed countries (Current and Garcia, 1991 and Nichols, 2000). *G. lamblia* (synonyms: *Giardia intestinalis* and *Giardia duodenalis*) is also considered as the main cause of non-viral non-bacterial diarrhea in developed countries (Hoque *et al.*, 2002). Similarly, human disease caused by *E. histolytica* is named amoebiasis. Infection with *E. histolytica* has been reported in many countries but the highest prevalence rates are reported in developing countries (Stanley, 2003). Due to the invasive potential of *E. histolytica*, approximately 100,000 cases from a global burden of 50 million cases are thought to result in death each year (Anonymous, 1997).

Diagnosis of these enteric protozoa relies entirely on laboratory diagnosis due to the frequently asymptomatic nature of infection and the high similarity of three clinical presentations (Thielman and Guerrant, 2004). Direct observation of protozoa by microscopy is frequently employed as a rapid and simple diagnostic method. However, it has frequently been shown to offer a low sensitivity and to depend to a great extent upon the skill of the person carrying out the analysis (Verweij *et al.*, 2004).

Currently, several antigen detection commercial kits are available and accepted as alternative cost-effective diagnostic methods (Garcia and Shimizu, 1997 and Mank *et al.*, 1997). However, the specificity and sensitivity of these kits have been reported to be lower than those obtained using the PCR based detection assays (Verweij *et al.*, 2003 and Fayer *et al.*, 2000). Although many PCR-based diagnostic assays have been reported, to the best of my knowledge none of these assays have been introduced into the clinical laboratory as routine diagnostic tests. This may be due to a number of reasons. First, most of these assays rely on multi-step procedures for the protozoal DNA extraction and subsequent PCR amplification. Second, most of these assays lack standardization and proper clinical evaluation. Finally, for poor countries where parasitic infections predominate, PCR is still considered an expensive technique in comparison to the conventional diagnostic methods.

1.1 Historical background

The first detailed description of *Cryptosporidium* was in 1907 when Ernest Tyzzer, an American parasitologist accidentally discovered a round organism with four naked internal sporozoites not surrounded by sporocysts in the gastric epithelium of laboratory mice and gave it a name of *Cryptosporidium muris* (Tyzzer, 1910). Five years later, he identified similar parasite in the small intestine and named it *Cryptosporidium parvum*. Over many years, numerous *Cryptosporidium* species were recognized in avian, animal and reptilian hosts. At that time, it was thought that *Cryptosporidium* was only a cause of avian and bovine diarrhea. In 1976, the first two human cases of cryptosporidiosis were reported (Nime *et al.*, 1976 and Meisel *et al.*, 1976). One case was in a three year-child living on a farm and the second was for

a woman undergoing chemotherapy and taking corticosteroids. Six years later, the pathogenic potential of *Cryptosporidium* was fully appreciated (Current *et al.,* 1983).

Giardia was discovered in year 1681 when a Dutch lens maker Antony Van Leeuweenhoek described a highly motile flagellated organism in his own watery stool (Thompson, 2000). In 1859, Vilém Dušan Lambl re-described the organism in more details and named it '*Cercomonas intestinalis'*. Many years later, it was named *Giardia lamblia* after him. Despite of the early description of *Giardia*, its clinical significance was not recognized until 1970. In this year, a number of visitors to Soviet Union acquired the infection (Brodsky *et al.,* 1974). Since then, *Giardia* has been considered as a major cause of traveler's diarrhea especially in the developing world (Wolfe, 1978).

Entamoeba histolytica was first identified in year 1875 when Fyodor Lösch identified amoebae in clinical samples from a case of severe dysentery in St Petersburg, Russia and gave the observed organisms the descriptive name '*Amoeba coli'*. Fifteen years later, William Osler identified a case of amoebic liver abscess. The next year, Councilman and Lafleur, at Johns Hopkins Hospital, confirmed the pathogenic potential of '*Amoeba coli'*. The organism was named as *Entamoeba histolytica* by Fritz Schaudinn. Several years later, other species of enteric amoeba were identified but the relationships of these species to *E. histolytica* were not recognised until 1925. Emile Brumpt suggested the presence of two morphologically identical species, one being pathogenic (*E. histolytica*) and the other non-pathogenic (*Entamoeba dispar*). The existence of these independent two species was the subject of debate for many years (Diamond and Clark, 1993). In 1997 the separation between the two species was accepted and given WHO approval (Anonymous, 1997).

1.2 General biology of the three protozoa

1.2.1 Morphology of the developmental stages

The target protozoa have two main developmental stages. The vegetative and motile form is termed trophozoite as in *G. lamblia* and *E. histolytica* or sporozoites as in *Cryptosporidium.* The other stage is dormant and recognised as cysts in *Giardia* and *Entamoeba* or oocysts in *Cryptosporidium.*

As can be seen in **Figure 1-1,** the *Cryptosporidium* oocyst is round in shape and has no visible nucleus. The mature oocyst is 4 – 6 µm in diameter and contains four crescent shaped parallel sporozoites (trophozoites) outlined with a smooth robust oocyst wall.

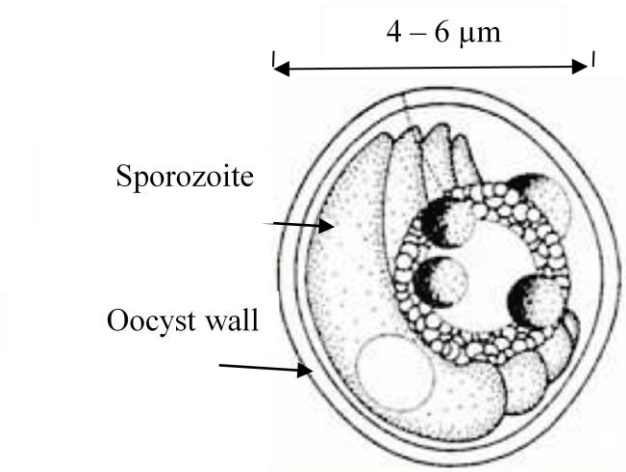


Figure 1-1: Schematic diagram of *Cryptosporidium* oocyst adapted from (Upton and Current, 1985).

The *Giardia* trophozoite is pear-shaped and dorsally convex. It has four pairs of posteriorly directed flagella. It measures 12 – 15 µm in length and 4 – 8 µm in width. It has a pair of pointed elongated median bodies with two apparently identical nuclei.

The central median body is considered to be an organelle unique to *Giardia* Trophozoite.

The trophozoite moves by flagella and attaches itself to the intestinal wall by a concave sucking disc present on the ventral surface. The *Giardia* cyst is oval or round in shape and 6 – 10 µm in size. It is surrounded by a tough hyaline cyst wall and includes four nuclei usually located at one end (**Figure 1-2**).

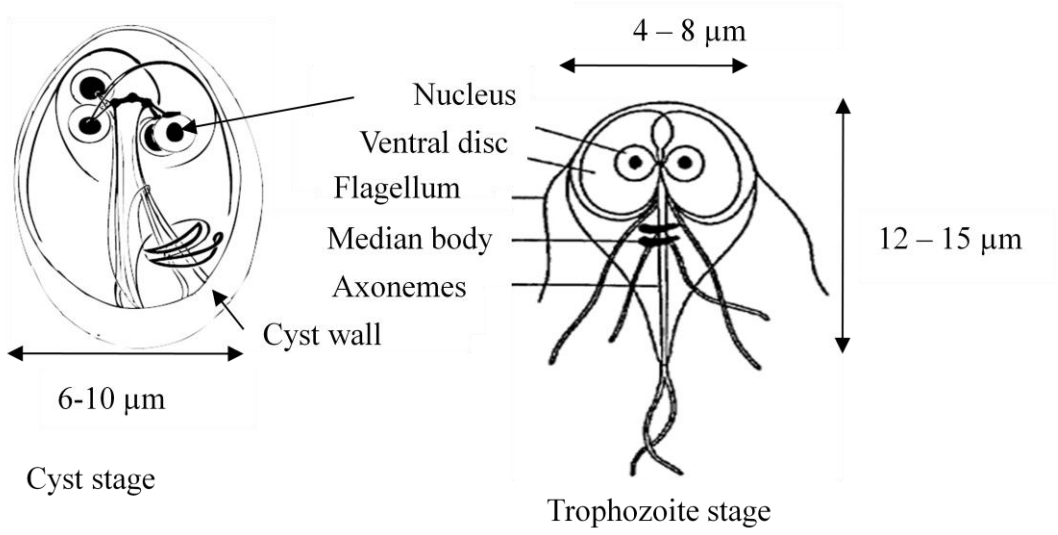


Figure 1-2: Schematic presentation of *Giardia* cyst and trophozoite stages adapted from (Filice, 1952).

The trophozoite stage of *E. histolytica* is highly dynamic and pleomorphic (i.e., takes many forms) with an average diameter of 25 µm (range 10 – 60 µm). It has a single nucleus 3 – 5 µm in diameter with fine granular peripheral chromatin and central compact karyosome (dark spot like in appearance). The trophozoite moves rapidly and unidirectional by extending its pseudopods.

The mature cyst of *E. histolytica* is spherical in shape and measures 10 – 15 µm in diameter with four nuclei (**Figure 1-3**). The nucleus is smaller than that of the trophozoite and has a smaller centrally located karyosome.

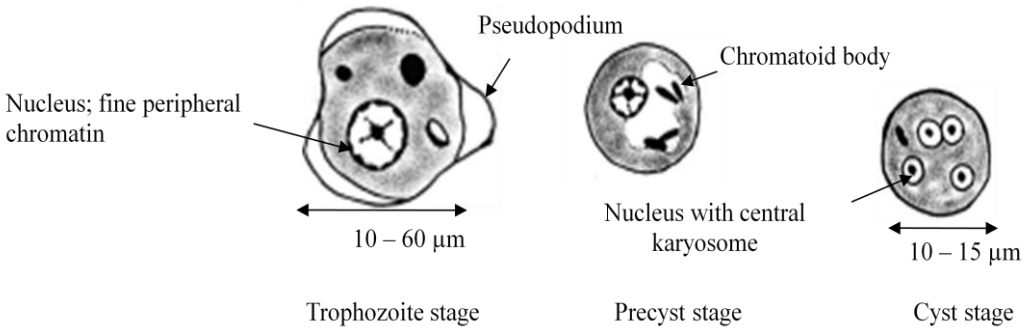


Figure 1-3: Schematic diagram for *E. histolytica*/*dispar* trophozoite and cyst stages adapted from (Tanyuksel and Petri, 2003).

1.2.2 Life cycles

The life cycles of these protozoa are characterized by being homoxenous. This means that each life cycle starts and ends in one host. Infectious cycles of *G. lamblia* and *E. histolytica* as can be seen in **Figure 1-4** are relatively simple. Infection is acquired by ingestion of mature cysts in contaminated drink or food. Sometimes, direct contact with diseased patient or his/her belongings may initiate the infectious cycle. Cell memberanes of *E. histolytica* cysts are usually dissolved by the effects of gastric acid and bile salts (Makioka *et al.*, 2006). *Giardia lamblia* cysts survive these effects and pass to the small intestine. In the small intestine, *G. lamblia* cysts are dissolved by the alkaline pH of intestinal secretions and the proteolytic activity of the duodenum (Gillin

et al., 1988). After that, nuclear as well as cytoplasmic divisions occur subsequently giving rise to eight uni-nucleated motile trophozoites in case of *E. histolytica* (metacystic trophozoites), and two trophozoites in case of *G. lamblia. Giardia lamblia* trophozoites stick to the intestinal wall by the ventral disc (Ortega and Adam, 1997) while

E. histolytica trophozoites settle unattached in the lumen of the colon. A sexual reproduction phase in *Giardia* life cycle was recently put forward (Cooper *et al.*, 2007 and Poxleitner *et al.*, 2008). Trophozoites usually settle in their final habitats which are the colon in case of *E. histolytica* or the small intestine in case of *G. lamblia*. In some cases, the trophozoites can invade the intestinal epithelial cells and disseminate to extra-intestinal sites. For example, *E. histolytica* trophozoites can reach to the liver, lung or the brain and those of *Giardia* can be found in the gall bladder and the biliary ducts. Lastly, as the trophozoites pass down to the lower ileum, they encyst and detach from the intestinal wall and are excreted with faeces to the environment to initiate another cycle.

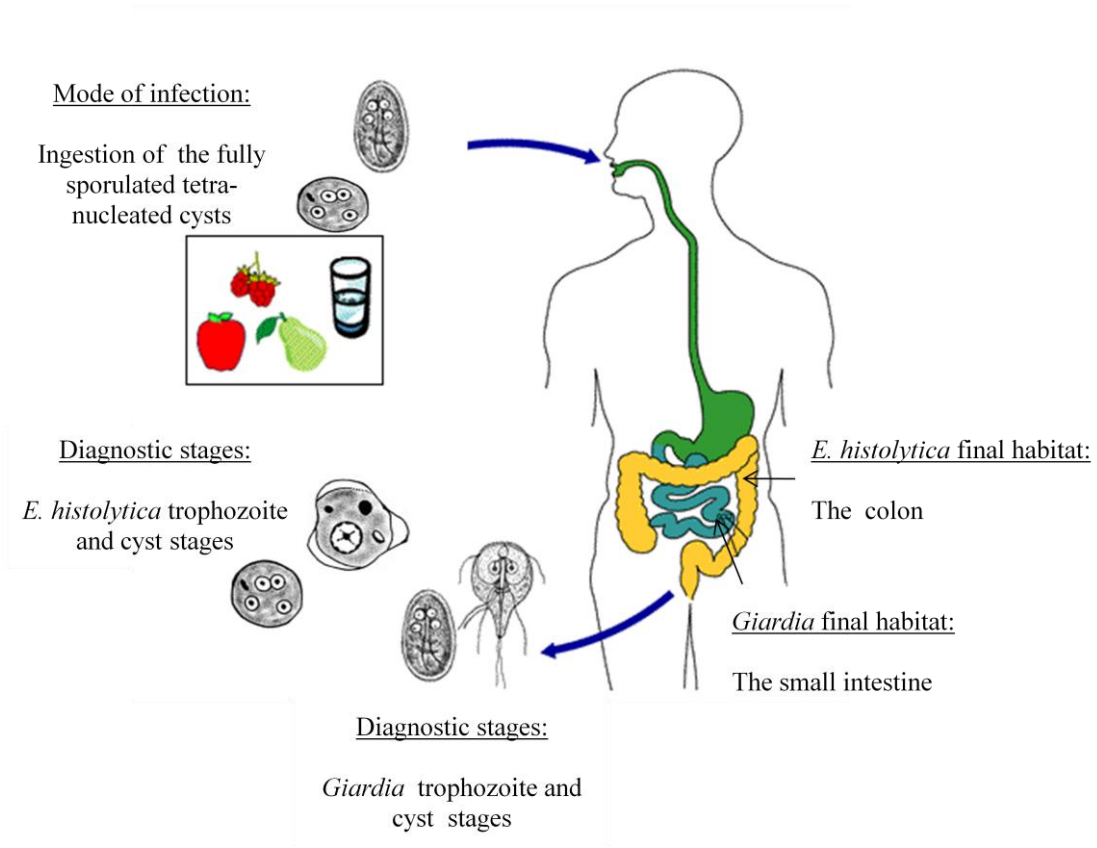


Figure 1-4: The life cycles of *E. histolytica* and *G. lamblia* taken and modified from CDC web site (www.dpd.cdc.gov/Amebiasis).

Contrary to *Giardia* and *Entamoeba*, the life cycle of *Cryptosporidium* is more complex as can be seen in **Figure 1-5**. The infectious cycle comprises exogenous and endogenous phases. The exogenous phase starts by the accidental consumption of the thick walled and the fully-sporulated oocysts and ends by invasion of the intestinal epithelium where the second endogenous phase starts (Tzipori and Ward, 2002).

Following ingestion of oocysts, the cell memberanes are dissolved by the effect of gastric secretions and pass to the small intestine. After that, both nuclear as well as

cytoplasmic divisions take place subsequently giving rise to four individual sporozoites (trophozoites). The sporozoites attach themselves to the enterocytes and enclose themselves within parasitophorous vacuoles to establish an intracellular but extra-cytoplasmic position (Fayer *et al.,* 1997). *Cryptosporidium* sporozoites multiply through non-sexual cycles (Schizogony or Merogony) as well as sexual cycles (Gametogony). Following the non-sexual cycle, two types of schizonts were formed (Schizogony). Type-1 schizonts develop eight nuclei each of them is incorporated into one merozoite (Merogony). Mature merozoites leave schizonts to infect another cell and develop into type-I or type-II schizonts which contains four merozoites (Fayer *et al.,* 1997). Type-II schizonts initiate the sexual cycle by differentiation into microgamete (or "male") or macrogamete (or "female") stages. Fertilized microgamete (zygote) then develops into thin-walled or thick-walled oocysts. In the small intestine, sporozoites emerge from the thin-walled oocysts (i.e., excyst) and initiate an auto-infection cycle while the thick-walled and fully-sporulated oocysts are shed to the environment with stool to start another cycle.

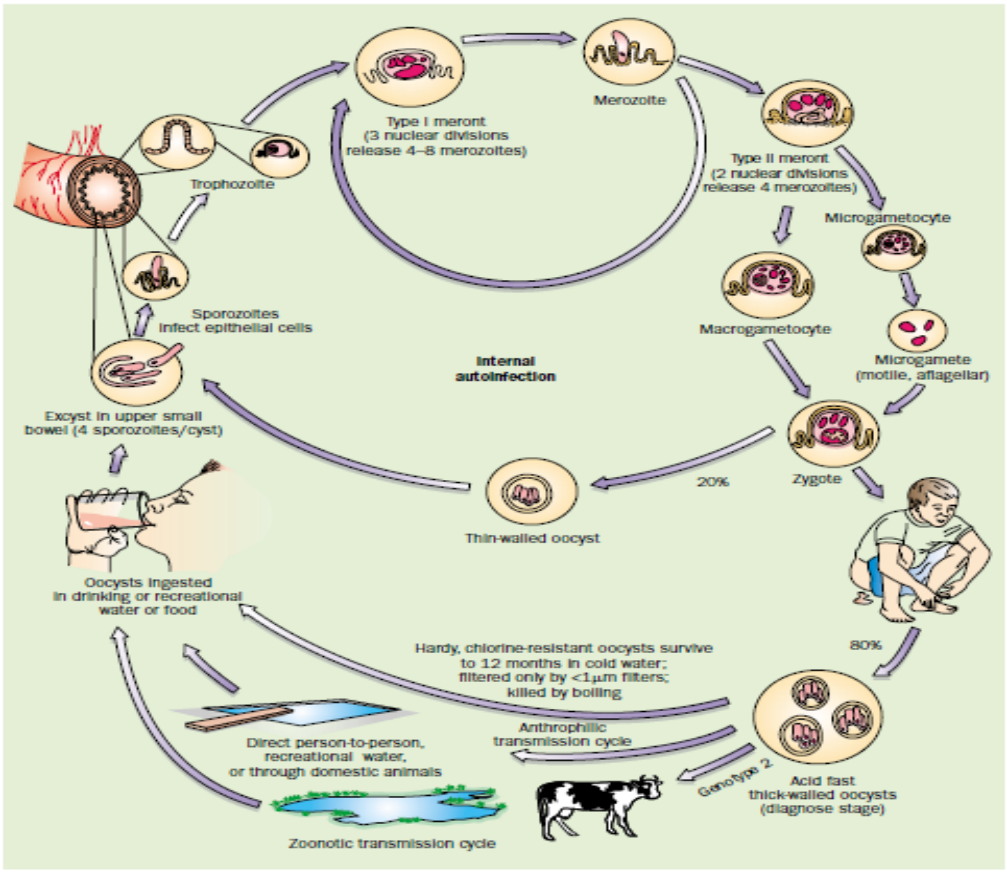


Figure 1-5: *Cryptosporidium parvum* life cycle (Kosek *et al*., 2001)

1.2.3 *In vitro* cultivation

Cultivation of enteric protozoa outside their living hosts (*in vitro*) has a long history. In contrast to bacteria, maintaining these protozoa in culture is not a simple task and requires labour-intensive effort. Also, elimination of unwanted contaminating organisms in the cultivation process is another problem. Furthermore, species identification relying on *in vitro* culture can be hindered by one species outgrowing another in cultures of specimens from mixed infections. Therefore, *in vitro* culturing for

these protozoa has been proved unreliable for routine laboratory practice (Sehgal *et al.,* 1995 and Arrowood, 2002).

Being an obligate intracellular parasite, *in vitro* growth of *Cryptosporidium* has been carried out using cell culture. *Cryptosporidium* oocysts can be initially purified from faecal specimens, excysted *in vitro* and inoculated into cell line culture. Numerous cell lines have been successfully used for culture, such as Mouse L929 fibroblasts (McDonald *et al.*, 1990), and colonic epithelial (T84) cells (Adam *et al.*, 1994).

Contrary to *Cryptosporidium,* both *G. lamblia* and *E. histolytica* are extra-cellular protozoa. Trophozoites of these two parasites have been grown *in vitro* from human stool samples containing infective cysts with high success rates. Purified cysts are excysted externally then cultured into axenic (i.e., free of all metabolizing cells) or xenic (i.e., one or more associated microorganism is present) culture media. For instance, Diamond's medium TYI-S-33 is frequently used for axenic cultivation of *E. histolytica* and *G. lamblia* (Diamond *et al.*, 1978; Bhatia and Warhurst, 1981) whereas Robinson medium (Robinson, 1968) and TYSGM-9 of Diamond (Diamond, 1982) are often used for xenic cultivation of *E. histolytica*.

1.2.4 Species and genotypes

1.2.4.1 *Cryptosporidium* species

There has been much debate regarding *Cryptosporidium* classification. Currently, the genus *Cryptosporidium* comprises around 20 well-characterized and widely-accepted species (see **Table 1-1**). Two of these species are of great importance, namely *C. parvum* and *C. hominis*. *C. parvum* infects humans as well as a wide range of other mammalian hosts (Rose *et al.,* 2002) while *C. hominis* primarily infects humans

(Morgan *et al*., 1999). Other species have been reported in rare cases of human infections including *C. meleagridis* (Pedraza-Diaz *et al*., 2001a), *C. felis* (Xiao *et al*., 2001), *C. muris* (Palmer *et al*., 2003), *C. canis* (Pedraza-Diaz *et al*., 2001b), *C. andersoni* and *C. suis* (Xiao *et al*., 2002).

Table 1-1: Currently known *Cryptosporidium* species

Species[1]	Major host (s)	Reference
C. hominis[2]	Humans, monkeys	Morgan-Ryan *et al.,* 2002
C. parvum[3]	Cattle, sheep, goats, humans	Tyzzer, 1910
C. meleagridis	Turkeys, humans	Slavin, 1955
C. muris	Rodents, camels	Tyzzer, 1910
C. andersoni	Cattle, camels	Lindsay *et al*., 2000
C. felis	Cats	Iseki *et al.*,1989
C. wrairi	Guinea pigs	Xiao *et al.,* 2004
C. canis[4]	Dogs	Fayer *et al.,* 2001
C. baileyi	Chicken, turkeys	Current *et al*., 1986
C. galli	Finches, chicken,	Ryan *et al.,* 2003
C. serpentis	Snakes, lizards	Levine, 1980
C. saurophilum	Lizards	Xiao *et al.,* 2004
C. molnari	Fish	Alvarez-Pellitero and Sitjà-Bobadilla, 2003
C. suis[5]	Pigs	Ryan *et al*., 2004a
C. bovis	cattle	Fayer *et al*., 2005
C. macropodum	eastern grey kangaroos	Power and Ryan, 2008
C. ryanae	cattle	Fayer *et al*., 2008
C. varanii	lizards	Modry *et al.*, 2000
C. fayeri	Red Kangaroo	Ryan *et al*., 2008

[1]*Cryptosporidium* species in **bold** have been identified in human infections.

[2]also known as *C. parvum* human genotype or genotype 1

[3]also known as *C. parvum* bovine genotype or genotype 2

[4]also known as *C.* dog genotype

[5]also known as *C.* pig genotype

Additionally, around 61 *Cryptosporidium* genotypes have been identified by molecular studies based on 18S rDNA gene sequences (Plutzer and Karanis, 2009). At least seven of these genotypes have been rarely found in human infection. These genotypes are *C. hominis* monkey genotype, *C. parvum* mouse genotype, *Cryptosporidium* cervine genotype (W4), chipmunk genotype I (W17), skunk, horse and rabbit genotypes (Ajjampur *et al*., 2007; Robinson *et al.,* 2008a and Xiao and Ryan, 2008).

1.2.4.2 *Giardia* species

Similar to *Cryptosporidium*, there has been much debate about *Giardia* classification. Based on the morphometric and ultrastructural characteristics of the trophozoite life stage, the *Giardia* genus currently comprises six well-known species. These species are *G. agilis*, *G. ardeae*, *G. lamblia*, *G. microti*, *G. muris* and *G. psittaci* (Adam, 2001). Nearly all of these species are host-specific except *G. lamblia* which has been found in more than one mammalian host including humans (Thompson *et al.,* 2000).

Recent molecular studies have shown that *G. lamblia* itself is a species complex with seven known genotypes or assemblages. These genotypes (assemblages) are named alphabetically from A to G (Meloni *et al.,* 1988; Hopkins *et al.,* 1997; Homan *et al.,* 1998 and Monis *et al.,* 1999). Both assemblage A and B have been isolated from humans as well as from a broad range of hosts including cats, dogs and wild animals. Isolates belonging to the assemblage A have been further grouped into subtypes I and II. Similarly, assemblage B isolates have been separated into subtypes III and IV (Thompson, 2000). On the other hand, infection with isolates belonging to assemblages C, D, E, F or G appear to be restricted to domestic livestock and wild animal hosts (Monis *et al.,* 2003). Assemblages C and D are canine specific (Hopkins *et al.,* 1997; Monis *et al.,* 1998; Thompson *et al.,* 2000), assemblage E seems

to be specific for livestock (Ey *et al.,* 1997), assemblage F for cats and assemblage G for rats (Monis *et al.,* 1999). All human isolates presently characterized belong to either assemblage A or assemblage B (**Table 1-2**).

Table 1-2: Currently recognised *Giardia* species and genotypes (assemblages)

Species	Genotype	Subgroup[1]	Major host (s)
G. lamblia	Assemblage A	AI/ AII/ AIII/ AVI	Humans, livestock
	Assemblage B	BI/ BII/ BIII/ BIV	Humans
	Assemblage C		Dogs
	Assemblage E		Cattle, other hoofed livestock
	Assemblage F		Cats
	Assemblage G		Rats
G. agilis			Amphibians
G. muris			Rodents
G. microti			Muskrats, voles
G. psittaci			Birds
G. ardeae			Birds

[1] This classification system was adapted from Caccio` *et al.,* 2005 and Read *et al.,* 2004.

1.2.4.3 *Entamoeba* species

The genus *Entamoeba* has many species. Six of these species have been reported as human enteric protozoa. These species include *E. histolytica, E. coli*, *E. hartmanni*, *E. gingivalis*, *E. polecki* and *E. chattoni* (Clark *et al.,* 2006). All of these species can be differentiated from each other by morphological characteristics. However, there are two species with closely similar morphological characteristics to *E. histolytica*, namely *E. dispar* and *E. moshkovskii.* For a long time, it was thought that *E. histolytica* was the

only species in the genus that could infect humans and cause disease (Clark and Diamond 1991). However, this has been challenged by some recent molecular studies after recovery of *E. dispar* and *E. moshkovskii* from patients with gastrointestinal symptoms (Parija and Khairnar, 2005; Fotedar *et al*., 2008).

1.2.5 Genomes and genomic sequences

1.2.5.1 *Cryptosporidium* genome

A random shotgun sequencing approach has been taken to obtain the complete DNA sequence of the Iowa "type II" isolate of *C. parvum* which is able to transmit disease among numerous mammals, including humans **(**Abrahamsen *et al*., 2004). Based on the assembled DNA sequence which has approximately 13X genome coverage, the genome, distributed on eight chromosomes, has a total length of about 9.1 Mb. The *C. parvum* genome seems quite compact as it contains short intergenic regions, few introns and a smaller number of genes than expected for a genome of this size. Only about 3800 protein-encoding genes have been identified within the *C. parvum* genome which exhibits a GC-content of 30 %.

Additionally, a draft sequence of *C. hominis* strain TU502 has been produced and analyzed. According to this draft blueprint, the genome of *C. hominis* strain TU502 contains around 3950 genes and displays a GC-content of 30 % (Abrahamsen *et al*., 2004).

1.2.5.2 *Giardia lamblia* genome

There are two *G. lamblia* laboratory strains that have been successfully cultured *in vitro*. The WB strain belongs to assemblage A and the GS strain is a member of

assemblage B. Assemblages A and B are the commonly encountered *G. lamblia* genotypes in human infections. Both laboratory strains have been fully sequenced to obtain their complete genome sequences (Morrison *et al.,* 2007). Based on the generated sequences, the genome of WB clone C6 (ATCC 50803) is 11.7 Mb in length distributed on five chromosomes. This genome includes approximately about 6580 genes with a GC-content of 49 %. Similarly, a draft genome sequence of the second strain (GS) has also been produced. A comparison on the two genomes at a genome level demonstrated 77 % nucleotide identity and 78 % amino-acid identity within protein coding regions, and revealed the presence of several unique genes in each isolate. The identified genomic differences between these two strains, representing assemblages A and B, has helped explain why several researchers through earlier studies have suggested that the two isolates belonged to two separate species (Nash *et al.,* 1985 and Mayrhofer *et al.,* 1995).

1.2.5.3 *Entamoeba histolytica* genome

Only one strain of *E. histolytica* named HM-1: IMSS has been successfully grown *in vitro* and used for complete genome sequencing (Loftus *et al.,* 2004). Based on the generated sequence, its genome length is around 24 Mb distributed on 14 chromosomes with a GC-content of 24 %. The genome is highly repetitive and has approximately 8340 genes. By contrast with the genome of *Cryptosporidium* and *G. Lamblia,* approximately 40 % of the genome sequence reads have been assigned to repetitive elements.

1.3 Epidemiology

1.3.1 Clinical presentations

The clinical presentations of infections caused by *Cryptosporidium* species, *G. lamblia* and *E. histolytica* are similar and could not be easily differentiated from bacterial or viral causes of gastroenteritis. Diseases range in seriousness from mild to sever and signs and symptoms depend on the site of infection, nutritional status, immune status of the person who catche the infection. Asymptomatic infection is frequently reported especially in countries where these protozoa are endemic where up to 75 % of amoebiasis (Haque *et al.*, 2006), 30 % of cryptosporidiosis (DuPont *et al.*, 1995) and 5 – 15 % of giardiasis (Ali and Hill, 2003) 'cases' are symptomless. On the other hand, infection may be symptomatic and producing distinct clinical pictures. The time interval between ingestion of oocysts/cysts and the appearance of clinical symptoms depends on the strain, infecting inoculum and immune status of the individual (DuPont *et al.*, 1995 and Read *et al.*, 2002).

Cryptosporidiosis is an acute self-limiting gastroenteritis in immune-competent humans. It occurs worldwide, and in all age groups, although children especially those under 2 years old are most frequently and severely affected. Diarrhoea can be of sudden onset and is generally watery and voluminous; between three and six stools (but sometimes many more) may be passed each day, which are sometimes offensive and may contain mucus (Huang and White, 2006). Other acute symptoms are abdominal pain, nausea or vomiting, pyrexia, anorexia, malaise and fatigue (Fayer and Ungar, 1986). Weight loss can be considerable. Bloating and gas production may be reported. Cough has also been reported in some cases but is not explained. Pus, blood and faecal leukocytes are not typically present in the stool. Symptoms usually last up to three

weeks, and are resolved through stimulation of immune responses. Some patients experience chronic diarrhoea of a month or longer. Oocysts may continue to be shed for a mean period of 7 days (range 1–15 days) after symptoms have ceased, although exceptionally for up to 2 months (Jokipii and Jokipii, 1986).

Whilest the majority of the healthy, well nourished, immune-competent patients will usually spontaneously recover from cryptosporidiosis, albeit after sometimes lengthy illness, some groups of immunecompromised patients such as those with poorly-controlled HIV infection can suffer prolonged, chronic disease, sometimes with devastating effects. In such patients, in addition to typical but severe intestinal disease, atypical and extra-intestinal disease can also develop. The whole gastrointestinal tract including the gall bladder, pancreatic duct and even the bronchial tree can be affected (Gomez-Morales *et al.*, 1996; Shrikhande *et al.*, 2009).

Giardiasis has a wide range of unpredictable symptoms; individual variability and the intermittent nature and changing of the symptoms are characteristic (Hill, 1993). Although some acute infection may clear spontaneously, a long-standing subacute or chronic infection may develop. This phase may involve two or more years of intermittent diarrhea. In individuals returning from endemic areas, the acute stage may not be remembered, and these patients can present with persistent or recurrent mild to moderate symptoms. During this chronic phase, lassitude, headache, and myalgia may occur with continued weight loss, anorexia, and malabsorption. Chronic infection in children may present as failure to thrive. Urticaria, cholecystitis, and pancreatitis have been reported with *Giardia* infections (Aronson *et al.*, 2001).

Amoebiasis, in the majority of infected persons is symptomless. However, some of these cyst carriers may develop colitis after a period of months. Symptoms commonly attributed to *E. histolytica* colitis or dysentery are abdominal pain or tenderness and diarrhea (watery, bloody, or mucous). Diarrhea can occur with up to 10 (or even more) bowel movements per day, and fever may occur in one-third of the patients. Patients are often reluctant to eat, and may loose some weight (Haque *et al*., 2003). Clinical diagnosis of amebiasis is difficult because of the nonspecific nature of symptoms. It is easily confused with shigellosis (*Shigella dysenteriae* and *S. flexneri*) and a number of other bacterial dysenteries (*Salmonella, Campylobacter*, and enterohemorrhagic and enteroinvasive *Escherichia coli*) that are common in tropical and subtropical countries (Stanley, 2003). In addition, it is very important and difficult to differentiate the symptoms of noninfectious intestinal diseases (ischemic colitis, inflammatory bowel disease, diverticulitis, and arteriovenous malformations) from infectious diseases, in part because of the lack of fever in patients with amebic colitis. Unfortunately, chronic non-dysenteric intestinal amebiasis, which is characterized by intermittent diarrhea, flatulence, presence of seropositivity, and amebae in the stool, can resemble ulcerative colitis, resulting in misdiagnosis and treatment with corticosteroids. The development of fulminant colitis, ameboma, cutaneous amebiasis, and rectovaginal fistulas can occur as complications of intestinal amebiasis (Stanley, 2003). In 0.1 to 1 % of symptomatic amoebiasis, *E. histolytica* trophozoites reach the liver causing amoebic liver abscesses and a clinical presentation of right upper quadrant abdominal pain and low grade fever (Pritt and Clark, 2008).

1.3.2 Transmission

Many different routes are involved in transmission of these infections (O'Donoghue, 1995; Stanley, 2003). Most commonly, infections are acquired through accidental ingestion of the fully sporulated oocysts/cysts contaminating water or raw food. Direct or indirect person-to-person transmission of infection is also a well recognised source of infection. Finally, zoonotic transmission has been widely recognised as an important route for *Cryptosporidium* infection particularly with *C. parvum* species. *Cryptosporidium* parvum, which has zoonotic potential, has been reported in cattle and, to a lesser extent, in sheep (Elwin *et al*., 2007).

Zoonotic transmission for *Giardia lamblia* is still an open topic of debate while for *E. histolytica* it has been ruled out as a likely possibility (Monis and Thomson, 2003).

1.3.3 Infectivity

In addition to the many routes of transmission, there are five major characteristics that make these protozoa highly infectious pathogens. First, both oocysts/cysts are shed into the environment in relatively high numbers and in fully sporulated forms. The number of oocysts shed by a *Cryptosporidium* infected person is ~ 3.3×10^6 oocysts per ml of stool during symptomatic infection and ~ 3×10^5 oocysts per gram of stool for asymptomatic cases (Bushen *et al.,* 2007). Similarly, a *Giardia*-infected individual excretes ~ 8.7×10^4 cysts per gm of stool in a symptomatic infection and ~ 6.9×10^5 cysts per ml of diarrheal stool (Kohli *et al.,* 2008).

The second feature is that oocysts/cysts are highly resistant to the commonly used disinfectants. Unlike most of other pathogenic agents like bacteria and viruses, protozoan cysts/oocysts are highly protected from most of the antimicrobial agents used

for water treatment. Moreover, the small sizes of these stages (4 – 15 µm in diameter) hinder their removal by the traditional filtration methods (Logsdon, 1998). High concentrations of free chlorine, ozone or prolonged UV light exposure is required for decontamination to be effective (Jarroll *et al.,* 1981 and Li *et al.,* 2009). Another factor to be considered is that *Cryptosporidium* oocysts, and to a lesser extent *Giardia* and *Entamoeba* cysts, can retain their infectivity for six months after being shed into the environment. Furthermore, oocysts/cysts continue to be shed by infected persons for a long period (Jokipii and Jokipii, 1986). Finally, ingestion of small number of oocysts/cysts can produce infection. Infection can be acquired following ingestion of as few as 10 *Giardia* cysts or 30 *Cryptosporidium* oocysts or even one *E. histolytica* cyst (Rendtorff, 1954; DuPont *et al.,* 1995 and Ravdin, 1995).

1.3.4 Worldwide distribution

Due to the high infectivity of protozoa and the availability of various modes of transmission, these three protozoa are globally distributed. Although the reported prevalence rates vary among studies, the largest burden of infections is in developing countries (O' Donoghue, 1995; Ortega and Adam, 1997 and Stanley, 2003). Populations at high risk of infections include children, the elderly and travelers to countries where infection is endemic (Casemore, 1990; Siwila *et al.,* 2009 and Weinke *et al.,* 1990). Furthermore, individuals with animal contacts and those with low immunity such as HIV-infected patients are more prone to infection with *Cryptosporidium* (Kurniawan *et al.,* 2009).

1.3.5 Outbreak potential

Most of these protozoal infections usually occur as sporadic cases (Hunter *et al.*, 2004; Ali and Hill, 2003 and Haque *et al*., 2003). However, many outbreaks of gastroenteritis caused by these protozoa have been reported in different countries (**Table 1-3**). The majority of these outbreaks (90 %) were typically associated with contaminated central water supplies (Rose and Slifko, 1999). Generally, outbreaks due to *Cryptosporidium* infection are more frequently reported than those caused by *G. lamblia* or *E. histolytica* (Karanis *et al.*, 2007).

Table 1-3: Examples of recent outbreaks of protozoal gastroenteritis

Country, Year (Reference)	Protozoa (Number of cases)
Recreational water as the source of infections:	
USA, 2003 (Boehmer *et al.,* 2009)	*Cryptosporidium* (16)
USA, 2006 (Eisenstein *et al.,* 2008)	*G. lamblia* (38) and *Cryptosporidium* (11)
UK , 2002 (CDR[1], 2003)	*Cryptosporidium* (50)
UK, 2001 (CDR, 2002)	*Cryptosporidium* (152)
USA, 1994 (Kramer *et al.,* 1996)	*G. lamblia* (80)
UK, 2000 (CDR, 2001)	*G. lamblia* (17)
Drinking water as the source of infections:	
USA, 2007 (Daly *et al.,* 2009)	*Cryptosporidium* (31)
Norway, 2004 (Nygard *et al.,* 2006)	*G. lamblia* (1,300)
France, 2001 (Dalle *et al.,* 2003)	*Cryptosporidium* (563)
Ireland, 2001 (Glaberman *et al*, 2002)	*Cryptosporidium* (230)
Canada, 2001 (CDR, 2001)	*Cryptosporidium* (6,000)
USA, 2002 (Lee *et al.,* 2002)	*G. lamblia* (27)
Georgia 1998 (Barwick *et al.,* 1998)	*E.histolytica* (177)
Taiwan, 1993 (Chen *et al.,* 2001)	*E.histolytica* (730)
Sweden, 1986 (Andersson and De Jong, 1989)	*G. lamblia* (1,480) and *E.histolytica* (106)
Food as the source of infections:	
USA, 1993 (Millard *et al.,* 1994)	*Cryptosporidium* (154)
Denmark, 2005 (Ethelberg *et al.,* 2009)	*C. hominis* (99)
Finland, 2009 (Ponka *et al.,* 2009)	*C.parvum* (72)
USA, 1986 (Rose and Slifko, 1999)	*G. lamblia* (88)
Thailand, 1988 (De Lalla *et al.,* 1992)	*E.histolytica* (42)

[1]; Centers for Disease Control Report

1.3.6 Rationale for treatment

Asymptomatic infections with *Cryptosporidium* or *G. lamblia* require no treatment as most cases will resolve spontaneously within 2 – 4 weeks (Rossignol, 2009). However, asymptomatic carriers of *E. histolytica* should be treated to reduce the risk of onward transmission via faecally-shed amoebic cysts and to minimize the possibility future

tissue invasion by *E. histolytica* trophozoites (Anonymous, 1997). Asymptomatic intestinal colonization of *E. histolytica* infection is usually treated by multi-dose therapy with paromomycin.

The mainstay of therapy for symptomatic cases of giardiasis and amoebiasis is multi-dose metronidazole. Alternatively, a single dose of the chemically-related tinidazole can be used as an acceptable substitute (Escobedo *et al.*, 2009). In severe cases of invasive amoebiasis, parasites persist in the intestine in as many as 40 to 60 % of patients who receive metronidazole or tinidazole as a sole treatment. Therefore, paromomycin or the second-line agent diloxanide furoate should be taken to cure luminal infection.

Conversely, there are no, currently available, drugs or vaccines that can cure or protect against *Cryptosporidium* infection. Conservative therapy such as fluid replacement and electrolyte balance maintenance are very important in severe or protracted diarrhea. Furthermore, highly active anti-retroviral therapy (HAART), paromomycin, azithromycin and nitazoxanide have been tried in HIV patients with cryptosporidiosis and shown to be effective in improving diarrhea but not in reducing parasite burden (Rossignol, 2009).

1.4 Clinical laboratory diagnosis

As mentioned above, it is not possible to rely on the clinical picture alone for the accurate diagnosis of these infections. There are considerable variations among clinical laboratories regarding the methods used for detection of intestinal protozoa (see **Figure 1-6**). The choice of diagnostic method used depends mainly on the availability of the required resources and the suitably trained personnel, and the extent

of infection in the population. Clinical laboratories with limited resources usually rely solely on microscopic examination of stool samples for ova/parasites or sometimes on serological assays (particularly for amoebiasis). Clinical laboratories with greater resources depend mainly on commercially available kits for protozoal antigen detection in stool samples (copro-antigen detection kits). More sensitive and expensive PCR-based assays for parasite DNA detection in stool samples (copro-DNA detection PCR assays) are still confined to research laboratories and a few specialized reference laboratories.

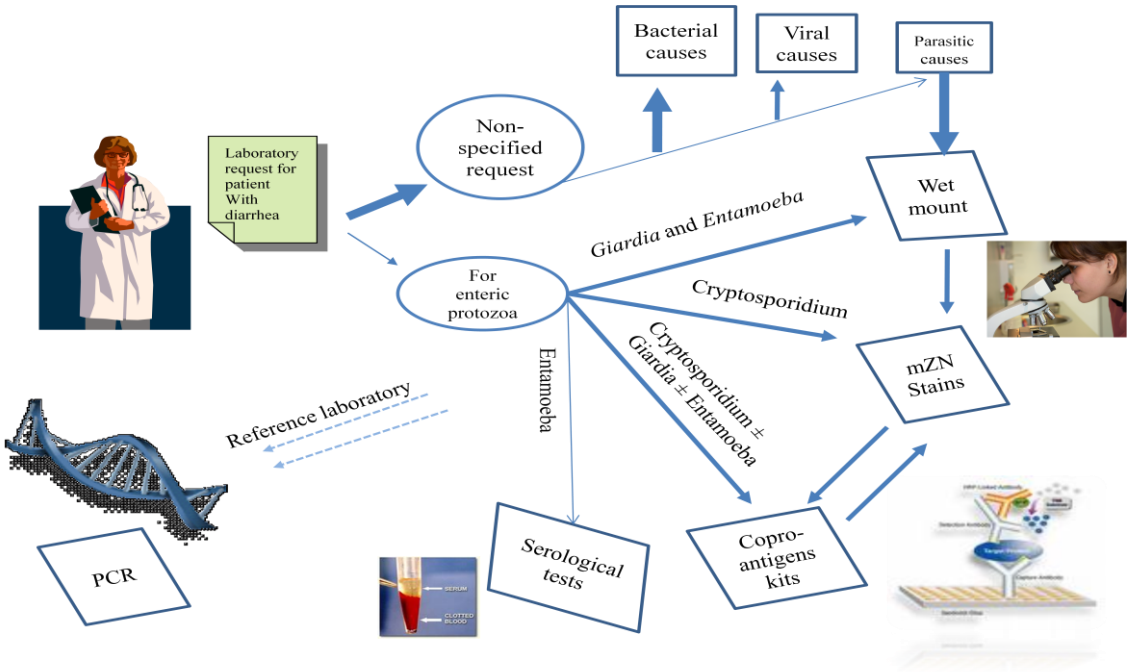


Figure 1-6: Flowchart diagram showing algorisms adopted for diagnosis of *Giardia*, *Entamoeba* and *Cryptosporidium* in clinical laboratories.

1.4.1 Microscopic diagnosis

Microscopic examination is the most common technique employed for identification of enteric parasites including protozoa. This is because it is simple and cost-effective method in most cases. However, the sensitivity of the microscope varies from 10 – 60 %. This low sensitivity is due to a number of reasons. Firstly, oocysts/cysts are shed intermittently in stool and therefore oocysts/cysts numbers vary greatly between samples (van Gool *et al.,* 2003). Secondly, many species like *E. histolytica* and *E. dispar* are morphologically similar and cannot be differentiated easily under the microscope. Finally, microscopic diagnosis is subjective and relies mainly on the skills of the person carrying it out (Libman *et al.,* 2008).

Direct wet mount preparation is usually used for diagnosis of *Giardia* and *Entamoeba*. However, *Cryptosporidium* species cannot be easily detected by this routine microscopic test without staining. Modified Ziehl-Neelsen and Kinyoun acid fast are the most widely stains used for *Cryptosporidium* oocysts identification (Clark, 1999). Similarly, concentration techniques such as formol-ether sedimentation and salt flotation can be used prior to microscopic examination to improve detection of these protozoa.

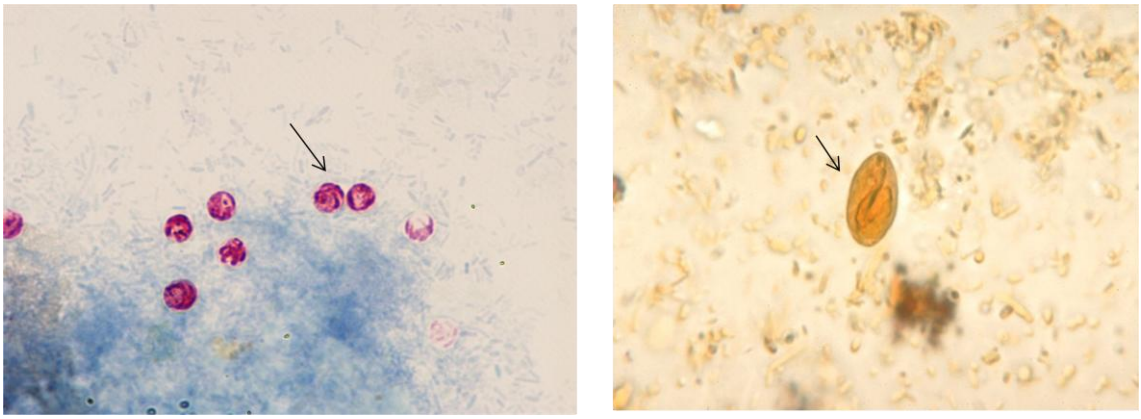


Figure 1-7: Representative bright field microscopic pictures for the *Cryptosporidium* oocysts stained with Modified Ziehl-Neelsen dye (left) and the *Giardia lamblia* cysts stained with iodine (right) with x200 magnification.

1.4.2 Serological assays

Serological tests may help to identify recently infected individuals by examining, in serum samples, seroconversion, a significant rise in anti-protozoal antibody titers, or the identification of antibody response to certain parasite antigen. Assays of this nature are usually not useful in the diagnosis of acute infections. It therefore appears that, these assays are mainly confined to seroepidemiological studies (e.g., Cedillo-Rivera *et al*., 2009; Haque *et al.*, 2006 and Elwin *et al*., 2007).

1.4.3 Copro-antigen detection assays

In copro-antigen detection assays, protozoal surface antigens present in faecal samples are used as targets for detection. Compared to microscopy, multiple stool specimens can be screened using these assays with higher sensitivities, less technical skill and faster turn-around times (Church *et al.*, 2005; Haque *et al.*, 1995 and Garcia *et al.*, 2000). However, when compared to PCR based detection methods, the sensitivity of

several assays were found to be suboptimal (Lebbad and Svard, 2005 and Gonin and Trudel, 2003).

Several protozoal antigens with variable degrees of antigenicity have been used to develop a range of commercially available copro-antigen detection kits. Two main types of copro-antigens assays are commercially available. The first type is an enzyme immunosorbent assay (EIA or ELISA kits) such as *E. histolytica* II, *Giardia* II, *Cryptosporidium* II test (TechLab, Blacksburg, VA) and Triage Parasite Panel (BIOSITE Diagnostics). The other type is a lateral flow immunochromatographic assay such as RIDA® QUICK *Cryptosporidium* and RIDA® QUICK *Giardia* (R-Biopharm).

1.5 PCR-based molecular assays

Over the past several years, nucleic acid amplification assays particularly polymerase chain reaction (PCR)-based assays have initiated a revolution in detection and characterization of many pathogens including enteric protozoa. Due to the incredible sensitivity, specificity, and speed of amplification, PCR has been widely used for detection of *Cryptosporidium*, *G. lamblia* and *E. histolytica* DNA in stool specimens. However, the majority of these PCR-based assays were originally developed as research tools only.

Protozoal DNA extraction directly from stool is a challenging procedure due to a number of factors. One factor to be considered is the complexity and non-uniformity of stool specimens **(**Stephen and Cummings, 1980 and Albaugh *et al.*, 1989). Furthermore, in contrast to many other enteric pathogens, the genetic material of protozoa, to be isolated, is enclosed mainly in oocysts/cysts which posses very robust cell memberanes. Lastly, some constituents of stool such as heme, bilirubins, bile salts,

and complex carbohydrates interfere with DNA amplifications. These substances impair cell lysis, degrade the nucleic acid and/or inhibit polymerase activity if co-extracted with the target pathogen DNA (Limor *et al.*, 2002). Accordingly, pre-PCR samples processing procedures have been frequently adopted for protozoal DNA amplification from stool specimens (see **Table 1-4**).

1.5.1 Stool sample preparation

Prior to protozoal DNA extraction, many different procedures have been employed as preparatory steps for stool specimens. Purification of protozoal oocysts/cysts from the complex faecal matrix has been approached using a variety of techniques such as flotationand concentration techniques (e.g., Cheun *et al.*, 2007; Hunter *et al.*, 2007 and Lee *et al.*, 2006). Other preparatory procedures were employed to facilitate oocysts/cysts walls disruption and nucleic acid isolation such as exposing the extract samples to variable number of freeze-thaw cycles or mechanical agitation with Fast Prep® instrument or bursts of sonication (e.g., Cheun *et al.*, 2007 and Miller and Sterling, 2007). In most of the studies, more than one preparatory step has been frequently used before protozoal DNA extraction.

These procedures are proved useful in reducing the carry-over of faecal material that impaired the target nucleic extraction and helped facilitate oocysts/cysts disruption. However, these procedures typically added significant more cost and time to the DNA extraction methods and caused some loss of the oocysts/cysts in the primary stool specimen.

1.5.2 Protozoal copro-DNA extraction

Various methods used for protozoal DNA extraction from human stool specimens. Most of these methods were frequently preceded with one or more preparatory procedure (**Table 1-4**). Few extraction methods have been employed for protozoal DNA extraction directly from stool specimens (**Table 1-5**).

Modified phenol-chloroform extraction method (Sambrook *et al.,* 1989) was frequently tried in earlier studies. It was adopted as a main DNA extraction method in some studies or as one step of a lengthy extraction protocol in others (Sulaiman *et al.,* 2003; Hooshyar *et al.,* 2004 and Santos *et al.,* 2007). Guanidine thiocyanate/silica (GuSCN/Silica) is another extraction method which has been extensively approached for protozoal DNA extraction directly from stool sample (Pedraza-Diaz *et al.,* 2001 and Amar *et al.,* 2003). Commercially available DNA extraction kits have been recently used for protozoal DNA extraction from stool specimens. Although the majority of these kits were originally designed for nucleic acid extraction from pathogens other than enteric protozoa, these kits were tried for protozoal copro-DNA extraction (Limor *et al.,* 2002 and Sulaiman *et al.,* 2003).

Table 1-4: Examples of pre-PCR stool samples processing procedures

Citation	Target protozoon	DNA purification	• Sample preparatory procedure (s)
Robinson *et al.,* 2008	*Cryptosporidium* spp.	QIAamp® DNA Stool Mini Kit (Qiagen)	• Saturated salt flotation technique + Boiling for 1h
Amar *et al.,* 2001	*G. lamblia*	Guanidine Thiocyanate/Silica	• Preparation of stained faecal smears on glass microscope slides
Van der Giessen *et al.,* 2006	*G. lamblia*	Puregene® kit (DNA Genotek)	• Sucrose density gradient flotation technique + Addition of anti-*Giardia* magnetic beads + freeze/thaw cycles
Kebede *et al.,* 2004	*E.histolytica*	QIAamp®Tissue Kit (Qiagen)	• Faecal suspensions preparation with PBS containing 2 % PVP + Boiling specimen for 10 min in SDS + Proteinase K treatment (2 h at 55 °C)
Guy *et al.,* 2004	*G. lamblia*	QIAmp DNA stool mini kit with minor modifications (Qiagen)	• Three washes with dH2O + Freeze-thaw cycles in lysis buffer + three bursts of sonication
Caccio *et al.,* 2002	*G. lamblia*	FastDNA® SPIN kit (Q-BIOgene)	• Formol-ether concentration technique + Mechanical agitation with Fast Prep® instrument
Coupe *et al.,* 2004	*Cryptosporidium* spp.	Alkaline lysis buffer (Na OH /SDS / PVP) + Qiagen spin column	• One-step ethyl-ether concentration + Pellets dilution in 2.5 % dichromate solution or distilled water
Hooshyar *et al.,* 2004	*E.* histolytica	Modified Phenol-Chloroform	• Culturing stool specimens in Robinson's medium

Table 1-5: Example of methods used for protozoal DNA extraction directly from human stool specimens

• DNA extraction method	Target protozoon	Citations
• Guanidine Thiocyanate/Silica	*Cryptosporidium* spp. *G. lamblia*	McLauchlin *et al.*, 1999 and Pedraza-Diaz *et al.*, 2001 Schuurman *et al.*, 2007 and Amar *et al.*, 2003
• Qiagen standard DNA kit	*Cryptosporidium* spp.	Meamar *et al.*, 2007
• Hexadecyl trimethyl ammonium bromide (CTAB)	*E. histolytica*	Khairnar and Parija, 2007
• GuSCN/Silica+ phenol/chloroform/isoamyl alcohol	*E. histolytica*	Santos *et al*., 2007
• Guanidine Thiocyanate/Silica/PVP	*Cryptosporidium* spp.	McLauchlin *et al.*, 2003 and Leoni *et al.*, 2006
• High Pure PCR Template Preparation kit (Roche)	*E. histolytica*	Calderaro *et al.*, 2006
• QIAamp® DNA stool mini kit (Qiagen)	*Cryptosporidium* spp. *G. lamblia* *E. histolytica*	Magi *et al.*, 2005 Read *et al.*, 2004 and Berrilli *et al.*, 2006 Paglia and Visca, 2004 and Fotedar *et al.*, 2007

1.5.3 Protozoal DNA target gene loci

1.5.3.1 *Cryptosporidium* species

Various genetic loci, such as the small subunit ribosomal DNA (18S rDNA), *Cryptosporidium* oocyst wall protein (*cowp*), the thrombospondin-related adhesive protein (*trap*) and 70 kDa heat shock protein (*hsp*70) genes have been targeted for the identification and/or differentiation of the predominant species of *Cryptosporidium* infecting humans. These target genes together with the various PCR strategies adopted are outlined in **Table 1-6**.

Selection of the target locus depends on the objectives of the PCR assay. Assays developed for *Cryptosporidium* detection and species identification frequently relied on 18S rDNA or *cowp* as targets for PCR amplifications (e.g., Pedraza-Diaz *et al.*, 2001 and Coupe *et al.*, 2004). PCR assays proposed for parasite characterization were often based upon non-coding DNA sequences. These sequences display a higher level of polymorphism than those obtained from using either 18S rDNA or *cowp* genes as PCR targets. PCR assays aimed at intra-species genotyping and subtyping are frequently based on sequence repeats occurring as minisatellite and microsatellite DNA markers within the *Cryptosporidium* genome (Mallon *et al*, 2003; Hunter *et al*., 2007 and Caccio, 2003) or sometimes on highly variable gene loci such as glycoprotein 60 (*gp*60) (Chalmers *et al*., 2008 and Hunter *et al*., 2007).

1.5.3.2 *Giardia lamblia*

A variety of genetic loci including 18S rDNA, triose phosphate isomerase (*tpi*), glutamate dehydrogenase (*gdh*), *β*-giardin and elongation factor 1- alpha (*ef1*-ά) have been tried as targets gene loci for many PCR assays (**Table 1-6**).

G. lamblia 18S rDNA gene is considered to be the shortest known gene in all eukaryocytes and has mainly used in PCR detection assays (Hopkins *et al.,* 1997). This is due to its highly conserved sequence among the major *G. lamblia* assemblages (Berrilli *et al.,* 2006 and Sulaiman *et al.,* 2003). Conversely, the *gdh*, *tpi* and *β*-giardin genes are frequently used in parasite detection and characterization studies (Caccio *et al.,* 2005; Bertrand *et al.,* 2005 and Guy *et al.,* 2004).

1.5.3.3 *Entamoeba histolytica*

Various genes including serine-rich *E. histolytica* protein (*SREPH*), gene encoding a 29-kDa/30-kDa surface antigen, chitinase, hemolysin *(HLY*6), extra-chromosomal circular DNA, episomal repeat sequences and *18S* rDNA gene are used as target loci for different PCR assays. Some of these PCR assays were originally developed to amplify target gene loci from a previously cultured stool sample (e.g., Stanley *et al.,* 1990; Tachibana *et al.,* 1991 and Hooshyar *et al*., 2004).

The first PCR assay developed for direct parasite detection in stool specimens targeted multi-copy, extra-chromosomal circular DNA sequences (Acuna-Soto *et al.,* 1993). After that, numerous PCR assays have been developed to amplify *E. histolytica* target gene DNA sequences directly from stool specimens. Most of these assays rely on the 18S rDNA as a target locus for amplification (**Table 1-6**). This is due to its high copy number in the parasite genome and its sequence diversity among different species (Liang *et al.,* 2009 and Gonin and Trudel 2002).

Table 1-6: Protozoal DNA target genes, DNA extraction methods and assay types that have been adopted in previously-developed PCR assays.

Citation	PCR target (s)	Assay type (s)	DNA extraction	Sample preparatory procedure (s)
<u>*Cryptosporidium* species:</u>				
Spano *et al.*, 1997	*cowp*	Standard PCR- RFLP[1]	GuSCN/Silica[2]	Formol-ether concentration technique → treatment of the pellet with 2 % PVP
Da Silva *et al.*, 1999	18S rDNA (18S)	Standard PCR	FastDNA® SPIN kit (Q-BIOgene)	Faecal suspension preparation with lysis buffer containing PBS/EDTA → agitation with Fast Prep® instrument
McLauchlin *et al.*, 1999	*cowp* + *trap* + 18S rDNA	Standard PCR	GuSCN/Silica	None
Patel *et al.*, 1999	18S rDNA + *cowp*	Multiplex PCR+ Standard PCR-RFLP	GuSCN/Silica	None
McLauchlin *et al.*, 2000	*cowp* + *trap* -C1+ 18S rDNA	Standard PCR	GuSCN/Silica	None
Pedraza-Diaz *et al.*, 2001	*cowp*	Nested PCR	GuSCN/Silica	None
Millar *et al.*, 2001	18S + *trap*-C2+ ML	Standard PCR	Alkaline wash + Freeze-thaw cycles + Boiling step	Faecal suspensions preparation with sterile saline → serial dilution preparation

Citation	PCR target (s)	Assay type (s)	DNA extraction	Sample preparatory procedure (s)
Amar *et al.*, 2001	*cowp*	Standard PCR- RFLP	GuSCN/Silica	Faecal material was smeared on glass microscope slides → staining slides by conventional procedures → positive slides were subjected to DNA extraction
Amar *et al.*, 2002	*cowp*	Standard PCR -RFLP	GuSCN/Silica	Stained fecal smears preparation or from concentrated samples with salt flotation technique
Limor *et al.*, 2002	18S rDNA	Real-time (qPCR) (Hybridization probes)	Phenol-Chloroform-Isoamyl alcohol extraction + QIAamp® DNA stool mini kit (Qiagen)	Sucrose-Percoll centrifugation or alkaline digestion of the whole stool specimens
McLauchline *et al.*, 2003	18S rDNA + *cowp*	Standard PCR + Nested	GuSCN/Silica + PVP	None
Coupe *et al.*, 2004	18S rDNA	Nested PCR-RFLP	Alkaline lysis buffer (Na OH /SDS / PVP) + Qiagen spin column	One-step ethyl-ether concentration → pellets dilution with 2.5 % dichromate solution or distilled water
Nichols *et al.*, 2006	18S + *cowp* + *dhfr*	Standard PCR + nested PCR-RFLP + multiplex allele specific PCR	Freeze-thaw cycles + vortexing in between + Proteinase K	Water-ether or formol-ether concentration technique

Citation	PCR target (s)	Assay type (s)	DNA extraction	Sample preparatory procedure (s)
Magi *et al.*, 2005	*cowp*	Nested PCR	QIAamp® DNA stool mini kit (Qiagen) with modifications	None
NAVARRO-i-MARTINEZ *et al.*, 2006	18S rDNA + *cowp*	Two nested PCR	FastDNA® SPIN kit (Q-BIOgene)	Purification of oocysts with lysis buffer containing PBS/EDTA → Fast Prep® instrument (Q-BIOgene)
Stroup *et al.*, 2006	18S rDNA	qPCR (Scorpion probes)	Freeze-thaw cycles + Qiagen standard DNA kit with modifications	None
Bushen *et al.*, 2007	18S rDNA	Nested PCR-RFLP	Freeze-thaw cycles + QIAamp® DNA stool mini kit (Qiagen)	None
Meamar *et al.*, 2007	18S rDNA	Nested PCR-RFLP	Qiagen standard DNA kit	None
Leoni *et al.*, 2006	18S rDNA + *cowp*	Standard PCR-RFLP	GuSCN/Silica + PVP	None
Soba *et al.*, 2006	18S rDNA + *cowp*	Nested PCR + Standard PCR	Qiagen standard DNA kit	None
Llorente *et al.*, 2007	18S rDNA + *cowp*	Two nested PCR-RFLP	GuSCN/Silica + PVP	None
Hunter *et al.*, 2007	18S rDNA + *cowp*	Nested PCR-RFLP + Standard PCR-RFLP	100°C for 60 min+ proteinase K + lysis buffer + Qiagen spin column	Oocyst purification through saturated salt flotationtechnique

Citation	PCR target (s)	Assay type (s)	DNA extraction	Sample preparatory procedure (s)
Leoni *et al.,* 2007	*cowp* + Microsatellite loci *ML*1,*GP*15, *MS*5	Nested PCR- RFLP+ Standard PCR	GuSCN/Silica + PVP	None
Cheun *et al.,* 2007	18S rDNA	Nested PCR- RFLP	Qiagen standard DNA kit	Preservation 2.5 % phosphate dichromate solution→Sucrose density gradient flotationtechnique → freeze-thaw cycles → sonicated on ice with Cell Disrupter
Wielinga *et al.,* 2008	18S + *cowp* + *hsp*70 + *gp*60 + ML1+ *ML*2	Standard PCRs	Nucleosens kit or DNA purification system	Ethyl ether concentration → Two washes with lysis buffer including Tris/HCl/ EDTA → Adding anti-*Cryptosporidium* magnetic beads → heating for 10 min at 100 °C in lysis buffer
Kaushik *et al.,* 2008	18S rDNA	Nested PCR	Boiling for 10 min + QIAamp® DNA stool mini kit (Qiagen)	None
Robinson *et al.,* 2008	18S + *cowp* + *hsp*70	PCR-RFLP	QIAamp® DNA Stool Mini Kit (Qiagen)	Saturated salt flotationtechnique + Boiling for 1h

Citation	PCR target (s)	Assay type (s)	DNA extraction	Sample preparatory procedure (s)
Giardia lamblia:				
Hopkins *et al.,* 1997	18S rDNA	Standard PCR	Phenol–Chloroform-Isoamyl alcohol	Modified sucrose density concentration technique → In vitro culturing using TYI-S-33 medium → SDS lysis buffer → freeze-thaw cycles
Ghosh *et al.,* 2000	18S rDNA (IGS) Intergenic spacer	Nested PCR	CTAB/NaCl solution + Phenol-Chloroform-Ethanol	In vitro culturing using TYI-S-33 medium → lysis buffer containing (Tris HCl/EDTA /sarcosyl/ proteinase K)
Amar *et al.,* 2001	*tpi*	Semi-nested-RFLP	GuSCN/Silica + mechanical agitation with high speed-vortex mixer	Stained fecal smears preparation on glass microscope slides
Caccio *et al.,* 2002	*β*-giardin+18S rDNA	Standard PCR-RFLP	FastDNA® SPIN kit (Q-BIOgene)	Formol-ether concentration technique → agitation with Fast Prep® instrument (Q-BIOgene)
Sulaiman *et al.,* 2003	*tpi* +18S rDNA	Nested PCR	Modified Phenol-Chloroform + QIAamp® DNA stool mini kit (Qiagen)	Three washes with distilled water → Samples suspension preparation with lysis buffer containing (KOH/DTT/HCl)
Amar *et al.,* 2003	*tpi*	Standard+ 2 nested qPCR -	GuSCN/Silica + mechanical	DNA extraction was done from the whole

Citation	PCR target (s)	Assay type (s)	DNA extraction	Sample preparatory procedure (s)
		RFLP (LightCycler and SYBR Green)	agitation with high speed-vortex mixer	stool or from stained faecal smears on glass microscope slides
Ng *et al.*, 2005	18S rDNA	qPCR (Scorpion probes)	QIAamp® DNA stool mini kit (Qiagen) with minor modifications	Samples were subjected to two washes with PBS → Exposure to number of freeze-thaw cycles
Read *et al.*, 2004	*gdh*	Semi-nested PCR	QIAamp® DNA Stool Kit or glass milk matrix method	DNA extractions were done from the whole stool specimens or from concentrated cysts with saturated salt and glucose gradients techniques
Guy *et al.*, 2004	*β*-giardin	qPCR	QIAamp® DNA stool mini kit (Qiagen) with minor modifications	SAF-fixed stool were used → three washes with dH2O → freeze-thaw cycles in lysis buffer → three bursts of sonication
Bertrand *et al.*, 2005	*tpi* + *gdh*	Two Standard PCR	QIAamp® DNA stool mini kit (Qiagen) with minor modifications	Sample purification through gel filtration chromatography or ethyl acetate concentration procedures
Nantavisai *et al.*, 2006	18S rDNA	Nested PCR	FTA® filter paper kit (Whatman)	*Giardia* cysts concentration by saturated sodium nitrate flotation

Citation	PCR target (s)	Assay type (s)	DNA extraction	Sample preparatory procedure (s)
Berrilli *et al.*, 2006	18S rDNA	Nested PCR	QIAamp® DNA stool mini kit (Qiagen)	None
Lee *et al.*, 2006	18S rDNA (IGS)	Standard PCR	Modified Phenol-Chloroform	*Giardia* cysts purification with sucrose density gradient flotationtechnique → freeze-thaw cycles
Van der Giessen *et al.*, 2006	*gdh* +18S rDNA	Standard PCR	Puregene® kit (DNA Genotek)	Sucrose density gradient flotationtechnique → addition of anti-*Giardia* magnetic beads→ freeze/thaw cycles
Schuurman *et al.*, 2007	18S rDNA	qPCR (*Taq*Man probes)	GuSCN/Silica	None
Entamoeba histolytica :				
Gonin and Trudel, 2002	18S rDNA	Standard PCR	QIAamp® DNA stool mini kit (Qiagen)	None
Verweij *et al.*, 2003	Episomal repeat sequences + 18S rDNA	Two qPCR (*Taq*Man probes)	QIAamp® Tissue Kit (Qiagen)	Faecal suspensions preparation with ethanol, PBS and 2% PVP → lysis buffer containing SDS→ proteinase K treatment (2 h at 55 °C)
Hooshyar *et al.*, 2004	Encoding a 30 kDa surface antigen	Standard PCR -RFLP	Modified Phenol-Chloroform	In vitro culturing stool specimens in Robinson's medium

Citation	PCR target (s)	Assay type (s)	DNA extraction	Sample preparatory procedure (s)
Kebede *et al.*, 2004	18S rDNA	PCR-SHELA[3]	QIAamp® Tissue Kit (Qiagen)	Faecal suspensions preparation with PBS containing 2 % PVP → boiling for 10 min in SDS → proteinase K treatment (2 h at 55 °C)
Paglia and Visca, 2004	18S rDNA	Nested PCR	QIAamp® DNA stool mini kit (Qiagen)	DNA extractions were applied on formalin-fixed whole stool specimens
Furrows *et al.*, 2004	NM[4]	PCR-SHELA and a commercial LightCycler PCR	QIAamp® DNA stool mini kit (Qiagen) or Qiagen blood mini kit with some modifications	DNA extractions were employed on frozen stool or formol-ether concentrated specimens.
Roy *et al.*, 2005	18S rDNA	Nested PCR / qPCR (molecular beacon)	QIAamp® DNA stool mini kit (Qiagen)	Two washes steps with PBS
Calderaro *et al.*, 2006	18S rDNA	Standard PCR / qPCR (hybridization probes)	High Pure PCR Template Preparation kit (Roche)	None
Hung *et al.*, 2005	18S rDNA	Nested multiplex	Diatom beads+ GuSCN+	None

Citation	PCR target (s)	Assay type (s)	DNA extraction	Sample preparatory procedure (s)
			mechanical agitation with high speed-cell-disruptor + 10% Chelex 100 resin	
Solaymani-Mohammadi *et al.*, 2006	18S rDNA	Nested	QIAamp® DNA stool mini kit (Qiagen)	Formol- ether concentration technique
Santos *et al.*, 2007	Extrachromosomal DNA	Multiplex qPCR	GuSCN/Silica + Phenol-Chloroform-Isoamyl alcohol	None
Fotedar *et al.*, 2007	18S rDNA	Nested	QIAamp® DNA stool mini kit (Qiagen)	None
Khairnar and Parija, 2007	18S rDNA	Nested multiplex	CTAB[5]	None

[1]; Restriction Fragment Length Polymorphism
[2]; Guanidine thiocyanate/silica
[3]; Solution Hybridization Enzyme Linked immuno-Assay
[4]; not mentioned
[5]; Hexadecyl trimethyl ammonium bromide

1.5.4 Simultaneous detection of the three enteric protozoa

PCR methodology has proven to be a valuable tool for differentiation, genotyping and sub-typing, of enteric protozoa. The majority of previously developed PCR assays targeted one or more gene loci for one specified enteric protozoon (**Table 1-6**). As mentioned previously, PCR as a diagnostic laboratory test is limited by cost and sometimes the availability of adequate test sample volume. To overcome these shortcomings and also to increase the diagnostic capacity of PCR, a variant termed multiplex PCR has been described. In multiplex PCR format more than one target sequence can be amplified by including more than one pair of primers in the reaction. Multiplex PCR has the potential to produce considerable savings of time and effort within the laboratory without compromising test utility. Given these advantages of multiplex PCR usage along with realizing the public health importance of enteric protozoa, two real-time multiplex PCR assays has been successfully developed for identification of *Cryptosporidium* spp., *G. lamblia, E. histolytica* in human stool specimens (Verweij *et al.*, 2004 and Haque *et al.*, 2007).

The first assay included three primer pairs developed from the corresponding protozoal 18S rDNA gene with *Taq*Man probes specific for the corresponding protozoon. Additionally a fourth primer pair plus a *Taq*Man probes specific for *phocine herpesvirus*1 (PhHV-1) which was used as an internal standard control. Faecal suspensions were prepared using phosphate buffered saline (PBS) containing 2 % of polyvinylpyrrolidone (PVP). Then each faecal suspension was subjected to boiling for 10 min. After that, both sodium dodecyl sulfate (SDS) and proteinase K were added. Lysate solutions were incubated for 2 hours at 55°C before DNA isolation with QIAamp® Tissue Kit (Qiagen). This multiplex assay has been clinically evaluated

using only 20 positive samples for each protozoon plus 25 negative clinical samples with sensitivity and specificity rates reaching 100 %.

The second assay included two primer pairs developed from 18S rDNA gene of *E .histolytica* and *G. lamblia* while the third pair developed from *cowp* gene of *C. parvum*. Similarly, one *Taq*Man probe for each protozoon is involved in the reaction. Two different preparatory steps were followed prior to DNA extraction with QIAamp® DNA Stool Mini Kit (Qiagen).

For *Giardia* and *Entamoeba* positive stool samples, 0.2 g was washed twice with PBS then subjected to six freeze-thaw cycles using liquid nitrogen and a 95°C water bath. However, for *Cryptosporidium*-positive samples, 1 g of stool was initially purified through a modified ether-phosphate-buffered saline sedimentation technique and separated with density gradient centrifugation. After that, the purified oocysts were subjected to sonication five times on the ice bath and six rounds of freeze-thaw cycles using liquid nitrogen and a 95°C water bath. Subsequently, oocysts/cysts samples were subjected to DNA isolation and purification using the kit following the manufacturer's protocol with minor modifications. The analytical sensitivity of the multiplex PCR assay has been evaluated through a range of clinical samples including 42 *E. histolytica*, 33 *G. lamblia* and 25 *Cryptosporidium* positive control clinical stool samples whereas the specificity of the assay was estimated on 29 parasite-free stool samples only. This multiplex PCR assay has achieved sensitivity of 86 % versus 95 % for singleplex PCR assays and specificity of 98 % compared to 99 % of the uniplex assays.

To sum up, many PCR based methods have been developed for detection of enteric protozoa from human stool specimens. However, the majority of these PCR assays

have not reached the point of reliability to be accepted as routine laboratory tests. There are a number of reasons explaining this low reliability. Firstly, PCR is still more expensive than the conventional diagnostic methods especially for clinical laboratories in developing countries where parasitic diseases are most prevalent (Louie *et al.*, 2000). Secondly, nearly all of these assays lack proper standardization and clinical evaluation. Another important reason is that, many PCR assays have employed multi-steps procedures for target DNA extraction which add more costs, time and effort to the assay. Finally, in spite of the acceptable preliminary results achieved by the two previously developed multiplex PCR assays, real-time PCR technology has significant drawbacks as it requires high levels of expertise and is still too costly in terms of equipment and consumables for many routine microbiology laboratories particularly those in countries with limited resources.

1.6 Aim of the study

The key aim of this study was to develop a reliable diagnostic multiplex PCR for simultaneous detection of the predominant pathogenic species/strains of *Cryptosporidium*, *Giardia* and *Entamoeba* infecting humans. The multiplex PCR developed would need to be simple, rapid, cost-effective, specific and adequately sensitive to be endorsed as a screening tool in clinical laboratories particularly those of limited resources. As a preliminary step to achieve this goal, several step-by-step objectives were pursued:

1. Development of a simple copro-DNA extraction method which aimed to be applicable for a broad range of enteric parasites and which had the potential for future automation.
2. Development of simple multiplex PCR amplification assay with high diagnostic capacity which could accept inclusion of other enteric pathogens as targets for detection in the future.
3. Proper standardization of the assay by incorporation of appropriate uniform controls and clinical evaluation with a wide range of clinical stool samples.

2 Materials and methods

2.1 Study population and epidemiological settings:

This study was conducted in the form of collaborative work between the Clinical Microbiology laboratory, Leicester Royal Infirmary (LRI), University Hospital of Leicester (UHOL) and Infection, Immunity and Inflammation (3Is) department, University of Leicester. All clinical stool samples were collected from samples that are usually submitted to the Microbiology laboratory unless otherwise stated.

Prior to conducting our study, data stored at the Microbiology laboratory during the period between January 2007 and January 2008, were analysed to get a rough idea on the prevalence of the target protozoa in the population of the study. During the specified period, approximately 15302 stool samples, from hospitalized as well as from community-based patients, have been submitted to the laboratory for parasitological diagnosis. *Cryptosporidium* and *Giardia* diagnosis were carried thorugh identification of the protozoal copro-antigens in stool specimens using certain algorithm (see, **section 2.3.3**) while that of *Entamoeba* was done through microscopic identification.

Cryptosporidium copro-antigen was identified in 100 stool samples with prevalence rate of 0.65 % while *Giardia* copro-antigens were detected in 201 clinical samples with prevalence rate of 1.3 %. Importently, no *Entamoeba* infection was reported during the same period. The majority of *Cryptosporidium*-positive samples were for childrens below 13 years while most of the *Giardia*-positive samples were for patients between 25-45 years. High infection rates were reported for both protozoa during the period

between June and December but these periodic variations were more evident for *Cryptosporidium* much more than *Giardia* infection (see **Figure 2-1**).

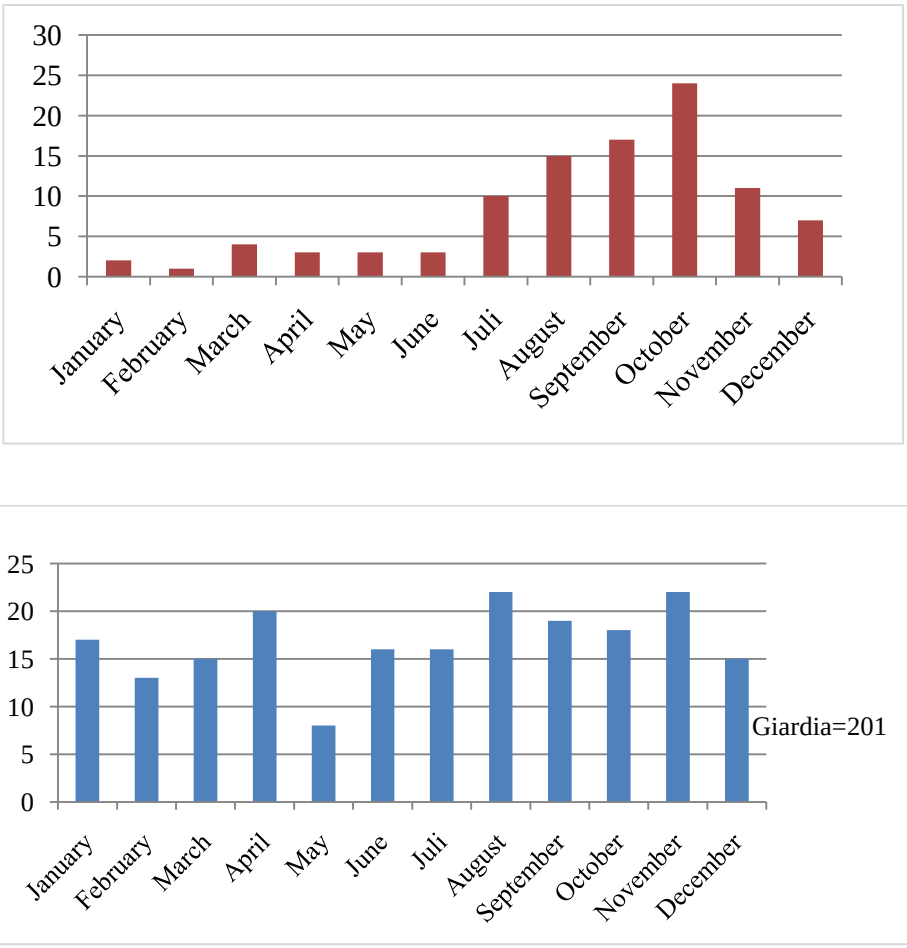


Figure 2-1: Monthly variations of *Cryptosporidium* (upper) and *Giardia* (lower) infections in Leicester based in data analysis at the Clinical Microbiology laboratory, LRI between January 2007 and January 2008

2.2 Materials

2.2.1 Control samples

As can be seen in **Table 2-1**, a range of different samples were used in this study as controls while developing the PCR assays. *Cryptosporidium* DNA control samples of the predominant species involved in human cryptosporidiosis were obtained from the

Cryptosporidium Reference Unit (CRU) at Swansea, Wales, UK. DNA samples were extracted from *Cryptosporidium*-positive human stool samples (i.e., crude DNA) after oocysts purification by salt-flotationconcentration technique. Positive clinical isolates were previously identified by *Cryptosporidium cowp*- or *hsp70*-targeted PCR assays (Spano *et al*., 1997 and Morgan *et al*., 2001, respectively). Species and genotype identification were carried out through restriction fragment length polymorphism (RFLP) and/or DNA sequencing.

The *Giardia lamblia* genomic DNA samples obtained from Royal Holloway University of London (RHUOL) were extracted from cultured trophozoites of WB and GS strains, as representatives of *G. lamblia* assemblage A and B that commonly encountered in human giardiasis. Molecular weight and the concentration (~170 – 200 ng/µl) were confirmed by gel electrophoresis. The *E. histolytica* genomic DNA samples were extracted from cryopreserved trophozoites of HM-1: IMSS laboratory strain. Molecular weight and concentration (~50 ng/µl) were confirmed by gel electrophoresis.

Table 2-1: Control samples used in the study

Sample	Source
Faecally derived *Cryptosporidium*-positive crude DNA[1]	
C. parvum	Dr. Chalmers, (CRU)[2]
C. hominis	Dr. Chalmers, (CRU)
C. meleagridis	Dr. Chalmers, (CRU)
C. Felis	Dr. Chalmers, (CRU)
C. andersoni	Dr. Chalmers, (CRU)
C. cervine genotype	Dr. Chalmers, (CRU)
Control clinical stool samples	
Cryptosporidium spp. positive (*n* = 50)	Current study
G. lamblia positive (*n* = 50)	Current study
E. histolytica positive (*n* = 15)	NLI, Egypt[3]
Parasite–free samples (*n* = 70)	Current study
Oocysts/cysts suspensions	
C.parvum oocyst suspension	Dr. Chalmers, (CRU)
G .lamblia cyst suspension	Current study
E.histolytica cyst suspension	Current study
Genomic DNA samples	
E.histolytica (HM-1: IMSS strain)	Dr. Kelvington, (UO L)[4]
G .lamblia (GS strain)	Helen, PhD student, (RHUOL)[5]
G .lamblia (WB strain)	
Recombinant plasmid DNA[6]	
pCR4®-TOPO:: EC455, EAC for *G. lamblia* PCR	Current study
pCR4®-TOPO:: IC300, IAC for *G. lamblia* PCR	Current study
pGEM®-T Easy::EC553, EAC for *Cryptosporidium* PCR	Current study
pGEM®-T Easy::IC375, IAC for *Cryptosporidium* PCR	Current study
pCR4®-TOPO:: EC167, EAC for *E. histolytica* PCR	Current study
pCR4®-TOPO:: IC375, IAC for *E. histolytica* PCR	Current study

[1]; DNA samples retrieved from positive stool samples
[2]; *Cryptosporidium* Reference Unit, Swansea, Wales, UK
[3]; National Liver Institute, Egypt (samples were collected as a whole stool preserved in 95% ethanol)
[4]; University of Leicester, UK
[5]; Royal Holloway University of London, UK
[6]; three external amplification controls (EACs) and three internal amplification controls (IACs) plasmids were constructed to monitor amplification of the corresponding individual target-uniplex PCR
(*n*) stands for the number of samples

PCR positive controls plasmids were constructed in this study and are described in more details in **Chapter-4**. Parasite-free faecal aliquots spiked with different concentrations of genomic, recombinant plasmid DNA or oocysts/cysts were prepared to address the analytical sensitivity of the multiplex PCR and the corresponding individual target-uniplex PCR assays (see **Chapter-5** for more details). In addition, one hundred eighty-five human stool samples including 70 negative and 115 positive controls were prepared for estimating the diagnostic performance of the different PCR assays developed in this study (see **Chapter-6** for more details**)**.

The oocysts/cysts suspensions, genomic DNA and faecally-derived DNA samples were aliquoted and stored at −20°C while the constructed plasmids were transformed into chemical competent bacterial cells that were subsequently stored as a glycerol stocks at −80°C until future use.

2.2.2 Clinical samples collection

Besides the aforementioned 185 clinical control samples, 212 diarrheal stool samples were randomly collected at LRI from patients with sporadic cases of gastro-enteritis for further evaluation of individual and multiplex PCR assays developed in this study. Samples were divided into 2 – 3 aliquots, labeled and stored without any preservative as whole faeces at 4°C until used. Additional information for these samples was obtained through the individual laboratory reports and outlined in a table that can be found in the **Appendix A**.

2.2.3 Reagents and supplies

All reagents, kits, chemicals and other laboratory supplies were outlined in a large table that can be followed at **Appendix B**.

2.2.4 Oligonucleotides

Basically four sets of oligonucleotides (primers) were used for PCR amplifications in this study. One set was adopted for developing the diagnostic multiplex PCR assay (diagnostic PCR primers). A second set was used for reference PCR assays (reference PCR primers). The third set of primers was designed for construction of PCR internal controls (inverse PCR primers). Finally, broad-range bacterial universal primers were occasionally used in the study during the optimization stage of the DNA extraction protocol (see **Chapter-3** for details). All primers are listed below in **Table 2-2**.

Oligonucleotides have on average a length of ~20 bases with some exceptions having 30 – 40 bp, a GC-content of ≥45 % and a T_m of about 50 – 60°C. Primers were synthesized by the VHBio (Gateshead, UK). All primers were dissolved in dH_2O for stock preparation (100 pmol/µl) and stored at −20˚C until use.

Table 2-2: Oligonucleotides (primers) used in the study

Primer name	Sequence (5' – 3')	Amplification target	Reference
Primers used in the diagnostic PCR assays			
Cry-9 (F)	GGACTGAAATACAGGCATTATCTTG	*Cryptosporidium* spp. *cowp* gene	Spano *et al.*, 1997
Cry-15 (R)	GTAGATAATGGAAGAGATTGTG	*Cryptosporidium* spp. *cowp* gene	Spano *et al.*, 1997
GDHeF[1] (F)	TCAACGTYAAYCGYGGYTTCCGT	*G. lamblia gdh* gene	Read *et al.*, 2004
GDHiR[1] (R)	GTTRTCCTTGCACATCTCC	*G. lamblia gdh* gene	Read *et al.*, 2004
GDHiF[1] (nested)	CAGTACAACTCYGCTCTCGG	*G. lamblia gdh* gene	Read *et al.*, 2004
EntaF (F)	ATGCACGAGAGCGAAAGCAT	*E. histolytica* 18S rDNA gene	Hamzah *et al.*, 2006
EhR (R)	GATCTAGAAACAATGCTTCTCT	*E. histolytica* 18S rDNA gene	Hamzah *et al.*, 2006
Primers used in the reference PCR assays			
RH11 (F	CAT CCG GTC GAT CCT GCC	*G. lamblia* 18S rDNA gene	Hopkins *et al.* 1997
RH4 (R)	AGTCGA ACC CTG ATTCTC CGCCAG G	*G. lamblia* 18S rDNA gene	Hopkins *et al.*, 1997
YH1 (nested)	TCC CTG CTA GCC GGC GGA CAC	*G. lamblia* 18S rDNA gene	Current study
XF1 (F)	TTCTAGAGCTAATACATGCG	*Cryptosporidium* 18S rDNA gene	Xiao *et al.*, 1999
XR1 (R)	CCCTAATCCTTCGAAACAGGA	*Cryptosporidium* 18S rDNA gene	Xiao *et al.*, 1999
XF2 (F)	GGAAGGGTTGTATTTATTAGATAAAG	*Cryptosporidium* 18S rDNA gene	Xiao *et al.*, 1999
XR2 (R)	AAGGAGTAAGGAACAACCTCCA	*Cryptosporidium* 18S rDNA gene	Xiao *et al.*, 1999
E-1 (F)	TAAGATGCACGAGAGCGAAA	*E. histolytica* 18S rDNA gene	Khairnar and Parija, 2007
E-2 (R)	GTACAAAGGGCAGGGACGTA	*E. histolytica* 18S rDNA gene	Khairnar and Parija, 2007
EH-1 (F)	AAGCATTGTTTCTAGATCTGAG	*E. histolytica* 18S rDNA gene	Khairnar and Parija, 2007

Primer name	Sequence (5' – 3')	Amplification target	Reference
EH-2 (R)	AAGAGGTCTAACCGAAATTAG	*E. histolytica* 18S rDNA gene	Khairnar and Parija, 2007
Primers used in construction of PCR internal amplification control[2]			
Inv PF-1 (F)	**AT**AAGCTTATTGATATGGTCTGCCCACC	pGEM®-T Easy::EC553, EAC	Current study
Inv PR-1 (R)	**TT**AAGCTTAAAACCAGAAGGACAAACGG	pGEM®-T Easy::EC553, EAC	Current study
Inv PF-2 (F)	**AG**AAGCTTCTCACAGGCAAGAACGTCAA	pCR4®-TOPO:: EC455, EAC	Current study
Inv PR-2 (R)	**AT**AAGCTTATGACCTCGTTGTCGGACTT	pCR4®-TOPO:: EC455, EAC	Current study
Inv PF-3 (F)	**CT**AAGCTTAGAGAAGCATTGTTTCTAGATC	pCR4®-TOPO:: EC167	Current study
Inv PR-3 (R)	**AT**AAGCTTATGCTTTCGCTCTCGTGCAT	pCR4®-TOPO:: EC167	Current study
catR (F)	TCTCATGAGTATGCATTCCCGGCCCACAGCGGATTATG	pJKO-3b[3]	Obtained from John
fimK2_rf_Kn5 (R)	TCCGGTTCGCATGCATTCCCGGCCCACAGCGGATTATG	pJKO-3b	Obtained from John
Broad range bacterial universal primers			
Bact-8F (F)	AGAGTTTGATCCTGGCTCAG	16S rDNA	Edwards *et al.,* 1989
1391R (R)	GACGGGCGGTGTGTRCA	16S rDNA	Lane *et al.,* 1985

(F) stands for forward and (R) stands for reverse

[1]; primers with degenerate bases; 'Y' indicates a 50:50 mix of 'C' and 'T' while 'R' is an equivalent mix of 'A' and 'G' in the degenerate primer mixes produced.

[2]; nucleotide sequence of *Hin*dIII restriction sites is underlined while the nucleotides written **in bold** are extra nucleotides inserted at 5'-terminus of the inverse primers

[3]; plasmid pJKO-3b was constructed by John, one member of our laboratory.

2.3 General methods

All stool-related procedures such as microscopic examination, oocysts/cysts purification, counting and DNA extractions were carried out at the Microbiology Laboratory, LRI while all DNA downstream applications such as PCR amplification, and cloning experiments were accomplished at the Infection, Immunity and Inflammation department, UOL, unless otherwise stated.

2.3.1 Microscopic diagnosis of *Cryptosporidium*

Detection of *Cryptosporidium* in diarrheal stool samples was carried out using the Modified Ziehl-Neelsen stain (Casemore *et al*., 1985). Two moderately thick smears were prepared for each test sample by applying a small amount of well homogenized and carefully diluted fecal specimen on a glass slide and left to air dry. Slides were fixed by adding few drops of methanol, allowed 3 min to air dry and subjected to staining procedure. Initially, few drops of strong carbol-fuchsin (Sigma, UK) was added on the slide and left for 10 min before washing with tap water. Subsequently, few drops of 1 % hydrochloric acid in methanol (v/v) were added with a brief agitation to the slide for decolorisation. Following a second washing step with tap water, 0.4 % malachite green (Sigma, UK) was added for 30 sec followed immediately by another wash step with tap water. Finally, slides were left to air-dry, then a drop of immersion oil was added and immediately scanned with Leica DC2000 light microscope (Leica Microsystems) using x40 objective lens.

2.3.2 Microscopic detection of *G. lamblia* and *E. histolytica*

Diarrheal stool samples were examined for *Giardia* and *Entamoeba* trophozoite and/or cyst stages following simple wet mount technique (Loughlin and Spitz, 1949). A small drop of liquid specimen was used directly in preparing a thin smear. A drop of freshly prepared iodine was added to the slide, covered with a cover slip and subjected to microscopic examination. The slide was scanned immediately for parasites with x10, x20 and x40 objective lenses.

2.3.3 Copro-antigens detection

Diarrheal stool specimens are tested for *Cryptosporidium* spp. and *Giardia* copro-antigens using three kits based immunoassay as the routine diagnostic test adopted at the Clinical Microbiology laboratory, LRI, UHOL. This testing procedure relies on screening all test samples through a fully automated system using Triturus® EIA analyser for protozoal copro-antigens with *Giardia*/*Cryptosporidium Check*™ enzyme immunoassay (EIA) screening test (TechLab) as an initial step. Then, pre-screened specimens positive for *G. lamblia* or *Cryptosporidiu*m in the EIA assay are subsequently subjected to the RIDA® Quick *Giardia* and RIDA® Quick *Cryptosporidium* (R-Biopharm) immunochromatographic discriminatory assays. All copro-antigen immunoassays were carried out following the manufacturers' protocols.

2.3.4 Preparation of *Cryptosporidium* oocysts suspension

Purification of the *Cryptosporidium* oocysts was carried out using saturated salt flotationconcentration method (Gardner *et al*., 1991). An aliquot of *Cryptosporidium* positive clinical control sample (~2 ml) was transferred into 15 ml polypropylene

centrifuge tube. Saturated salt (sodium chloride) solution with specific gravity of 1.2 was prepared as mentioned in **Appendix B**. Eight milliliters of a previously prepared saturated salt solution were added and thoroughly mixed by shaking. Then, 3 ml of dH_2O was dropped along the edge of the tube to form a layer above the salt/faecal mixture. The tube was capped and immediately centrifuged for 8 min at 3,000 rpm. The top water layer was swirled with the tip of a Pasteur pipette just below the surface producing a "tornado effect" and the oocysts at the interface and in the water layer were aspirated and transferred into a clean 15 ml centrifuge tube. Consecutively, the oocysts were washed with 10 ml of dH_2O, centrifuged for 5 min at 3,000 rpm and resuspended in 1 ml of PBS.

20 µl of oocysts suspension was smeared over microscopic slide and left for 5 min at RT to dry. Then a drop of immersion oil was added immediately, covered with a coverslip, and scanned with the ×40 objective lens.

Cryptosporidium oocysts appear as highlighted round organisms (4 – 6 µm), and their margins lead to a characteristic refraction of the light of the microscope (Kimmig, Hartmann, 1986). The average oocyst count per slide was identified and accordingly, the oocysts count per ml was calculated following this equation:

Number of oocysts per 1 ml = average count per slide (20 µl) × 50

2.3.5 Preparation of *Giardia* and *Entamoeba* cysts suspension

Initially, *G. lamblia*- and *E. histolytica*-positive stool samples were concentrated with modified formol-ether concentration technique (MFEC) (Loughlin and Spitz, 1949). 1 ml of microscopically positive *Giardia*/*Entamoeba* diarrheal stool samples was emulsified in a glass universal container containing 10 % formal-saline (E and O

Laboratories) and left to fix for 20 min at RT. After a thorough mix, the samples were strained individually with a nylon tea strainer (420 µm) and the filtrate was collected in a new container. Three milliliters of the ethyl-acetate (Fischer Scientific) was added to the filtrate and the mixture was briefly agitated. Samples were transferred into ether-resistant conical polypropylene tubes to be centrifuged at 1,500 x g for 1 min. A wooden stick applicator was used to loosen the debris layer (faecal plug) and the supernatant was decanted into a biohazard waste container. Each tube was cleaned from any hanging debris with cotton tipped applicators and the sediment was resuspended with the remaining fluid in the bottom of the tube.

A drop of the sediment was smeared onto a glass slide to be scanned for parasites using bright field microscopy. Modified formol ether-purified and concentrated samples were further purified by the sucrose density gradient centrifugation technique (Heyman *et al*., 1986) as follows: the pre-concentrated stool sediment was resuspended in 3.5 ml dH2O and layered along the side walls of 10 ml polypropylene tube containing 5.5 ml 1 M sucrose (specific gravity 1.11). The tube was spun at 650 x g for 10 min and the water-sucrose interface was aspirated carefully into another tube and washed twice with dH_2O.

Finally, *Giardia*/*Entamoeba* cysts suspensions were counted with the modified Fuchs Rosenthal haemocytometer. The haemocytometer chamber has nine large squares each made of 16 small squares. One large square has a surface area of $1mm^2$ and a volume of 0.2 µl. *G. lamblia* and *E. histolytica* cysts were counted in five large squares in a volume of 5 × 0.2 µl (1 µl). ~15 µl of the diluted cyst suspension was filled into the chamber and counted with x20 objective lens of the bright field microscope. Number of cysts present in 1 ml was counted following the equation:

Number of cysts per 1ml = no of cysts in 5 large squares × DF × 1000 where DF = dilution factor

2.3.6 DNA extraction

Fresh and/or frozen clinical stool samples were subjected to DNA extraction within two weeks after collection using QIAmp® Stool Mini Kit (Qiagen, UK). Initial experiments were carried out following the manufacturer's protocol. After a series of optimisation experiments (see **Chapter-3** for more details), all subsequent extractions were accomplished using a modified QIAmp® Stool Mini Kit DNA extraction protocol.

This protocol includes the following modifications: Firstly, the lysis temperature adopted was 100˚C for 10 minutes instead of 97°C for 7 minutes. Secondly, the incubation time for the InhibitEX tablet, included in the kit, in the DNA lysate solution was increased to 3 – 5 minutes instead of 1 minute. Furthermore, the 95 % ethanol was pre-cooled at −20°C for ~10 min before use. Finally, 50 – 100 µl of elution buffer instead of 200 µl as specified were used to elute the nucleic acid sample after a three-minute incubation time at room temperature.

2.3.7 DNA amplification

2.3.7.1 Standard PCR technique

Development of diagnostic PCR assay required a lot of optimisation experiments in order to achieve acceptable sensitivity and specificity levels. To facilitate presentation, all of these optimisation experiments and the fully-optimised protocols are described in **Chapter-5**.

Generally, PCR amplifications were carried out with Techne™ TC-4000 thermal cycler. GoTaq® Hot Start Polymerase (Promega) and other PCR reagents were used in amplification reactions with the final concentrations given below (**Table 2-3**).

Table 2-3: The final concentration of PCR reaction mix

PCR reagent	**Final concentration**
5X Green or Colorless GoTaq® Flexi Buffer	1 – 2X
$MgCl_2$ Solution, 25mM	1.5 mM
PCR Nucleotide Mix, 10 mM each	0.2 mM each dNTP
Upstream primer	10 – 20 µM
Downstream primer	10 – 20 µM
GoTaq® Hot Start Polymerase (5u/µl)	1 – 2 u
Template DNA	Variable
Nuclease-free water up to 20 – 50 µl reaction volumes	

Sometimes, this final concentration was scaled up and down to achieve success in PCR amplifications.

The cycling conditions for all standard PCRs were closely similar except for the annealing temperatures. The standard thermal cycling conditions are listed in **Table 2-4**.

Table 2-4: Thermal cycling conditions

PCR cycling conditions with GoTaq® Hot Start Polymerase	
Initial denaturation step	2 – 4 min at 94 – 95°C
Second denaturation step	30 – 60 sec at 94 – 95°C
Primers annealing step	30 – 60 sec at the primers annealing temperature (1 – 5°C below the calculated T_m of the primers)
Primers extension step	30 sec to 4 min (1 min for every 1kb of DNA) at 72°C
Final extension step	7 – 10 min at 72°C
Number of cycles	25 – 40 cycles

2.3.7.2 Variations in the standard PCR technique

Several PCR cycling profiles other than the aforementioned standard PCR were adopted in the study as follows;

2.3.7.2.1 Nested and semi-nested PCR

Nested PCR, one of these cycling profiles in which, a second fragment of the protozoal target DNA internal to the first primer pair sequences was amplified with an additional PCR round. Sometimes, this round was guided by one nested primer together with the reverse primer that used in the first round (i.e., semi-nested PCR) or by two nested primers (i.e., nested-PCR). Semi-nested PCR was adopted in the initial experiments prior complete optimisation of *G. lamblia* uniplex PCR assay as a single-round PCR. Three nested PCR assays were used in the study as a references for the individual target-uniplex diagnostic PCR assay developed in the study (for more details, see **Chapter-5**).

2.3.7.2.2 Touchdown PCR

Touchdown PCR was used to increase the sensitivity as well as the specificity of *G. lamblia* uniplex and the three-enteric protozoon multiplex PCR assays developed in the study (see **Chapter-5**). Ten cycles of a touchdown PCR were introduced over the standard PCR protocol. Touchdown procedure was initiated by setting up a higher annealing temperature ten degrees above the Tm of the primers and then for the subsequent ten cycles, the annealing temperature was decreased by 1°C per cycle. The remaining 20 – 30 cycles were completed following the standard PCR protocol.

2.3.7.2.3 Multiplex PCR

Three protozoal target genes were simultaneously amplified in the same PCR reaction under the same cycling conditions. According to the length of the amplified sequences, the concentration of each primer pair, the balance between the deoxynucleotides and magnesium chloride concentration, cycling temperatures all were adjusted and optimized. At first, PCRs were run to amplify two targets in individual PCR tubes but under the same cycling protocol. Then, these two targets were amplified in a duplex reaction using the same PCR tube ad under the same thermal cycling condition. Finally, the three targets were simultaneously amplified in the same PCR reaction under the same cycling conditions as a multiplex assay (see **Chapter-5** for more details).

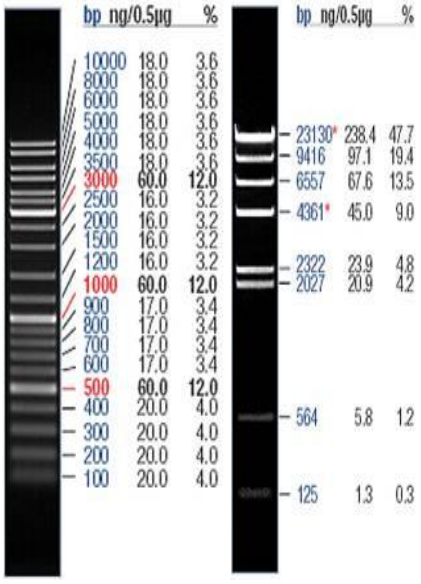
2.3.7.2.4 Inverse PCR

In the process of internal amplification controls (IACs) construction (see **Chapter-4**), inverse PCR was used as a tool to delete DNA fragment from a recombinant plasmid. The deleted DNA sequence was internal to the flanking primers of known sequences.

For each recombinant plasmid, two inverse primers carrying *Hin*dIII restriction sites were designed to amplify DNA sequence through the vector backbone sequence

2.3.8 Agarose gel electrophoresis

Agarose concentration of 0.8 – 2 % was used in gel electrophoresis relying on the sizes of the DNA fragment. The agarose gels were made in 1X TAE buffer (Sigma, UK) containing 0.5 µg / ml ethidium bromide (Sigma, UK) to visualize DNA. Gels were submerged in 1X TAE buffer and DNA applied to wells in 6X gel loading buffer (see **Appendix B**) in order to monitor migration of the DNA. Electrophoresis was performed in an in-house electrophoresis tank. Gels were viewed by UV illumination and photographed using a gel documentation system (Kodak). For sizing of the separated DNA fragments λ-*Hin*dIII marker (Fermentas) and/or GeneRuler™ ladder (Fermentas) were run on the same gels (**Figure 2-2**).



The left hand columns of numbers represent the band sizes in (bp). The right hand columns of numbers represent the equivalent (ng) per 0.5 µg to approximately quantify PCR amplicons. The **left** picture is for the GeneRuler™ ladder and the **right** hand picture is for the λ-*Hin*dIII marker.

Figure 2-2: Molecular DNA markers

2.3.9 Cloning of PCR products

Cloning of PCR products were adopted for construction of the external and internal standard PCR controls. Steps employed in PCR product cloning can be seen in **Figure 2-3**.

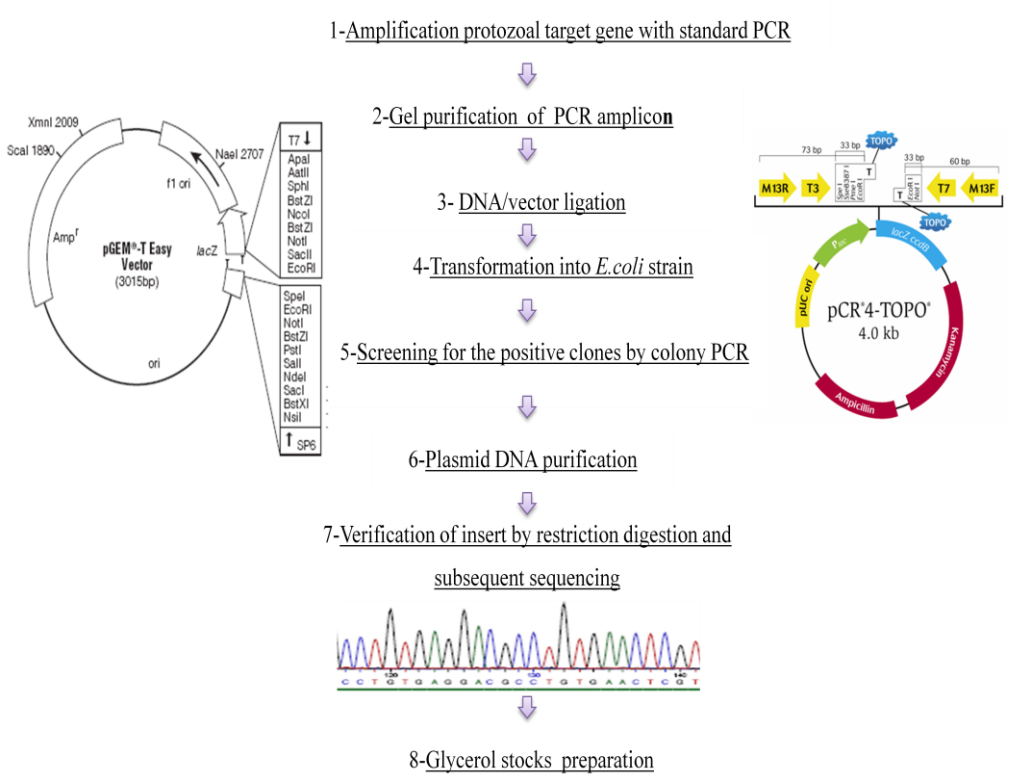


Figure 2-3: Schematic diagram showing steps adopted for cloning a PCR product

2.3.9.1 DNA gel extraction and purification

DNA purification was performed through YORBIO Gel/PCR DNA Purification Kit (Yorkshire Bioscience) following the enclosed protocol. Briefly, DNA fragments were excised from the agarose gel with a scalpel, transferred to a clean 1.5 microfuge tube

and weighed. The gel slice was dissolved by heating at 65°C for 10 min in the presence of double its volume binding buffer. The mixture was decanted into spin column and let to stand at RT for 2 min before spinning at 13,500 rpm for 1 min. Spin column-trapped DNA sample was washed once with 500 µl of wash buffer and centrifuged for 15 sec followed by another centrifugation step for 1 min to remove any residual ethanol present in the washing buffer. Lastly, DNA was recovered by 30 – 50 µl of the elution buffer and stored at −20˚C for subsequent procedures.

2.3.9.2 DNA/ vector ligation

Ligation reactions were performed with two different vector systems. Reactions with pCR®4-TOPO vector (Invitrogen) were incubated for 5 min and for pGEM-T Easy vector (Invitrogen) were incubated for 30 min at RT. 1 to 2 µl of a fresh PCR product were mixed with 1 µl of the vector (10 ng/µl). In the case of the pGEM-T Easy system, 3U of T4 DNA Ligase (Promega) in a 2X ligation buffer (Promega) was used. All ligation reactions were placed on ice for subsequent transformation (see **Section** 2.3.9.4).

2.3.9.3 Preparation of chemical competent cells

A single colony of *E. coli* Top10 or DH5α strain was transferred into 5 ml of Luria-Bertani (LB) medium and the cells were grown o/n at 37°C, 200 rpm in a shaking incubator. 500 µl of the o/n culture was inoculated into 300 – 400 ml sterile LB broth. Cells were grown at 37°C, 200 rpm until they reached an optical density (OD) at 600 nm of 0.3 to 0.4 and cells were harvested by centrifugation at 3,000 x g for 10 min at 4˚C. Pellet was resuspended on ice with 50 ml of ice cold 100 mM $MgCl_2$ and spun at 3,000 x g for 10 min at 4˚C. On ice, the pellet was dissolved with 50 ml of ice cold 100

mM $CaCl_2$, 10 min incubated on ice and centrifugation step was repeated. Cells were resuspended on ice with 4 ml of ice cold 100 mM $CaCl_2$ in 10 % glycerol (w/v) and divided into 50 µl aliquots to be stored immediately at −80°C for future transformations.

2.3.9.4 Heat shock transformation

Chemical competent *E. coli* TOP10 and DH5α strains were used for the transformation reactions. Competent cells were removed from −80°C freezer and placed straight on ice to thaw slowly. Then 2 µl of the ligation reaction and the control empty vector were added to 50 µl of the competent cells and mixed gently by flicking the tube. Reactions were incubated on ice for 30 min and subsequently subjected to heat-shock for 45 sec at 42˚C using either heat block or water bath and immediately returned back on ice for 2 min. Consequently, 250 µl of 'in-house' or commercially prepared SOC medium (see **Appendix B**) was added to cells and the tube was incubated in a shaking incubator at 200 rpm at 37˚C for 1 hour. Lastly, 50 – 100 µl of cell suspension were plated on the appropriate selective LB agar plates and placed in 37˚C incubator for o/n growth.

2.3.9.5 Screening of the transformants by colony PCR

Single colony was picked up from a freshly grown LB plate, using sterilized pipette tip and rolled into a PCR tube containing 5 µl of nanopure water. Using the same pipette tip, one fresh LB plate with selective antibiotic was streaked on and incubated o/n at 37˚C. Each insert was amplified with the corresponding set of target or plasmid primers. PCR amplification was performed in 20 µl volumes with 5 µl of the colony suspension as a template with 1.5 mM MgCl2, 200 µM of each dNTP (Bioline, UK), 10 µM of each primer, and 1.25U of Go Taq® Hot Start DNA Polymerase (Promega). The

reactions were subjected to 35 cycles of 94˚C for 30 sec, 58˚C for 30 sec, and 72˚C for 60 sec followed by a final extension at 72˚C for 10 min. All PCR products were analysed on ethidium bromide-stained 1 % agarose gel. Colonies that were PCR positive was selected for subsequent plasmid purification and glycerol stock preparation.

2.3.9.6 Growth and storage of bacteria containing plasmids

Bacterial strains and cultures of bacteria containing plasmids were stored for short-term use (2-3 weeks) at 4˚C on LA plate containing the appropriate antibiotics. For long term storage, 800 µl of a freshly saturated bacterial culture grown in LB-medium with the appropriate antibiotics was added to a screw capped vial containing 200 µl of sterile 99 % glycerol solution. Vials were carefully mixed, labeled and immediately stored at −80˚C. Stored cells were recovered from storage by scraping off splinters of solid ice with a toothpick or sterile pipette tip and streaking these splinters onto an LB plate containing the appropriate antibiotic.

2.3.10 Plasmid DNA purification

Large-scale Plasmid DNA extraction and purification was carried out using QIAGEN Plasmid *plus* Midi Kit (Qiagen, UK). Small-scale purifications were done through QIAprep Spin Miniprep Kit (Qiagen, UK) or by alkaline lysis. All plasmid DNA extractions based on the commercially available kits were employed following the manufacturers' protocols.

2.3.10.1 Alkaline lysis

Small-scale plasmid DNA extraction was accomplished following the alkaline lysis method (Birnboim and Doly, 1979) as follows; a single colony was inoculated into 5 ml of LB with 50 µg/ml ampicillin and incubated o/n in 37˚C, 200 rpm. Bacteria culture was pelleted by spinning down at 1,200 x g for 10 min at RT. The pellet was resuspended in 200 µl of freshly prepared lysozyme solution (50 mM Glucose, 25 mM Tris-HCl, 10 mM EDTA, 4 M Lysozyme) and incubated 5 – 10 min at RT. 400 µl of fresh alkaline solution (0.2 M NaOH, 1 % SDS) and 300 µl of ammonium acetate (7.5 M) were added, mixed by inversion and incubated on ice for 5 min. The lysate was spun down at 12470 x g for 30 min at RT and the supernatant was decanted. The pellet was washed in 500 µl of 70 % cold ethanol before centrifugation again at 12470 x g for another 10 min at RT. Nearly all of the supernatant was removed but a little bit was left behind and dried down in vacuum dryer. Pellet was dissolved in 30 – 50 µl of sterile water and 2 µl of RNase A was added and DNA was stored at −20°C.

2.3.11 Restriction digestion of the recombinant DNA

Restriction digestion with *Eco*RI, *Hin*dIII and *Dpn*I restriction enzymes (Roche or New England Biolabs) were carried out according to the manufacturers' instructions. Volumes of 20 – 50 µl were used for digestion of 1 – 5 µg DNA sample with 2 – 5 units of the enzyme in the presence of a 1X restriction buffer. Digestion reaction were performed at 37˚C and completed within 2 – 4 hr, 5 µl of the digest was loaded into a gel for visualization and estimation of plasmid DNA concentration through comparing the resolution of DNA band (s) with those of λ *Hin*dIII DNA marker.

2.3.11.1 DNA quantification with spectrophotometer

The purity of DNA sample is usually estimated by calculating the ratio of absorption spectra reading at wavelengths of 260 nm and 280 nm (OD_{260}/OD_{280}). Pure preparations of DNA have OD_{260} : OD_{280} values of 1.8 to 2.0 respectively. The sample was 1 : 10 diluted in dH_2O in order to obtain a linear A_{260} reading in a range of 0.2 – 1.0. The concentration of DNA samples was calculated as follow:

DNA concentration = OD_{260} × DF × 1 OD

A_{260} of 1 OD = ~50 µg/ml for double stranded DNA.

2.3.12 DNA sequencing and sequence analysis

Plasmids containing the correct sized inserts were sent to MWG (Germany) for bidirectional automated sequencing. Recombinant pTOPO® DNA was sequenced with primers T3 and T7 and pGEM®-T EASY with M13 forward and M13 reverse primers provided in the corresponding cloning kit (Invitrogen). Obtained DNA sequences were trimmed from the sequence of the cloning vectors and subjected to similarity searches of the non-redundant GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/).

Similarly, PCR amplification products of 24 samples including samples that showed discordant results among uniplex PCR, multiplex PCR and the combined copro-antigen detection test were purified from the agarose gel and sent for DNA sequencing. The retrieved target DNA sequences were compared to those available in the GenBank database using the same free online bioinformatic tool.

2.4 Data storage and statistical analysis

Results obtained from examination of the clinical stool samples as well as some relevant clinical data for the clinical samples were stored and analyzed through Microsoft Excel TM (see the clinical samples data table in **Appendix A**). The diagnostic sensitivity, specificity, positive predictive value and negative predictive value of various diagnostic assays were determined by standard formulae (Galen and Gambino, 1975). For further confirmation of the calculated values, occasionally, the performance characteristic of the diagnostic PCR assay was carried out through online free software (http://ilm.medicine.arizona.edu/EBDM/DTPC/calculator.html).

3 Development of a QIAamp® Stool Mini Kit-based DNA extraction protocol

3.1 Introduction

In this chapter, the use of the QIAamp® DNA Stool Mini Kit (Qiagen) was investigated as a common DNA extraction method for the three target protozoa. The QIAamp® DNA Stool Mini Kit was originally developed for DNA isolation from metabolically active cells found in faecal specimens. It has a buffer system which permits direct cell lysis and optimal binding of nucleic acids to a silica gel membrane. Inclusion of an initial heating step, InhibitEX tablets and two successive wash steps are employed in the manufacturer's instructions to remove contaminants that are commonly found in stool.

Recent studies have investigated the utility of the kit as a DNA extraction tool for a range of entero-pathogenic bacteria directly from human stool. Both spore-forming (Subrungruang *et al*., 2004) and non-spore forming bacteria (LaGier *et al*., 2004) were targeted for DNA extraction and subsequent PCR amplifications. The kit has also been frequently employed for DNA extraction from purified protozoa oocysts/cysts suspensions (Stroup *et al.*, 2006 and Furrows *et al.*, 2004). Only a few studies have reported the use of the kit for *E. histolytica* and *G. lamblia* DNA extraction directly from whole stool specimens (e.g., Fotedar *et al.*, 2007 and Berrilli *et al.*, 2006). For *Cryptosporidium* spp. DNA extraction directly from faecal specimens, the standard kit protocol is usually preceded by several preparatory steps. This chapter focuses on development and optimization of a DNA extraction protocol based on the QIAamp®

DNA Stool Mini Kit (Qiagen) to be used as a common extraction method in the diagnostic multiplex PCR. Clinical evaluation of the extraction protocol was carried out as a part of the multiplex PCR assay investigation described in **Chapter-6**.

3.2 Optimization of Qiagen stool kit protocol

3.2.1 Preliminary experiments using the manufacturer's standard DNA extraction protocol

Initially at the start of the study, six stool samples identified as *Cryptosporidium* positive by the copro-antigen combined test (see **Materials and Methods**, Section 2.3.3). These *Cryptosporidium* positive samples had been collected and stored unpreserved at −20°C for variable periods of time (2 days – 2 weeks) prior to DNA extraction with QIAamp® DNA Stool Mini Kit following the manufacturer's protocol. The faecally-derived DNA samples were then subjected to PCR amplification using the *Cryptosporidium* diagnostic PCR (see **Chapter-5** for details). This PCR assay targeting the *cowp* gene generates an amplicon of ~550 bp with template DNA from the predominant *Cryptosporidium* species infecting humans. However, using the standard QIAamp® DNA Stool Mini Kit faecal DNA preparations, The *Cryptosporidium* target sought could not be detected on agarose gel for any of the six known-positive clinical samples. Accordingly, the faecally derived DNA samples were subjected to a series of experiments to rule in or rule out PCR inhibition as a cause of the PCR negative results.

Firstly, the DNA samples were diluted (1 : 10 and 1 : 100) with nanopure water and subsequently used as a template with the same diagnostic PCR. Once again, no amplification of the target DNA was detected by agarose gel electrophoresis.

Secondly, the same faecally-derived DNA samples were subjected to PCR amplification using 16S rDNA-broad range universal primers namely Bact-8F (Edwards *et al.,* 1989) and Bact-1391R (Lane *et al.,* 1985). PCR amplification was carried out using 20 µM of Bact-8F and Bact-1391R primers (see **Materials and Methods**, Table 2-2), in the presence of 1 µl of the template DNA, 1X Go Taq® green buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1 % DMSO (Sigma, UK), and 1 Uof Go Taq® Hot Start polymerase (Promega) final concentrations. PCR reactions were set up in 20 µl volumes and completed using the following cycling conditions:

The protocol was initiated by a pre-heating step for 4 min at 95°C, and followed by 20 cycles of 30 sec at 95°C, 30 sec at 56°C and 90 sec at 72°C. A final extension step of 8 min at 72°C was included.

Amplicons of ~1.5 kb were successfully detected on agarose gel for the six samples (see **Figure 3-1)**.

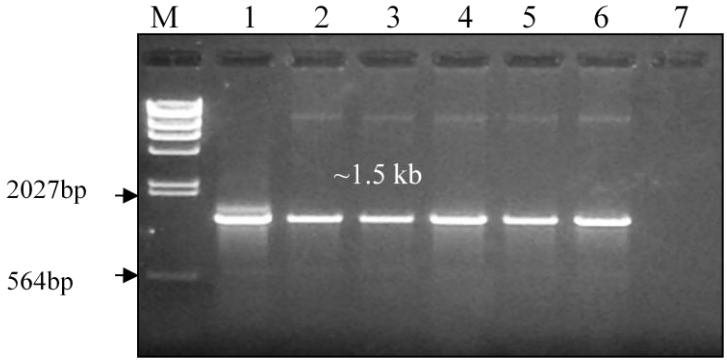


Figure 3-1: Representative ethidium bromide-stained 0.8 % agarose gel picture showing PCR amplification products for DNA samples recovered from six *Cryptosporidium* positive stool samples by the QIAamp® DNA Stool Mini Kit. Band of ~1.5 Kb of 16S rDNA gene sequence was amplified using Bact-8F/139-R (broad-range bacterial primers).M, λ *Hin*dIII DNA marker; Lane-1-6, *Cryptosporidium* positive clinical samples; Lane-7, no-template master mix sample (PCR negative control).

3.2.2 Modifications of the manufacturer's protocol

Several experiments were carried out subsequently for optimization of the kit's extraction protocol using *Cryptosporidium* positive specimens.

Different lysis temperatures, lysis duration, centrifugation time, incubation time and elution volumes were individually assessed in a series of experiments. Four modifications of the kit's instructions were carried out. (1) Lysis temperature of 100˚C for 10 min instead of 97°C for 7 min **(**see **Figure 3-2**, lane-4**)**. (2) Increasing the incubation time for the InhibitEX tablet in the DNA lysate solution to 3 – 5 min instead of 1 min. (3) Pre-cooling the 95 % ethanol before use. (4) Eluting the purified DNA sample with 50 – 100 µl of elution buffer instead of 200 µl as specified after a three-min incubation time at RT.

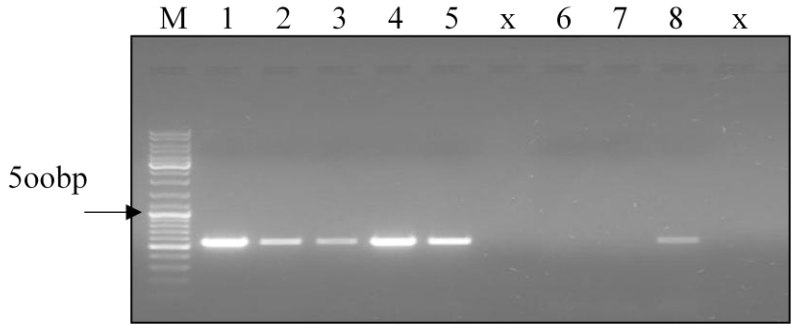


Figure 3-2: Representative ethidium bromide-stained 1 % agarose gel picture showing PCR amplification products for four aliquots of *Cryptosporidium* positive stool sample subjected to different lysis temperatures and for various durations. Amplicons of ~550 bp of the *cowp* gene sequence were generated using Cry-9/Cry-15 diagnostic primers. M, GeneRuler™ 100 bp DNA marker; Lane-1, DNA control sample extracted from purified oocysts suspension (for comparison); Lane-2, an aliquot of stool sample subjected to lysis at 97°C for 15 min; Lane-3, at 97°C for 20 min; Lane-4, at 100°C for 10 min; Lane-5, at 100°C for 15 min; Lane-6, *Cryptosporidium* negative stool sample (Extraction negative control); Lane-7, no-template master mix sample (PCR negative control); Lane-8, plasmid DNA sample (PCR positive control).

To gauge the efficiency of the boiling step on oocysts/cysts disruption, two *Cryptosporidium*- and two *Giardia*-positive stool samples were subjected to DNA

extraction following the QIAamp® DNA Stool Mini Kit (Qiagen) protocol with inclusion of the modifications mentioned above. After initial heating of the stool homogenate and the subsequent centrifugation, 20 µl of each cell lysate was mounted on a microscopic slide and examined by bright field microscope. Few oocysts/cysts with intact cell memberanes (0 – 3) were seen despite careful examination of the entire microscopic slide (see **Figure 3-3)**. Similarly, four microscopic slides were prepared, but this time, from the sedimented faecal pellet of each sample and examined by the bright field microscope. Few oocysts/cysts with intact cell memberanes (0 – 2) were also identified.

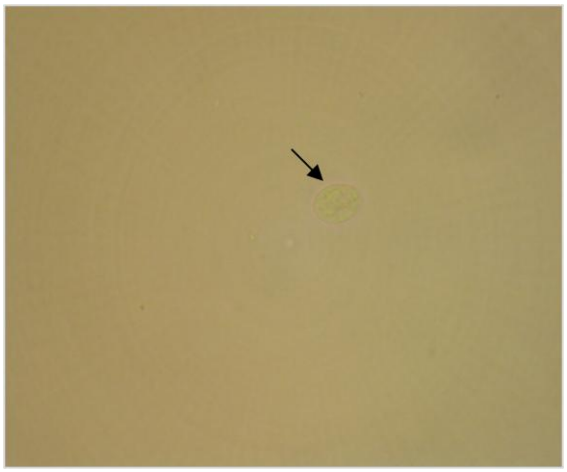


Figure 3-3: Microscopic picture showing a *G. lamblia* cyst with intact cell wall that was still present in cell lysate post the heating and sedimentation step (indicated by the black arrow)

To check the efficiency of using 50 – 100 µl elution buffer for recovering all the faecally-purified DNA samples from the spin column, a second elution step with another 50 µl elution buffer was adopted for a large batch of samples and subjected to amplification by the corresponding individual target-uniplex diagnostic and reference

PCRs. No amplification of the specified target DNA was shown in all DNA samples recovered by the second elution step.

3.2.3 Impact of prior mechanical disruption on the extraction protocol

To study the impact of prior oocysts purification and subsequent mechanical cell memberanes disruption on the modified DNA extraction protocol, two sets of extract material were prepared from five *Cryptosporidium* positive control stool samples. The first set was 200 µl whole stool aliquots directly sampled from the *Cryptosporidium* positive clinical samples. The second set of extract was 200 µl aliquots of purified and vigorously agitated oocysts suspension. Oocysts purification was carried out using salt flotationtechnique (see **Materials and Methods**, Section 2.3.4). Pre-purified oocysts suspensions were vigorously agitated with FastPrep® Instrument (Qbiogene). All aliquots were subjected to copro-DNA extraction using the QIAamp® DNA Stool Mini Kit (Qiagen) with the modified protocol as mentioned above and DNA amplification using the *Cryptosporidium* diagnostic standard PCR. Bands of the expected size (~550 bp) were detected on agarose gel from both sets of extracts with comparable results (see **Figure 3-4**). However, adoption of the oocysts purification and subsequent agitation steps increased the assay time by ~25 min.

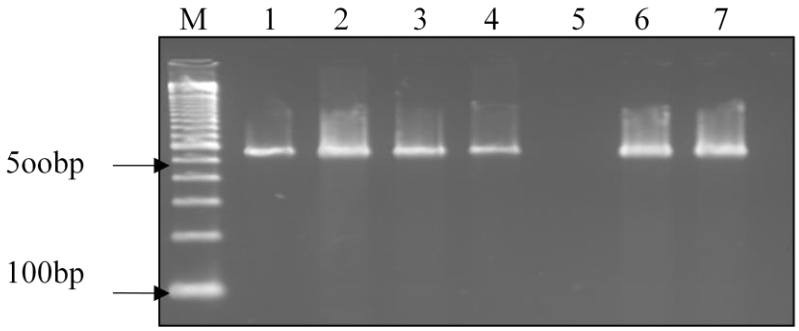


Figure 3-4: Representative ethidium bromide-stained 2 % agarose gel picture showing PCR amplification products for a *Cryptosporidium* positive stool samples subjected to DNA extraction by the modified Qiagen stool kit DNA extraction protocol using two different approaches. DNA fragments of ~550 bp of the *cowp* gene sequence were amplified using primers Cry-9/Cry-15. **M**: GeneRuler™ 100 bp DNA marker; Lane-1 & 3, aliquots of a *Cryptosporidium* stool samples subjected to direct DNA extraction; Lane-2 & 4; aliquots of the same *Cryptosporidium* stool samples subjected to oocysts purification and cell memberanes disruption prior to DNA extraction; Lane-5; a *Cryptosporidium* negative stool sample (extraction negative control); Lane-6; *Cryptosporidium hominis* crude DNA sample (PCR positive control); Lane-7; *Cryptosporidium parvum* crude DNA sample (PCR positive control).

3.2.4 Impact of prior freeze/thaw cycles on the extraction protocol

Similar to oocysts purification combined with mechanical agitation, exposing the whole stool extract to various rounds of freeze/thaw cycles has been frequently used prior to DNA extraction. Accordingly, the impact of this sample-processing procedure on the modified QIAamp® stool kit DNA extraction protocol was studied using the aforementioned *Cryptosporidium* positive control stool samples.

Two set of whole stool aliquots were prepared prior to DNA extraction. The first set was five aliquots, 200 µl each, prepared by direct sampling from the whole stool samples. The other set was another five aliquots prepared by direct sampling from the whole stool samples and exposed to six rounds of freeze/thaw cycles prior to DNA extraction. The freeze/thaw cycle was carried out by exposing stool extract (200 µl) to dry ice-ethanol bath and heating at 97˚C (1 min each). Faecal aliquots were then

subjected to DNA extraction using the modified QIAamp® DNA Stool Mini Kit (Qiagen) DNA extraction protocol and subsequently amplified using the *Cryptosporidium* diagnostic uniplex PCR.

Based on the intensity of the ethidium bromide-stained bands as seen on the agarose gel, the positive samples that were subjected to six rounds of prior freeze/thaw cycles showed more amplification products than samples that had not undergone this prior treatment (see **Figure 3-5**, lane 3 & 5). However, the freeze/thaw procedure increased the assay time by ~20 min and led to greater complexity.

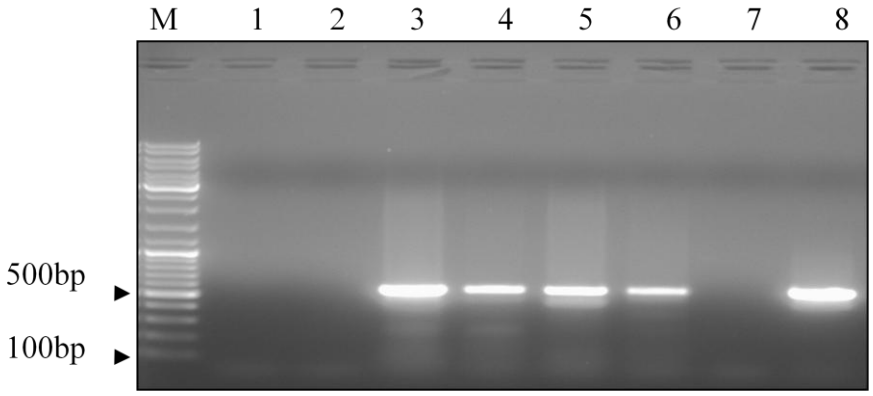


Figure 3-5: Representative ethidium bromide-stained 1 % agarose gel picture showing PCR amplification products for *Cryptosporidium* positive stool samples subjected to DNA extraction by the modified Qiagen stool kit DNA extraction protocol using two different approaches. Target DNA sequences of ~550 bp were amplified using primers Cry-9/Cry-15. **M**, GeneRuler™ 100 bp DNA marker; Lane-1 & 2, two aliquots of a *Cryptosporidium* negative stool sample subjected to direct DNA extraction; Lane-3 & 5, two aliquots of a *Cryptosporidium* positive stool sample subjected to six rounds of freeze-thaw cycles prior to DNA extraction; Lane-4 & 6, two aliquots of the same *Cryptosporidium* positive stool sample directly subjected to DNA extraction; Lane-7, no-template master mix sample (PCR negative control); Lane-8, plasmid DNA sample (PCR positive control).

Based on the optimisation experiments performed, all subsequent DNA extractions was carried out directly on stool samples using the QIAamp® Stool Mini Kit (Qiagen) DNA extraction protocol that incorporated the four modifications mentioned above.

Prior oocysts/cysts enrichment and freeze/thaw treatments were not included as part of the modified protocol developed.

3.3 Inhibitor-free faecally-derived DNA yield

To exclude any detectable inhibitory effects on DNA polymerase activity that may occur as a result of impurities present in the stool sample and co-extracted with the target DNA, the faecally-derived DNA samples, 50 µl each, were subjected to a series of experiments.

Initially, the effect of the faecally-derived DNA concentration on PCR amplification was assessed through the following experiment. Two aliquots of faecally-derived nucleic acid samples recovered from two *Giardia* positive stool samples were subjected to PCR amplification using the *G. lamblia* diagnostic PCR. Variable concentrations of the DNA samples were used as template for amplifications in a fixed PCR reaction volume of 20 µl. The *G. lamblia* specific DNA sequence (~455 bp) was successfully amplified from all concentrations tested (see **Figure 3-6**).

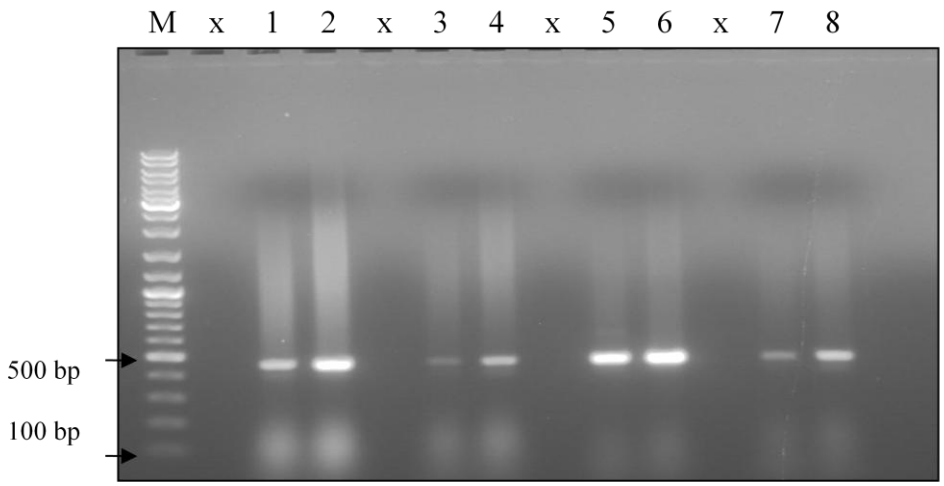


Figure 3-6: Representative ethidium bromide-stained 1 % agarose gel picture showing the *G. lamblia* diagnostic PCR amplification products for two *G. lamblia* crude DNA samples recovered by the modified Qiagen stool kit DNA extraction protocol. Amplification bands of ~450 bp of the *gdh* gene sequence were amplified using primers GDHeF/GDHiR in 20 µl PCR volume. **M**, GeneRuler™ 100 bp DNA marker; Lane-1 & 2, amplification products of 5 µl of the faecally-derived DNA; Lane-3 & 4, amplification products of 3 µl of the faecally-derived DNA; Lane-5 & 6, amplification products of 1 µl of the faecally-derived DNA Lane-7 & 8, amplification products of 0.5 µl of the faecally-derived DNA.

To investigate the presence of any inhibitory effect of the impurities present in the stool specimen on the sensitivity of the diagnostic PCR assay developed in parallel (see **Chapter-5** for more details), a second experiment was carried out. Two aliquot of faecally-derived nucleic acid samples recovered from *E. histolytica* and *G. lamblia* negative control samples were spiked with high concentrations of the corresponding target-matching plasmid or genomic control DNA samples. Decimal (i.e., ten-fold) serial dilutions were prepared from the original stocks using the matching faecally-derived target DNA-negative nucleic acid samples as a diluent. All dilutions were subjected to PCR amplification with the corresponding standard diagnostic assay. All dilutions showed the expected amplification bands down to the lower detection limits of the corresponding diagnostic PCR assay (**Figure 3-7**).

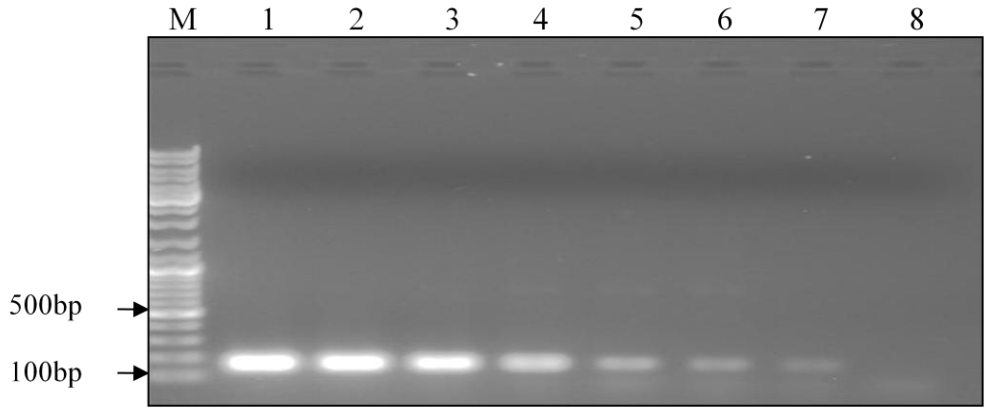


Figure 3-7: Representative ethidium bromide-stained 2 % agarose gel picture showing PCR amplification products for *E. histolytica*-negative stool sample extracted by the modified Qiagen® stool kit DNA extraction protocol and spiked with the positive control plasmid DNA (pCR4®-TOPO:: EC167). Amplification products of ~170 bp of the 18S rDNA gene sequence were amplified using primers EntaF/EhR. **M**, GeneRuler™ 100 bp DNA marker; Lane-1 to 8, an aliquot of the faecally-derived DNA sample spiked with the plasmid DNA and serially diluted to give the following concentration; 30 pg, 3 pg, 0.3 pg, 30 fg, 3 fg, 0.3 fg and 0.03 fg/µl respectively. The *E. histolytica* diagnostic PCR assay was able to detect 0.03 fg of the recombinant plasmid DNA (the same detection limit reported using the same serial dilutions of pure recombinant plasmid control DNA).

A third experiment was performed to rule out the presence of inhibitory effects in the faecally-derived DNA samples on the subsequent PCR amplification. In this experiment, a target-matching internal amplification control (IAC) was included with previously tested PCR-negative faecally-extracted DNA samples in a duplex PCR reaction to be amplified in the same PCR tube and under the same cycling conditions (see **Chapter-4** for more details). The internal control sequence was solely amplified in all negative samples. In other experiments, IAC was included with the target-matching faecally-derived positive DNA samples in one tube and subsequently amplified with the corresponding diagnostic PCR assay. The internal control was successfully detected in all samples tested except for ~10 samples with high target parasite loads. The negative IAC amplification in these instances was probably due to excessive direct target competition **(Figure 3-8)**.

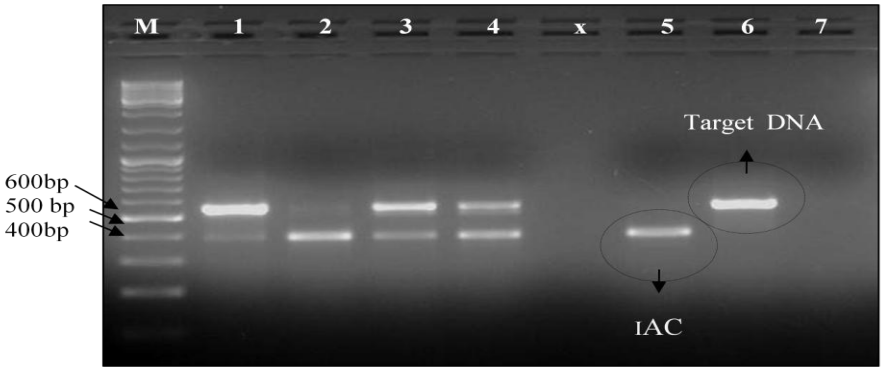


Figure 3-8: Representative ethidium bromide-stained 1 % agarose gel picture showing the *Cryptosporidium* diagnostic PCR assay amplification products in the presence of the internal amplification control (pGEM®-T Easy::IC375). **M**, GeneRuler™ 100 bp DNA marker; Lane-1 to 4, amplification products of four *Cryptosporidium* positive stool samples with variable parasite load showed the amplification products of the target DNA sequence (~550 bp) and the non-target internal control DNA (~370 bp); Lane-5, a *Cryptosporidium* negative clinical sample showed the PCR product of the internal control only; Lane-6, plasmid DNA sample (PCR positive control); Lane-7, no-template master mix sample (PCR negative control).

Finally, to test the effects of impurities present in stool on the integrity of the recovered DNA samples, DNA sequences of molecular weights higher than those targeted by the diagnostic PCR assays, were targeted for amplification using two reference PCRs. The *Cryptosporidium* reference PCR targeted ~1,325 bp of the 18S rDNA gene using primers X1F/X1R (Xiao *et al*., 1999) while the *E. histolytica* reference PCR targeted ~890 bp of the 18S rDNA gene using primers E-1/E-2 (Khairnar and Parija, 2007). Bands of the expected size for both PCRs were successfully amplified and detected on agarose gel from all positive samples (see **Figure 3-9**).

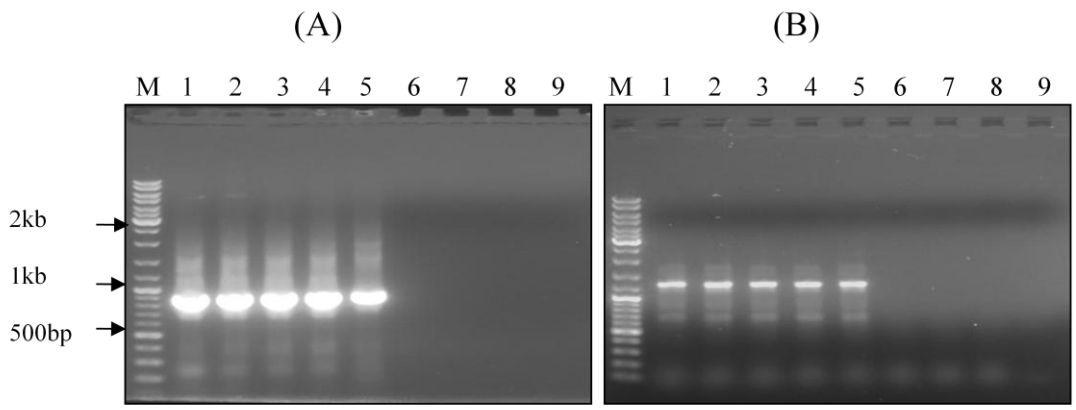


Figure 3-9: Representative ethidium bromide-stained 0.8 % agarose gel picture showing PCR amplification products of faecally-derived DNA samples extracted by the modified Qiagen stool kit DNA extraction protocol. PCR amplicons of ~1.3 Kb and ~890 bp were detected on gel. **(A)**, Amplification products of the *E. histolytica* reference PCR (~890 bp); Lane-1 to 5, *E. histolytica* positive stool samples; Lane-6 to 9, *E. histolytica* negative stool samples; **(B)**, Amplification products of the *Cryptosporidium* reference PCR (~1,3 Kb); Lane-1 to 5, *Cryptosporidium* positive stool samples; Lane-6 to 9, *Cryptosporidium* negative stool samples; **M**, GeneRuler™ 100 bp DNA marker.

3.4 Discussion

In this study, an extraction protocol based on QIAamp® DNA Stool Mini Kit was developed for protozoal DNA extraction directly from the diarrheic stool specimens. The protocol developed proved to be simple and economical as it did not require hazardous reagents such as phenol, or additional preparatory steps such as concentration techniques or application of mechanical force using instruments such as Fast Prep disruptor or Mini Beadbeater for oocysts/cysts disruption. The small volume of sample subjected to extraction (200 µl) allowed the extraction procedure to be carried out at 1 – 2 ml scale, hence permitting the use of inexpensive table-top microfuges and heating blocks.

In the initial optimization experiments, more attention was paid to the *Cryptosporidium* than to *G. lamblia* and *E. histolytica* as a target for DNA extraction because

Cryptosporidium oocysts have more robust cell memberanes. An extraction protocol which worked effectively with *Cryptosporidium* was predicted to also be effective for the other two protozoa. This assumption was supported in the literature describing the successfully use of the QIAamp® DNA Stool Mini Kit for extraction of *E. histolytica* and *G. lamblia* DNA directly from stool samples (Fotedar *et al*., 2007; Gonin and Trudel, 2002).

The cause(s) of amplification failure with the six initial *Cryptosporidium* positive samples subjected to the standard manufacturer's DNA extraction protocol remains speculative. Failure of amplification of target DNA from faecally-derived positive stool samples may be due to many factors. Factors related to the PCR assay such as low sensitivity of the assay or inhibition of the reaction by impurities present in the stool samples and co-purified with the target DNA were considered. Factors related to the extraction procedure such as inefficient nucleic acid isolation or purification were also considered. At this early stage of the study, two experiments were carried out to rule out PCR inhibition. PCR amplification of the 16S rDNA using two broad bacterial universal primers demonstrated that sufficient amounts of bacteria DNA was extracted using the protocol and this DNA was successfully amplified using the 16S rDNA primers. However, interpretation of these data is highly problematic as faecal samples contain massive numbers of bacteria, potentially yielding very high loads of bacterial 16S rDNA that are likely to exceed by many orders the quantities of protozoal DNA in the samples. Dilution of nucleic acid samples prior to PCR amplification did not change the results. Dilution of the nucleic acid sample can be useful in decreasing the burden of potential inhibitory substances on the *Taq* polymerase, but this procedure decreases the amount of the target DNA present in each PCR assay.

With the exception of the aforementioned six samples processed by the unmodified protocol, all positive samples were successfully extracted by the QIAamp® DNA Stool Mini Kit following the modified protocol developed in this study and amplified using corresponding matching PCR assays. A series of optimisation experiments were performed in an attempt to increase the DNA yield. Firstly, to facilitate the isolation of genetic material enclosed inside very robust cell memberanes, the lysis temperature was raised to boiling point for 10 minutes. Secondly, increasing the incubation time of the InhibitEX tablet step to five minutes was purposed to allow for enhanced adsorption of the DNA-damaging substances and PCR inhibitors present in faecal specimens. The use of pre-cooled ethanol for nucleic acid precipitation appeared to improve yields but no obvious explanation was apparent. Finally, use of the smaller elution volume (50 – 100 µl) without any apparent loss of elution efficiency, allowed for concentration of the final DNA sample by 2 – 4 fold.

The optimised protocol was tried on purified oocysts suspension and on stool samples exposed to six rounds of freeze-thaw cycles as previously reported in other studies (Guy *et al.,* 2004 and da Silva *et al.,* 1999). Based on the intensities of the ethidium bromide-stained DNA bands on the agarose gel, the results were comparable to those obtained from PCR amplification of DNA recovered directly from the whole stool samples. Use of freeze-thaw procedure appeared to enhance yields slightly but the diagnostic significance of apparent refinement remains to be explored. Importantly, the two additional preparatory steps added more time and costs to the extraction procedure without clear evidence of substantial gains. As such neither was included as part of the final developed QIAamp® DNA Stool Mini Kit modified DNA extraction procedure.

With faecally-derived DNA, the ratio of target DNA to background host-, food-, fungal- and prokaryote-derived DNA is often very low. It therefore appears that, the direct estimation of the DNA concentration present in the crude DNA samples was found to be minimally helpful. Accordingly, the concentration of the target DNA was indirectly estimated by analysis of its amplification product with agarose gel electrophoresis. Similarly, to exclude the presence of DNA-damaging substances and PCR inhibitors present in the DNA yield, a series of experiments were carried out. The faecally-derived DNA samples showed amplification of the specific target gene sequence in all of these experiments demonstrating that the QIAamp® DNA Stool Mini Kit effectively removed faecal impurities that can inhibit amplification or degrade DNA. These results are in line with previously published studies (e.g., Zaki *et al*., 2003 and Abbaszadegan *et al*., 2007).

In summary, on the basis of this evaluation, the QIAamp® DNA Stool Mini Kit (Qiagen), with the modifications introduced over the manufacturer's protocol, proved to be a very useful tool for protozoal DNA extraction directly from stool samples. The developed nucleic acid extraction procedure was carried out using a simple, relatively cheap and instrument non-intensive protocol. Critically, the DNA samples obtained supported the adequacy of this approach for subsequent sensitive faecal protozoal PCR diagnostic assays. The faecally-derived DNA obtained was perfectly compatible with clinical diagnostic PCR assays that were developed in parallel.

4 Construction and validation of PCR amplification controls

4.1 Introduction

The PCR is an extremely sensitive technique. Therefore it is very prone to contamination which can cause false-positive results. In addition, the different constituents in stool specimens can inhibit PCR assay causing false-negative results. Both of these diagnostic errors can lead to significant negative impacts on patient management. To ensure that diagnostic PCR assays are precise, these PCR assays should be properly controlled. Accordingly, general measures such as primers and reagents validation, use of physical containment facilities and/or separate rooms, duplicate testing and inclusion of multiple no-template controls should be taken to minimize the risk of contamination. Besides these measures, negative and positive standard controls have to be included in the diagnostic assay to monitor precision and accuracy of results.

In this chapter, two sets of plasmids were designed and constructed to monitor individual PCR assays. Each plasmid of the first set of plasmids comprises a cloning vector carrying the entire target DNA sequence within its multiple cloning site (MCS); these plasmids were used as external amplification controls (EACs). An EAC, similar to target DNA, is amplified by the same primers and with the same PCR protocol in a separate parallel test tube. In most cases, failure of amplification of an EAC requires re-testing of samples. Each plasmid of the second set of plasmids is a cloning vector harboring a modified target DNA sequence in its MCS; these plasmids were used as internal amplification controls (IACs). Modification of the target DNA sequence was carried out through deleting a certain DNA fragment or inserting a foreign DNA

fragment between the primer flanking sites. The IAC was amplified with the target DNA in one tube using the same primers and the same PCR protocol. In most cases, failure of IAC amplification indicates PCR inhibition and the primary test sample should be subjected to fresh DNA extraction and re-tested. Both the IAC and the target amplicons are clearly distinguishable by size on standard gel electrophoresis.

Several IAC methods have been adopted in previous studies. In this study, a strategy based on competitive PCR was employed (Siebert and Larrick 1992). Moreover, different procedures have been used previously for construction of competitive IAC substrates (Abdulmawjood *et al*., 2002 and Cubero *et al*., 2002). However, in this study, a new strategy based on inverse PCR and restriction digestion methods was used.

4.2 Construction of external amplification controls (EACs)

C. hominis (W16954), *G. lamblia* (WB) and *E. histolytica* (HM-1: IMSS) genomic DNA control samples were amplified using the corresponding individual target-uniplex diagnostic PCR assay developed in this study (**Chapter-5**). Amplicons of the expected sizes were excised from the agarose gel, purified using YORBIO Gel/PCR DNA Purification Kit (Yorkshire Bioscience) and subsequently cloned into the MCS of a cloning vector (see **Materials and Methods**, Section 2.3.9 for more details). The *Cryptosporidium cowp* target gene sequence (~550 bp) was cloned into the pGEM®-T-Easy vector (Invitrogen) while the *G. lamblia gdh* target DNA (~455 bp) and the *E. histolytica* 18S rDNA gene sequence (~170 bp) was cloned into the pCR®4-TOPO cloning vector (Invitrogen). Ligation reactions were transformed into either *E.coli* Top10 or *E.coli* DH5α chemical competent cells. ~500 white colonies per plate were obtained after overnight incubation and 10 randomly selected white colonies

were screened for the presence of the correct plasmid by colony PCR (see **Figure 4-1**).

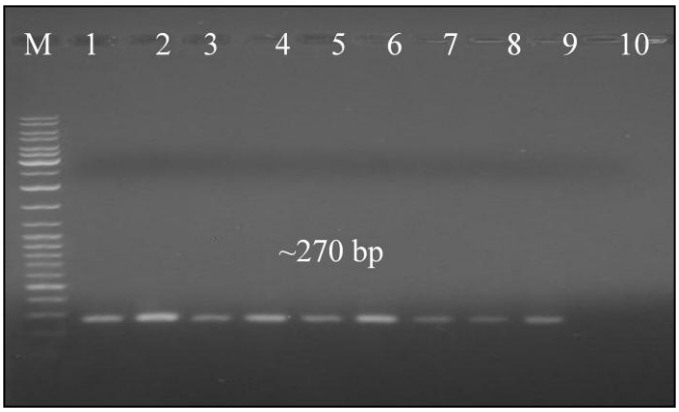


Figure 4-1: Representative ethidium bromide-stained 2 % agarose gel picture showing the colony PCR amplification products using T3/T7 plasmid primers and transformed colonies containing plasmid DNA (pCR4®-TOPO:: EC 167) as templates; **M**, GeneRuler™ 100 bp DNA marker; Lane-1 to 9; nine white colonies containing the correct plasmid; Lane-10, no-template master mix sample (PCR-negative control).

Inserts verification was carried out by *Eco*RI (New England Biolab) restriction digestion (see **Figure 4-2**). A single candidate recombinant plasmid for each of the EACs sought was sent for DNA sequencing and the retrieved DNA sequences confirmed 99 % identity with sequences stored in the GenBank for the original strains. Glycerol stocks of the *E. coli* host strains carrying each EAC plasmid were prepared, carefully labeled and stored at −80°C for future use. Each plasmid was given a name and recorded in our laboratory plasmid database with its important details and features (see **Table 4-1**).

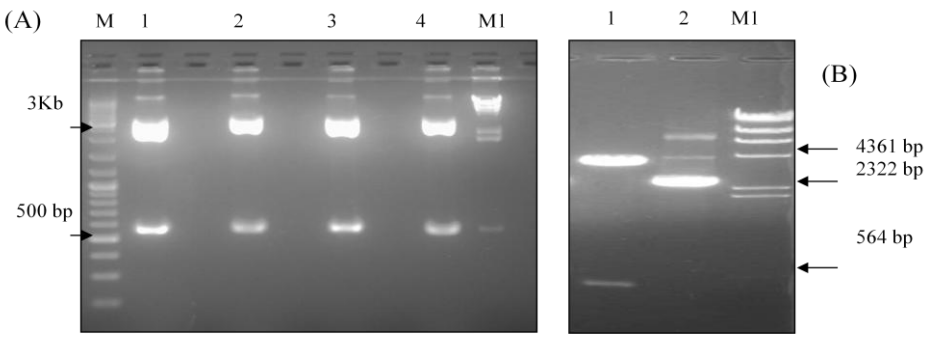


Figure 4-2: Representative ethidium bromide-stained 0.8 % gel picture showing products of *Eco*RI restriction digestion for two prepared plasmid DNA samples. **(A)** restriction digestion of pGEM®-T Easy::EC553(EAC constructed for the *Cryptosporidium* diagnostic PCR assay); **M**, GeneRuler™ 100 bp DNA marker; Lane-1 to 4; restriction digestion product of 5µl of plasmid DNA; **(B)** restriction digestion of pCR4®-TOPO:: EC 167 (EAC constructed for the *E. histolytica* diagnostic PCR assay); Lane-1, the digestion product of 5µl of the recombinant plasmid DNA; Lane-2, 5µl of an intact (undigested) plasmid; **M1**, λ *Hin*dIII DNA marker.

Table 4-1: The External Amplification Controls (EACs).

Important features	pGEM®-T Easy:: EC553	pCR4®-TOPO:: EC455	pCR4®-TOPO:: EC167
The diagnostic PCR assay:	*Cryptosporidium* spp. PCR assay	*G. lamblia* PCR assay	*E. histolytica* PCR assay
<u>Construction:</u>			
Cloned gene	*cowp*	*gdh*	18S rDNA
PCR primers	Cry-9/Cry-15	GDHeF/GDHiR	EntaF/EhR
Molecular weight	~ 550 bp	~ 455 bp	~170 bp
Cloning Vector	pGEM®-T-Easy (Invitrogen)	pCR4®-TOPO	pCR4®-TOPO (Invitrogen)
<u>Storage:</u>			
E. Coli strain	DH5α	Top10	Top10
<u>Important features:</u>			
Antibiotic resistance	ampicillin/erythromycin	ampicillin	ampicillin
Plasmid length	~3.5 kb	~4.4 kb	~4 kb
*Eco*RI digestion	~570 bp + 3 Kb (1: 6)	~ 475 bp + 3.9 Kb (1: 9)	~195 bp + 3.9 kb (1:19)
Insert copy number per 1 ng of plasmid DNA[1]	~2.65 x 10^8	~2.11 x 10^8	~2.32 x 10^8

[1]The copy number of the construct per 1 ng of plasmid DNA was calculated using this equation (Whelan *et al*., 2003):

$$\text{DNA (copy)} = \frac{6.02 \times 10^{23}(\text{copy/mol}) \times \text{DNA amount(g)}}{\text{DNA length(bp)} \times 660(\text{g/mol/bp})}$$

4.3 Construction of competitive internal amplification controls (IACs)

The previously constructed EACs were used to develop a matching set of internal amplification controls for the corresponding diagnostic PCR assay. Steps adopted in construction of different IACs are outlined in **Figure 4-3.**

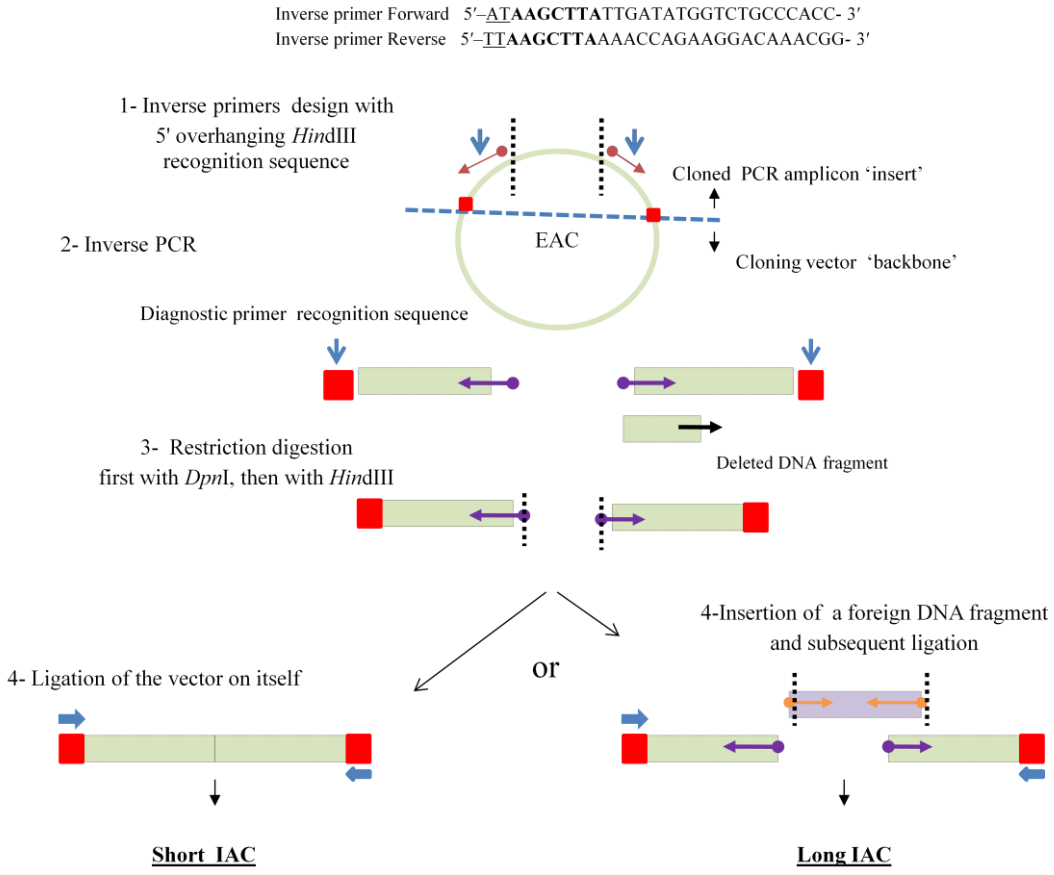


Figure 4-3: A schematic diagram showing the different steps adopted in IACs construction. Further details are provided in the main text.

Initially, using the full sequence of the recombinant plasmids, all the shared and unique restriction sites between each insert and the corresponding cloning vector were

searched for, identified and characterized using the free online web tool: (http://tools.neb.com/NEBcutter2/index.php). No unique restriction site for any of the three inserts was found. Therefore, introduction of a unique restriction site inside the DNA sequence of each insert was proposed. The method adopted for introducing restriction site is called inverse PCR. In inverse PCR, the primers are pointing away from each other and in the process amplify both the defined parts of the target sequence and the cloning vector backbone. In addition, a restriction enzyme cut-site sequence was introduced at each end of the amplified plasmid sequence by incorporation of the cut-site sequence towards the 5′ends of both primers. The *Hin*dIII restriction enzyme sequence was introduced into inverse PCR amplicons generated from each of the EAC plasmids following the same principal. Three sets of inverse PCR primers were manually designed from the retrieved sequence of the inserts in each EAC plasmid (see **Materials and Methods**, Table 2-2). The *Hin*dIII recognition sequence plus an additional 2 – 3 nucleotide bases were introduced in each primer sequence at the 5′ terminus. Each inverse PCR reaction was carried out using 10 µM of each inverse primer in the presence of 1 µl of the recombinant DNA (EAC), 1X Go Taq® green buffer (Promega), 0.2 mM of each dNTP (Bioline), 1.5 mM $MgCl_2$, 1 % DMSO (Sigma) and 1 Uof GoTaq® HotStart polymerase (Promega) as final concentrations in 50 µl volumes. PCR reactions were completed using the following temperature cycling protocol:

The protocol was started with a pre-heating step for 4 min at 94°C, and followed by 40 cycles of 30 sec at 94°C, 30 sec in a gradient block at 56 – 58°C and 4.30 min at 72°C. A final extension step of 7 – 10 min at 72°C was included. All the products of amplifications were loaded on ethidium bromide-stained 0.8 % agarose gel prior to gel extraction (**Figure 4-4 A**). As a result of the three inverse PCRs, a specified DNA

sequence was deleted from each construct as follows: DNA sequences of ~210 bp, ~155 bp and ~125 bp were deleted from the pGEM®-T Easy:: EC553, pCR4®-TOPO:: EC455 and pCR4®-TOPO:: EC 167, respectively. Each inverse PCR amplicon has *Hin*dIII recognition sequences located very close to both ends of the amplicons. Amplicons of the expected size were cut out and purified from gel using YORBIO Gel/PCR DNA Purification Kit (Yorkshire Bioscience, UK). Purified DNA samples were subjected to restriction digestion first with *Dpn*I (New England Biolab) and then by *Hin*dIII restriction endonuclease (Roche) **(**see **Figure 4-4 B**).

As a result of restriction digestion reactions, three linearized fragments with sticky *Hin*dIII restriction ends were produced. According to the proposed lengths of the desired IAC constructs, one of two ways was chosen as follows:

4.3.1 Construction of short competitive IACs

This type of IAC was constructed for *Cryptosporidium* spp. and *G. lamblia* diagnostic PCR assay. The inverse PCR-linearized truncated pGEM®-T Easy::EC553 ‘fragment’ was re-ligated on itself creating a new construct with a target insert of ~375 bp with flanking sequence for the Cry-9 and Cry-15 diagnostic primers at each end. Similarly, the inverse PCR-linearized truncated pCR4®-TOPO:: EC455 ‘fragment’ was re-ligated on itself creating a new construct with a target insert of ~300 bp with flanking sequence for the GDHeF and GDHiR diagnostic primers at each end.

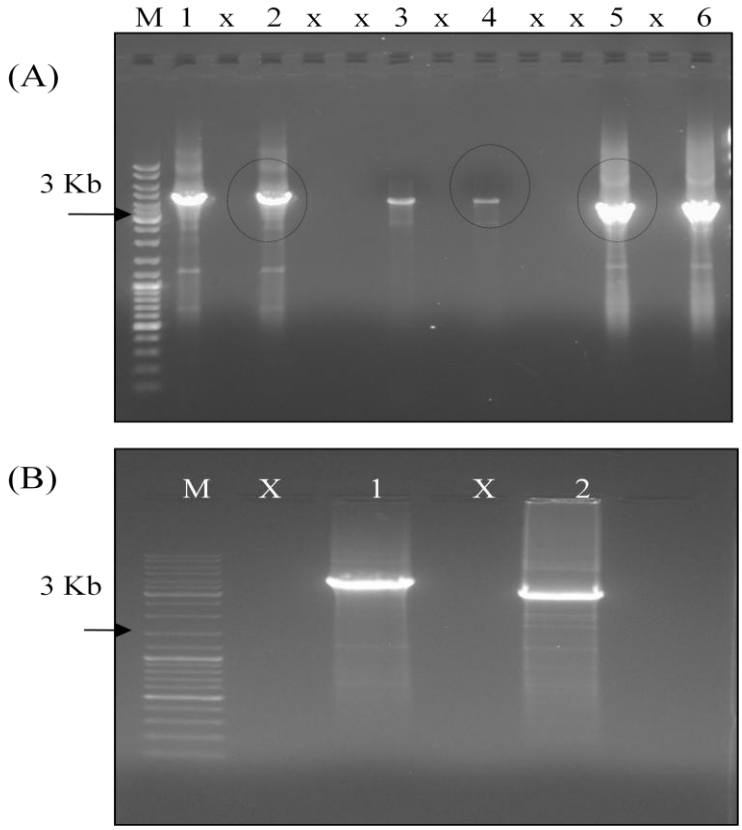


Figure 4-4: (A) Representative ethidium bromide-stained 0.8 % agarose gel picture showing products of inverse PCRs; Three inverse PCRs were individually applied on the corresponding EAC using target-matching inverse primers; Two annealing temperatures in a gradient block thermal cycler were used for each primer pair; Lane-1 & 2, pCR4®-TOPO:: EC455 (EAC of the *G. lamblia* diagnostic PCR assay) used as template for the first inverse PCR; Lane-3 & 4, pCR4®-TOPO:: EC 167 (EAC of the *E. histolytica* diagnostic PCR assay) as template for the second inverse PCR; Lane-5 & 6, pGEM®-T Easy:: EC553 (EAC of the *Cryptosporidium* diagnostic PCR assay) as template for the third inverse PCR, **M**, GeneRuler™ 100 bp DNA marker; Lane-1 & 3& 5, annealing temperature of 56°C; Lane-2 & 4 & 6 annealing temperature of 58°C. **(B)** Representative 1% agarose gel picture showing products of two successive restriction digestions, first with *Dpn*I and then with *Hin*dIII restriction enzymes as a preparatory steps prior to ligation. **M,** GeneRuler™ 100 bp DNA marker; Lane-1, pCR4®-TOPO:: EC455 inverse PCR products ; Lane-2, pGEM®-T Easy::EC553 inverse PCR.

4.3.2 Construction of long competitive IAC

This type of IAC was prepared for *E. histolytica* diagnostic PCR assay. Instead of ligating the linearized truncated EAC vector-derived fragment on itself, a foreign DNA

segment of ~300 bp bearing *Hin*dIII ends was prepared and ligated to the linearized truncated pCR4®-TOPO:: EC 167 'fragment'. As a result, a new construct with a target insert of ~375 bp carrying the DNA sequence for the EntaF and EhR diagnostic primers at each end was produced (see **Figure 4-5**). This approach was taken as the native *E. histolytica* amplicon was already very short and further truncation would have rendered resolution on conventional agarose gels problematic.

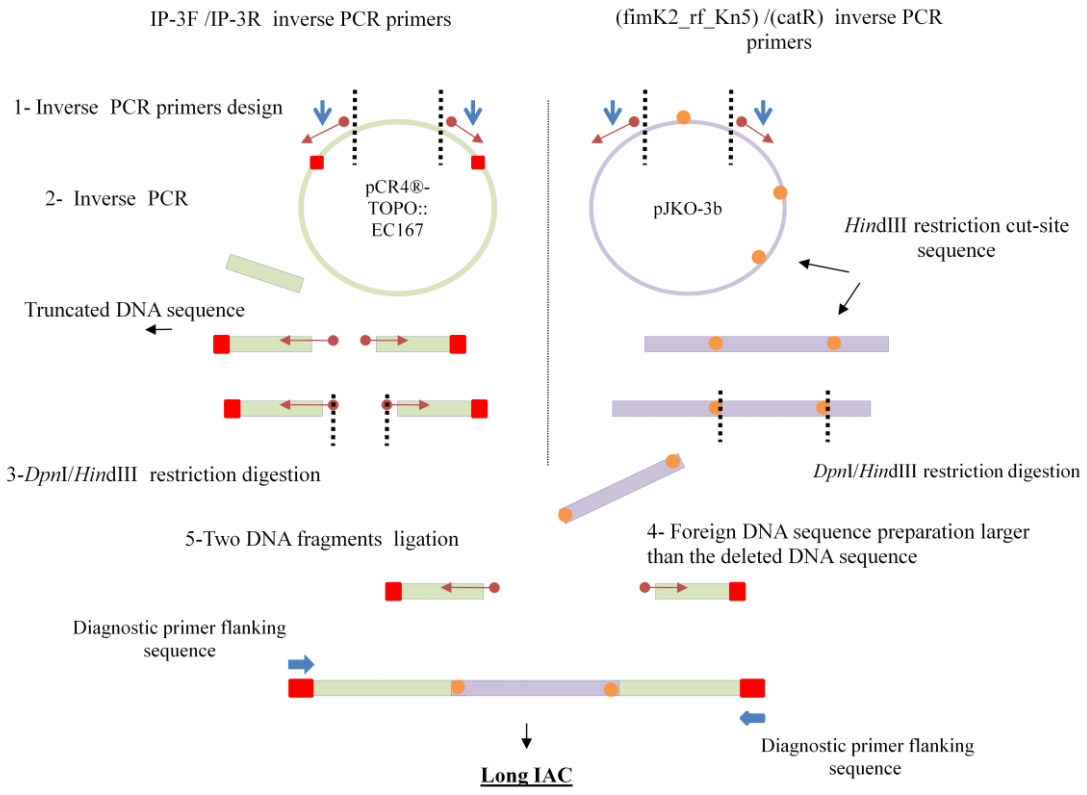


Figure 4-5: Schematic diagram showing strategy adopted for construction of the long competitive IAC. See main text for further details.

Plasmid (pJKO-3b), one of the plasmids constructed in our laboratory, has three sites for *Hin*dIII restriction endonuclease. Therefore, it was used as a source for the foreign DNA fragment. Similar to the truncated EAC vector-derived fragment, the foreign

DNA fragment was prepared as mentioned above through an inverse PCR reaction followed by restriction digestion first with *Dpn*I and then by *Hin*dIII restriction endonuclease. Inverse PCR was carried out to amplify ~4 kb fragment bearing two *Hin*dIII restriction sites from pJKO-3b (Figure 4-6A). Inverse PCR reaction was done using reagents-final concentration and thermal cycling conditions similar to that adopted for the aforementioned inverse PCR reactions but with forward primer catR 5′ and reverse primer fimK2_rf_Kn5 (see **Materials and Methods**, Table 2.2). As a result of restriction digestion, a fragment of ~300 bp with sticky *Hin*dIII restriction ends was produced. The specified fragment was purified from gel and ligated with the linearized pCR4®-TOPO:: EC 167 (**Figure 4-6 B & C**).

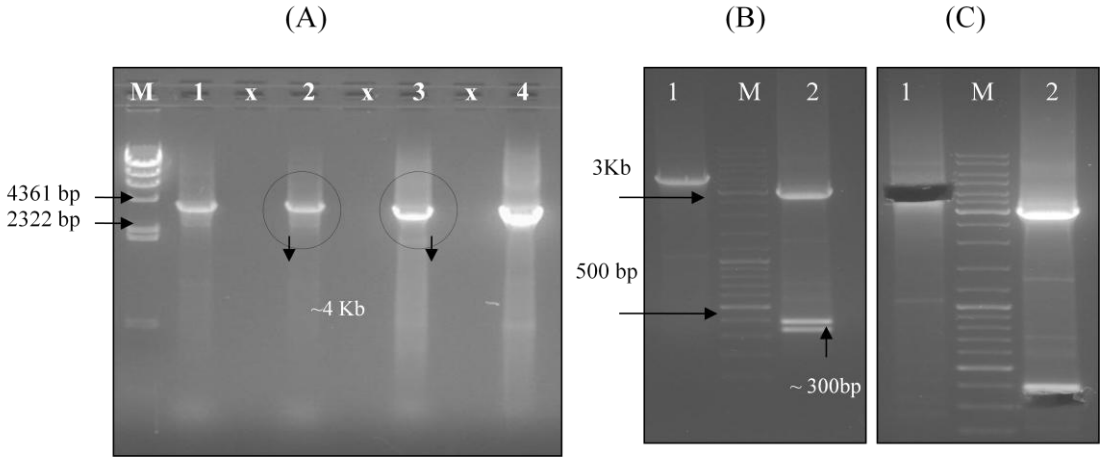


Figure 4-6: **(A)** Representative ethidium bromide-stained 0.8 % agarose gel picture showing amplification products of two inverse PCRs. Lane 1 & 2, inverse PCR amplification product of pCR4®-TOPO:: EC167 (EAC of the *E. histolytica* diagnostic PCR assay); Lane 3 & 4, inverse PCR amplification product of pJKO-3b (source of the foreign DNA fragment); **M**, λ *Hin*dIII DNA marker; **(B)** Representative ethidium bromide-stained 2 % gel picture showing products of digestion with *Hin*dIII restriction enzyme for two DNA fragments; Lane 1, pCR4®-TOPO:: EC167 inverse PCR amplification product (~4 kb); Lane 2, pJKO-3b inverse PCR amplification product; **M**, GeneRuler™ 100 bp DNA marker; **(C)** the same gel showing the excised foreign DNA fragment (~300 bp) and the prepared inverse PCR amplicon (~4 kb) for subsequent ligation and long IAC construction.

4.3.3 Storage of the constructed IACs

Short and long competitive IACs were transformed into chemically competent *E.coli* DH5α cells and selected with ampicillin. Approximately ten ampicillin resistant

colonies were screened for the presence of the desired new construct by colony PCR using the corresponding diagnostic PCR primers (**Figure 4-7**). Finally, glycerol stocks of host strains carrying each plasmid were prepared from the correct clones, carefully labeled and stored at −80°C for future use. Each host strain and plasmid was given a name and recorded in our laboratory strain and plasmid databases with important features detailed (**Table 4-2**).

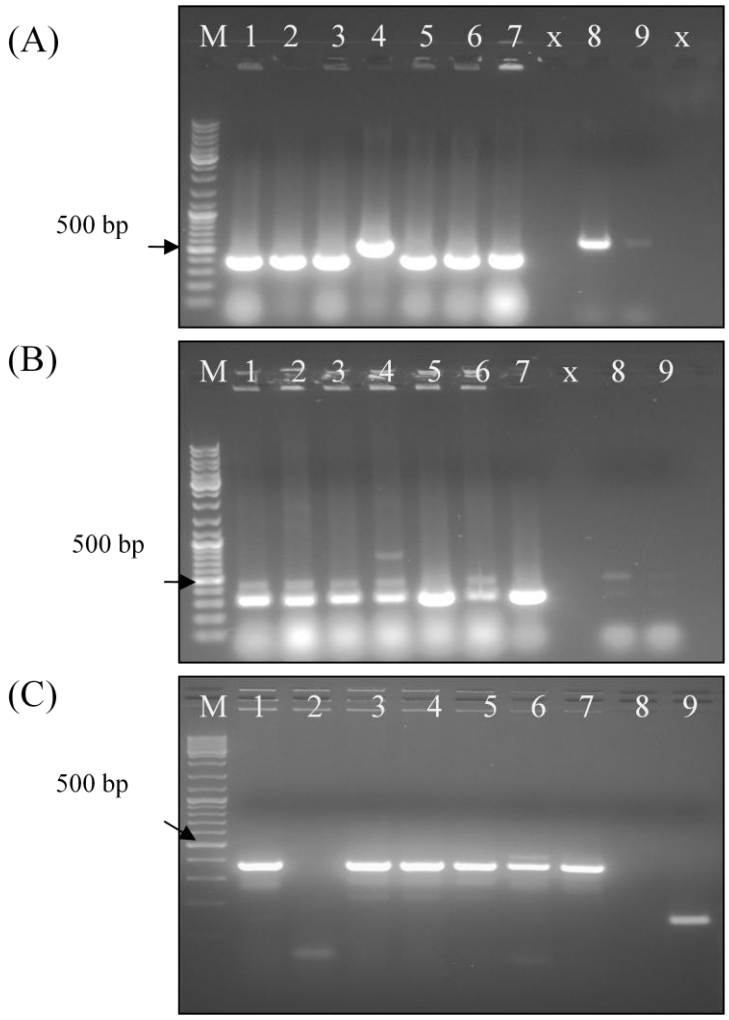


Figure 4-7: Representative ethidium bromide-stained 1.5 % agarose gel pictures showing amplification products of colony PCRs; **(A)** Screening transformants for pGEM®-T Easy::IC375; Lane 4, 8 & 9, transformants with incorrect IAC insert; Lane 1,2,3,5,6 & 7, transformants with the correct IAC insert (~375 bp); **(B)** Screening transformants for pCR4®-TOPO:: IC300 ; Lane 1 to 7, PCR products of transformants with the right IAC insert (~300 bp); Lane 8-9, transformants with the incorrect plasmid; **(C)** Screening transformants for pCR4®-TOPO:: IC375; Lane 1,3,4,5,6,7, PCR products of transformants with the right IAC insert (~375 bp), Lane 2, transformants with the incorrect plasmid; Lane-8, no-template master mix (PCR negative control); Lane 9, PCR positive control (~170 bp); **M**, GeneRuler™ 100 bp DNA marker.

Table 4-2: The competitive Internal Amplification Controls (IACs).

Features	pGEM®-T Easy:: IC375	pCR4®-TOPO:: IC300	pCR4®-TOPO:: IC375
Diagnostic PCR assay:	*Cryptosporidium* spp. PCR	*G. lamblia* PCR	*E. histolytica* PCR
Construction:			
Original plasmid	pGEM®-T Easy::EC553	pCR4®-TOPO:: EC455	pCR4®-TOPO:: EC167
Original insert length	~550 bp	~455 bp	~170 bp
Inverse Primers	IP-1F/R	IP-2F/R	IP-3F/R
Inverse PCR amplicon	~3.4 kb	~4.2 kb	~4 kb
Deleted sequence	~210 bp	~150 bp	~125 bp
Inserted sequence	none	none	~300 bp
IAC length	~375 bp	~300 bp	~375 bp
Storage:			
E. Coli strain	DH5α	DH5α	DH5α
Important features:			
Antibiotic resistance	ampicillin/erythromycin	ampicillin	ampicillin
New plasmid length	~3.4 kb	~4 kb	~4.3 Kb
*Hin*dIII digestion	One band of ~3.4 kb	One band of ~4 kb	~370 bp/ ~ 3.9Kb (1:10)
Insert copy number per 1 ng of plasmid DNA[1]	~2.6 x10^8	~2.1 x 10^8	~2.6 x 10^8 copies

[1]the copy number of construct present in 1 ng of plasmid was calculated following the equation mentioned previously (see it at the footnote of **Table 4-1**)

4.3.4 Estimation of the optimal concentration of each IAC

To estimate the quantity of each IAC to be added to the faecally-drived DNA samples, the following experiments were carried out. Plasmid DNA was purified and prepared using the alkaline lysis method (see **Materials and Methods**, Section 2.3.10). Approximately 5 µl of each plasmid DNA was digested by *Eco*RI restriction enzyme (New England Biolab). Plasmid DNA was roughly quantified on agarose gel using the known mass of the individual bands of the λ-*Hin*dIII molecular DNA marker (Fermentas) loaded in parallel with the digests as an approximate calibrator.

Ten-fold serial dilutions of 1 ng of each IAC plasmid DNA were prepared down to concentration of 0.1 fg/µl. The EAC plasmid DNA of the corresponding diagnostic assay with concentration equal to the lower detection limit was included with 1µl of each dilution in a duplex PCR. The optimum concentration of IAC was defined as the lowest dilution consistently detectable with the target amplicon on ethidium bromide-stained agarose gel (see **Figure 4-8**).

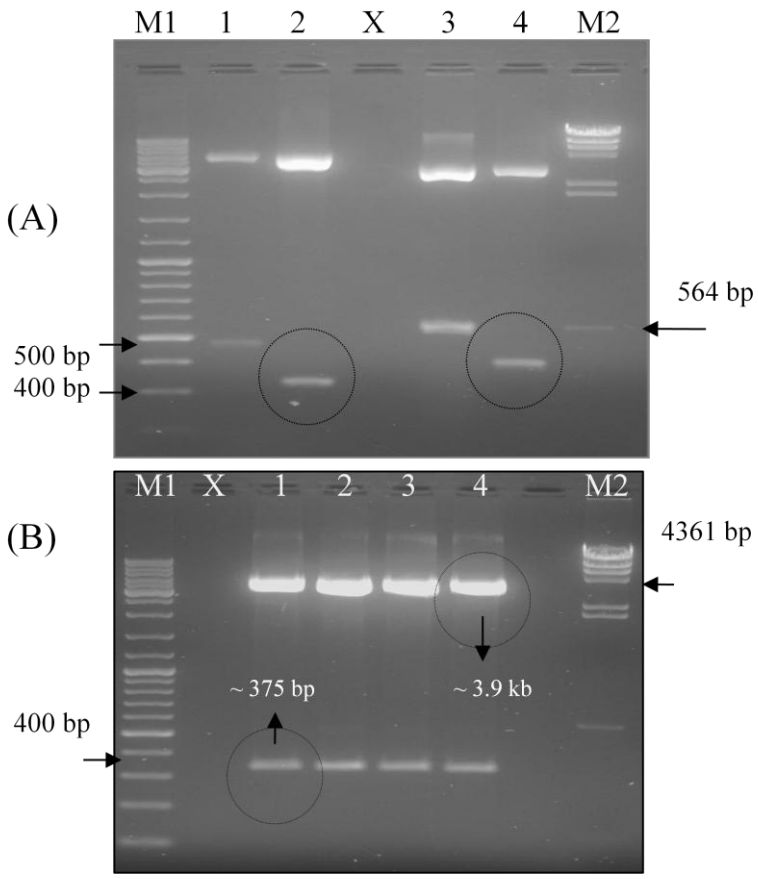


Figure 4-8: (A) Representative ethidium bromide-stained 1.5 % agarose gel picture showing *Eco*RI restriction digestion products of various plasmids; **M1**, GeneRuler™ 100 bp DNA marker; **M2**, λ *Hin*dIII DNA marker; Lane 1, digestion product of pCR4®-TOPO:: EC455 (the EAC of the *G. lamblia* diagnostic PCR assay); Lane 2, digestion product of pCR4®-TOPO:: IC300 (IAC of the *G. lamblia* diagnostic PCR assay); Lane 3, digestion product of pGEM®-T Easy::EC553 (EAC of the *Cryptosporidium* spp. diagnostic PCR assay); Lane 4, digestion product of pGEM®-T Easy::IC375 (IAC of the *Cryptosporidium* spp. diagnostic PCR assay); **(B)** Representative gel picture showing products of *Hin*dIII restriction digestion of the long IAC. **M1**, GeneRuler™ 100 bp DNA marker; **M2**, λ *Hin*dIII DNA marker; Lane 1 - 4, digestion products of pCR4®-TOPO:: IC375 (the IAC of the *E. histolytica* diagnostic PCR assay).

As a result the optimal concentration for each IAC was estimated as follows: the optimal concentration of pGEM®-T Easy:: IC375 DNA when included with 0.4 fg of the pGEM®-T Easy:: EC553 DNA in a duplex reaction using Cry-9 and Cry-15 primers was 0.4–20 fg/µl. The optimal concentration of pCR4®-TOPO:: IC300 DNA when included with 0.5 fg of the pCR4®-TOPO:: EC455 DNA in a duplex reaction using GDHeF and GDHiR primers was 0.9 – 3.5 fg/µl (**Figure 4-9**). Finally, the

optimal concentration of pCR4®-TOPO:: IC375 DNA when included with 0.3 fg of the pCR4®-TOPO:: EC167 DNA in a duplex reaction using EntaF and EhR primers was 3.5 – 35 fg/µl.

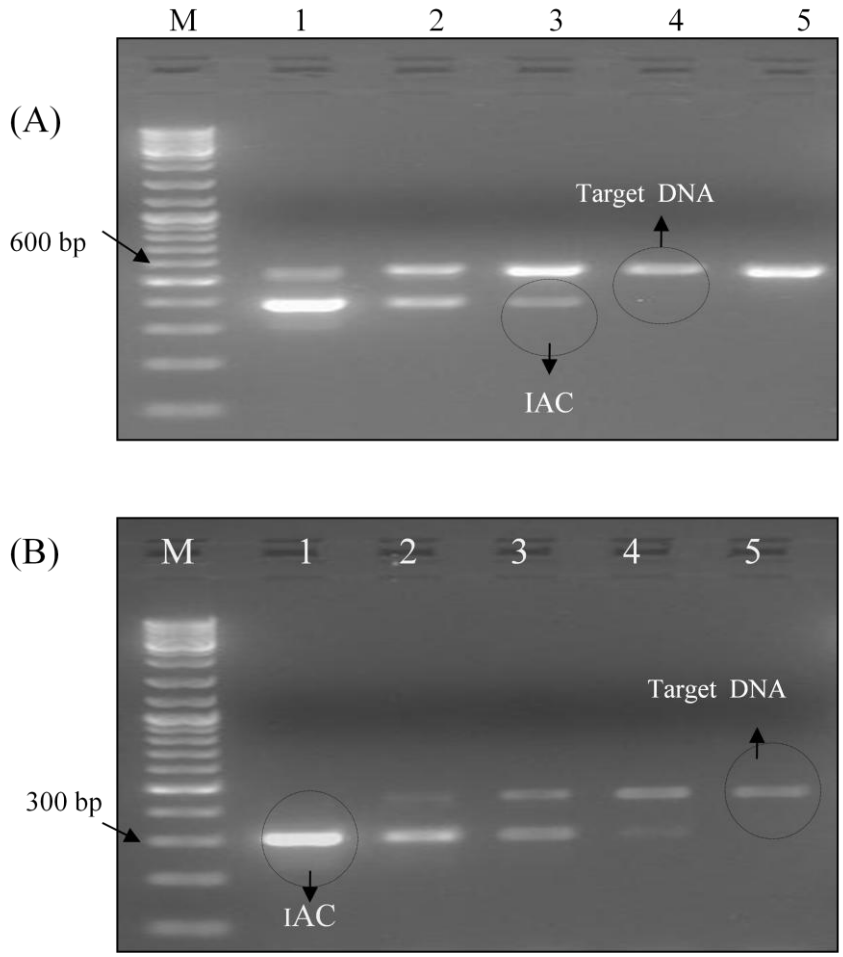


Figure 4-9: (A) Representative ethidium bromide-stained 1 % agarose gel picture showing the optimum concentration of IAC in the *Cryptosporidium* diagnostic PCR. **M**, GeneRuler™ 100 bp DNA marker; Lane 1, Target/IAC = (0.4 fg/20 fg); Lane 2, (0.4/10 fg); lane 3, (0.4/1 fg); Lane 4, (0.4/0.5 fg); Lane 5, (0.4/0.2 fg). It was shown that IAC with concentration of (0.4 – 20 fg) was optimum and did not alter the lower detection limit of the *Cryptosporidium* diagnostic PCR. **(B)** Representative 1.5 % gel picture showing the optimum IAC concentration for the *G. lamblia* diagnostic PCR. **M**, GeneRuler™ 100 bp DNA marker; Lane 1, Target/IAC = (0.5 fg/7.2 fg); Lane 2, (0.5/3.6 fg); lane 3, (0.5/1.8 fg); Lane 4, (0.5/0.9 fg); Lane 5, (0.5/0.4 fg). It was shown that IAC with concentration of (0.9 – 3.6 fg) was optimum and did not alter the lower detection limit of *G. lamblia* diagnostic PCR.

4.3.5 Validation of the constructed IACs

The IAC/target duplex PCR was applied on 24 representative samples of *Cryptosporidium*, *G. lamblia* and *E. histolytica* faecally-derived DNA samples. Each IAC DNA was used in the reaction with the optimal concentration previously estimated. The results were as follows:

For the *Cryptosporidium* diagnostic PCR, the *Cryptosporidium* specific target DNA (~550 bp) was successfully amplified from all positive samples. However, the expected IAC amplicon (~375 bp) could not be easily detected on agarose gel in three positive samples (**Figure 4-10**).

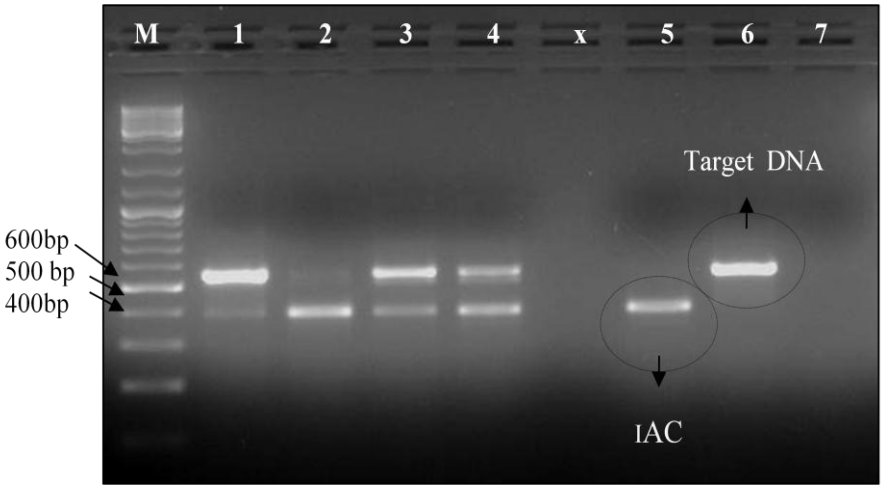


Figure 4-10: Representative ethidium bromide-stained 1 % agarose gel picture showing the PCR products for *Cryptosporidium* positive stool samples extracted by the modified QIAamp® Stool Mini Kit protocol and the recovered DNA sample was subjected to PCR amplification in the presence of internal standard control (IAC). Amplification bands of ~550 bp and ~375 bp were produced from the crude and the plasmid DNA (IAC) respectively by diagnostic primers Cry-9/Cry-15. **M**, GeneRuler™ 100 bp DNA marker; Lane-1 to 4, amplification products of four *Cryptosporidium* positive stool samples; Lane-5, *Cryptosporidium* negative clinical sample; Lane-6, EAC (PCR positive control); Lane-7, no-template master mix sample (PCR negative control).

For the *G. lamblia* diagnostic PCR, IAC (~300 bp) and target DNA (~450 bp) were successfully amplified from all positive samples (**Figure 4-11**).

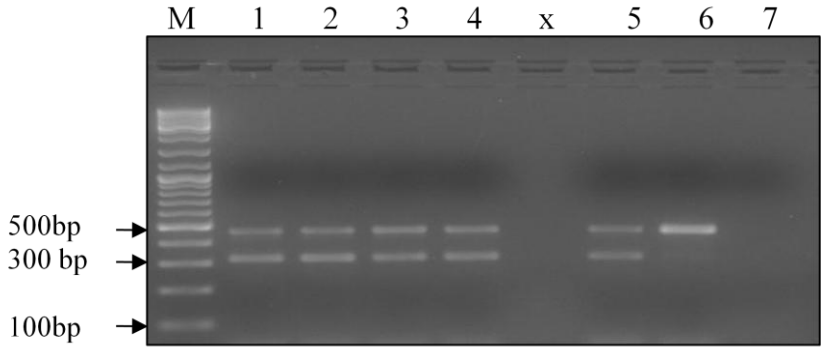


Figure 4-11: Representative ethidium bromide-stained 1.5 % agarose gel picture showing PCR amplification products for *G. lamblia* stool samples extracted by the modified QIAamp® Stool Mini Kit protocol and the recovered DNA sample was subjected to PCR amplification in the presence of internal standard control (IAC). Amplification bands of ~455 bp and ~300 bp were produced from the crude and the plasmid DNA (IAC) respectively by diagnostic primers GDHeF/GDHiR. **M**, GeneRuler™ 100 bp DNA marker; Lane 1 to 4, *G. lamblia* positive clinical samples; Lane 5, both external and internal plasmid recombinant DNA; Lane 6, plasmid recombinant DNA (PCR positive control); Lane 7, no-template master mix sample (PCR negative control)

For *E. histolytica* diagnostic PCR, the target DNA (~170 bp) could not be identified in 10 samples while the IAC DNA (~375 bp) was successfully detected on agarose gel in all samples. The target DNA was successfully identified when these 10 samples were subjected to re-amplification in absence of the IAC (**Figure 4-12**).

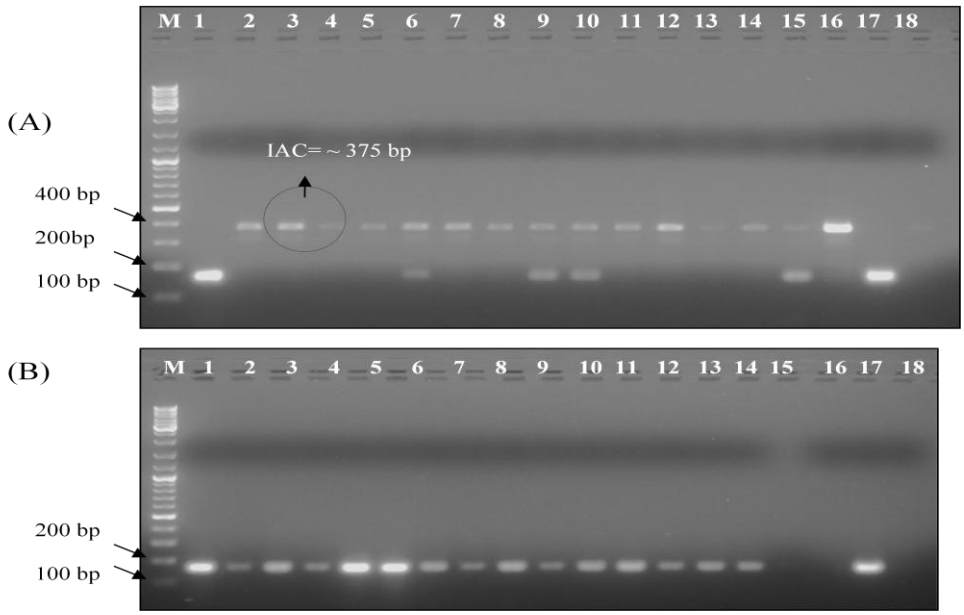


Figure 4-12: Representative ethidium bromide-stained 1.5 % agarose gel two pictures showing PCR amplification products for *E. histolytica* stool samples extracted by the modified QIAamp® Stool Mini Kit protocol and the recovered DNA samples were subjected to PCR amplification using two different approaches**(A)** Application of the full duplex PCR on *E. histolytica* positive clinical samples. The expected IAC amplicon size was ~375 bp while that of the target amplicon was ~170 bp; Lane 1 to 15, *E. histolytica* positive samples; Lane 16, *E. histolytica* negative sample (extraction negative control); Lane 17, PCR positive control; Lane 18, no-template master mix sample (PCR negative control); M, GeneRuler™ 100 bp DNA marker; **(B)** Application of the uniplex PCR on the same *Entamoeba histolytica* positive clinical samples (i.e., in absence of IAC). Lane 1 to 15, *Entamoeba* positive samples; Lane 16, *Entamoeba* negative sample (extraction negative control); Lane-17, pCR4®-TOPO:: EC167 DNA (PCR positive control); Lane 18, no-template master mix sample (PCR negative control); **M**, GeneRuler™ 100 bp DNA marker.

4.4 Discussion

One of the cornerstones of a diagnostic PCR is the quality assurance consideration. A lot of measures have to be taken to provide confidence in the validity of a diagnostic PCR test result. Development of internal quality controls is one of these important measures (Burkardt, 2000). In this study, an external and internal standard controls were constructed for each diagnostic PCR assay to monitor the occurrence of false negative results. The false negative result of a diagnostic PCR is unacceptable. It can

occur as a result of a technical error while setting up the reaction or running the amplification reaction under suboptimal cycling conditions (Erlich, 1989). Furthermore, poor quality DNA template or the narrow range of the designed primers may be the cause of these false negative results (Freed, 2002). In addition, many inhibitory substances found in the clinical samples that may be co-purified with the target DNA can inhibit amplification (Rådström *et al*., 2003). The majority of false negative results can be monitored by performing a separate PCR reaction in parallel to the PCR assay using the same PCR master mix but with the addition of an external amplification control (EAC) in this separate reaction. On the other hand, PCR inhibition can be monitored more directly by inclusion of an internal amplification control (IAC) in the same reaction mix to be detected alongside the target DNA as a duplex reaction (Hoorfar *et al*., 2004).

There are several ways can be adopted for constructing an IAC. However, the main difference lies in whether IAC DNA amplification has to be carried out through the diagnostic primers or requires a separate primer pair. In the first case, there is competition between the analyte target DNA and the IAC as both are detected by the sole primer pair present in the assay. This type of IAC is known as a competitive IAC. In the second case, both the IAC and the target DNA are individually amplified by two separate primer pairs present in the same reaction tube. These latter IACs are known as non-competitive IACs.

In this study, a competitive IAC was constructed based on modifying the cloned target DNA sequence internal to the primers attaching sequence. Modification of the target DNA sequence was carried out either by deleting or adding nucleotide sequences. Selection between the two methods was mainly related to the size of the cloned target

DNA sequence. For *E. histolytica* diagnostic PCR, the cloned target sequence was relatively small (~170 bp) and accordingly construction of long competitive IAC was preferred. On the other hand, the native sizes of the other two targets were ~500 bp permitting sequence deletion and production of short IACs.

To the best of my knowledge, the inverse PCR approach has only been used once previously for construction of a short competitive IAC (Oikonomou *et al*., 2008). In this study, the inverse PCR was also used as a strategy for IAC construction but with some modifications. The approach relied on the use of inverse PCR to delete a short DNA sequence from the internal span of the target sequence, whilst fully preserving the primer annealing sequences at each end. The length of the deleted DNA fragment was defined by the inverse PCR primers designed for the procedure. Due to the absence of suitable restriction enzymes cut sites located within the target sequence but not shared with the cloning vector, introduction of a unique restriction site towards the 5′end of each inverse PCR primer for a single enzyme that was not known to cut elsewhere on the inverse-PCR template was used as an alternative solution. The *Hin*dIII restriction enzyme sequence was inserted as an overhanging sequence at the 5′terminus of each inverse primer. Two additional nucleotide bases were added 5′distal to the enzyme recognition sequence to provide added DNA-enzyme complex stability and facilitate efficient cutting of sites located close to the ends of the linear DNA (Jung *et al.*, 1990). Prior to ligation, each inverse PCR amplicon was firstly treated with *Dpn*I that selectively cuts only the methylated DNA removing any remaining of the template plasmid DNA (Li *et al*., 1999) and secondly with the *Hin*dIII restriction endonuclease to facilitate ligation by producing sticky ends.

The inverse PCR reactions, restriction digestion, and cloning strategy used proved to be very efficient method for IAC construction. Although the approach requires several

days for completion, it offers the continuous availability of high quality IAC DNA with controlled stability, size and high copy number. The size of IACs was selected to be close to that of the native target DNA to reduce the primers preference towards one target over the other as previously reported (Sachadyn and Kur, 1998). Amplification of both targets DNA with the same primers has been successfully achieved without potentially altering the diagnostic efficiency of the PCR assay by the addition of extra primers.

In this study, the influence of IAC on the detection limit of the corresponding diagnostic PCR was investigated. Although there was some detectable competition between both targets towards primers, the analytic sensitivity for each diagnostic PCR was not altered by the presence of IAC. When the PCR assays were applied on positive clinical samples, the diagnostic sensitivity of the assays particularly the *E. histolytica* diagnostic PCR was reduced. The *E. histolytica* specific DNA sequence could not be detected on agarose gel from many positive stool samples in the presence of IAC. The reduced sensitivity of the diagnostic primers toward the target DNA may be due to the low parasite load in the clinical samples, under-estimation of the IAC concentration or the low purity of the faecally derived DNA relative to the IAC plasmid DNA. The successful identification of the *E. histolytica* specific DNA in all clinical samples in absence of IAC ruled out the low parasite load as a cause. Similarly, the successful identification of the *G. lamblia* specific DNA in all positive stool samples ruled out the reduced purity of the faecally-derived DNA as the cause as all DNA samples were purified by the same extraction protocol. Accordingly, under-estimation of the IAC concentration and consequent addition of excess IAC to reactions would be the likely cause for reduction of the *E. histolytica* diagnostic sensitivity in the presence of the matching IAC. In view of these results, all subsequent *E. histolytica* PCR

amplifications were initially performed in the absence of the IAC with the use of the *E. histolytica* IAC being restricted to a second round PCR for samples of questionable DNA quality that tested negative to the other enteric protozoa and IAC controls.

Similar to the competitive IAC types that were adopted in this study, the non-competitive types have their own disadvantages. In most of cases, amplification of the IAC may not accurately monitor the amplification of the native target DNA due the differences in primers sequences and the copy number of both target DNA sequences (Hoorfar *et al*., 2004). For example, the broad-range bacterial 16S ribosomal DNA-based primers have been used to amplify this DNA sequence found in faecally-derived crude DNA samples as an IAC for other diagnostic PCR assays (Persson and Olsen, 2005). However, due to the massively high copy number of the 16S ribosomal DNA (IAC) relative to the non-bacterial targets sought in these enteric PCR assays, the PCR reaction kinetics would be heavily biased towards IAC amplification. Hence, amplification of the IAC sequence could not rule out with any confidence the presence of inhibitory substances present in the faecally-derived DNA samples.

Finally, the phocin herpes virus 1 (PhHV-1) which is normally not found in stool has been recently used as an internal control for real-time based multiplex PCR assays (ten Hove *et al*., 2009; Haque *et al*., 2007 and Verweij *et al*., 2003). The phocin herpes virus 1 has been added to the stool specimen prior to DNA extraction and subsequent amplification by PhHV-1-specific primers to act as both an extraction control and a non-competitive IAC. However, it should be noted that PhHV-1 virus is unlikely to be a suitable extraction control for enteric micro-parasite DNA as oocysts/cysts are undoubtedly much more resistant to extraction than the lipid enveloped herpes virus.

In summary, an external and internal amplification controls were constructed for each diagnostic PCR assay to monitor the amplification. Each EAC proved to be very useful as an external standard control. Similarly, IACs proved to be very helpful in ruling out PCR inhibition for all the faecally-derived crude DNA samples. In addition, the inclusion of the IAC into the diagnostic PCR did not alter the analytic sensitivity of the assays. However, the diagnostic sensitivity of the PCR assays particularly that of *E. histolytica* was reduced by inclusion of IAC in the reactions. Accordingly, all subsequent *E. histolytica* PCR amplifications have to be initially done in absence of IAC and the clinical samples that showed doubtful negative results have to be re-tested for PCR inhibition by inclusion of the IAC in the reaction.

5 Development of a three-enteric protozoa-diagnostic multiplex PCR

5.1 Introduction

The use of PCR as a routine diagnostic laboratory test is limited by the lack of expert personnel, unsuitable laboratory design and the high costs of reagents, the thermocyclers and other equipment. One way to reduce the reagent costs would be through inclusion of more than one set of primers to simultaneously amplify multiple targets in one PCR assay of each clinical specimen (i.e., multiplex PCR).

In this chapter, a block-based multiplex PCR for simultaneous detection of the three most common pathogenic enteric protozoa, *Cryptosporidium* spp., *G. lamblia* and *E. histolytica*, directly from a single stool specimen is described. As far as can be identified from the published literature, this is the first block-based multiplex PCR developed for parallel detection of these three protozoa.

The development of this multiplex PCR assay was accomplished through three sequential stages as follows: firstly, three standard PCRs were developed, optimised and validated as uniplex single-round assays. Secondly, the three sets of primers were included in a single PCR assay in a multiplex PCR format and the multiplex PCR assay was optimised and validated using mock samples. Finally, the analytical performances of the individual uniplex PCR assays and the combined multiplex PCR assay developed were determined prior to subsequent clinical evaluation.

5.2 Selection of the target gene loci

Three gene loci, one for each protozoon, were selected as targets for PCR amplification. Based on published primers listed in Table 2-2 (**Materials and Methods**), three diagnostic PCR assays were developed. All DNA amplifications were carried out in Techne™ TC-4000 thermal cycler as single-round individual (uniplex) PCR assays before multiplexing in a subsequent stage.

In the *Cryptosporidium* diagnostic PCR, a 550 – 553 bp fragment of the *cowp* gene was targeted with primers Cry-15 and Cry-9 (Spano *et al.,* 1997). In *G. lamblia* diagnostic PCR, a specific DNA sequence of ~450 bp of the *gdh* gene was selected as a target locus for amplification by the GDHeF and GDHiR primers (Read *et al.,* 2004). Finally, ~170 bp DNA fragment of the *E. histolytica* 18S rDNA gene was used as a target for the diagnostic PCR using primers EntaF and EhR (Hamzah *et al*., 2006).

In addition, three nested PCRs were selected as additional reference PCR tests in this study (see **Materials and Methods**, Table 2-2). In the *Cryptosporidium* spp. reference PCR, a DNA sequence of 819 – 825 bp of the 18S rDNA gene is amplified by a nested PCR. First, ~1,3 kb is amplified by primers XF-1/XR-1. Then, 819 – 825 bp is amplified by nested primers XF-2/XR-2 (Xiao *et al.,* 1999). In the *G. lamblia* reference PCR, ~290 bp of the 18S rDNA target gene sequence is amplified using primer pair RH-11/RH-4 (Hopkins *et al*., 1997) and a second PCR product of ~170 bp is amplified by primers RH-4/YH1 as a semi-nested PCR. Primer YH1 is a nested primer designed in this study for a second round nested run if the product of the first round could not be detected on agarose gel. Finally, in the *E. histolytica*, a genus specific primer pair E1/E2 is used in the first round to amplify ~890 bp of the 18S rDNA target gene. The

second primer pair EH1/EH2 is species specific and amplifies ~440 bp of the first PCR product (Khairnar and Parija, 2007).

All primers sequences were checked for specificity by conducting BLASTN searches against a broad range of DNA sequences stored at the GenBank DNA sequence database using The National Center for Biotechnology Information (NCBI) free online tool http://blast.ncbi.nlm.nih.gov/Blast.cgi. All primers showed 100 % specificity for the target gene loci. As a next step, all PCR amplifications were modelled using the computer simulation (*in silico* PCR) using the free online web tool http://insilico.ehu.es/. The target species/genotype DNA sequence information was obtained from the NCBI database. DNA sequences stored in the GenBank carrying the accession numbers AAEL0100062 (*C. hominis*), Z22537 (*C. parvum*) and AF266266 (*C. meleagridis*) were subjected to *in silico* PCR amplification using Cry-9 and Cry-15 primer sequences. Sequences carrying the accession numbers of L40509 (*G. lamblia*, assemblage A) and L40508 (*G. lamblia*, assemblage B) were subjected to amplification using primers EntaF and EhR sequences to trigger the reaction. Finally, a DNA sequence carrying the accession numbers of X64142 (*E. histolytica*) was also subjected to *in silico* PCR by EntaF and EhR primers sequences. All the theoretically expected amplicons were produced by *in silico* PCR. Importantly, no other secondary products were produced by these 'assays'.

5.3 Optimization of PCRs components

Initially, each PCR assay was performed according to the previously published protocol using available genomic and crude DNA control samples (see, **Materials and Methods**, section 2.2.1). Results showed that each reaction required further optimisation. For this purposes, a series of optimisation experiments were sequentially

carried out. The optimal primer concentrations, annealing temperature (T_a), $MgCl_2$ concentration as well as other reagents concentrations were optimised as assessed by the amount of PCR products produced following separation of the completed PCR mixes on agarose gel.

5.3.1 *Cryptosporidium* spp. diagnostic uniplex PCR

Based on the results of the optimization experiments (**Figure 5-1**), all subsequent PCR amplifications were carried out using 10 – 20 µM of Cry-15 and Cry-9 primers in the presence of 1 µl of the template DNA, 1X Go Taq® green buffer (Promega), 0.2 mM of each dNTP (Bioline), 1.5 – 2.5 mM $MgCl_2$ (Promega), 1 % DMSO (Sigma), and 1 Uof GoTaq® HotStart polymerase (Promega) as final concentrations. PCR reactions were set up in 20 µl volumes and completed using the following cycling conditions:

The protocol was initiated by a pre-heating step for 4 min at 94°C, and followed by 40 cycles of 30 sec at 94°C, 30 sec at 56 – 57°C and 30 sec at 72°C. A final extension step of 7 – 10 min at 72°C was included.

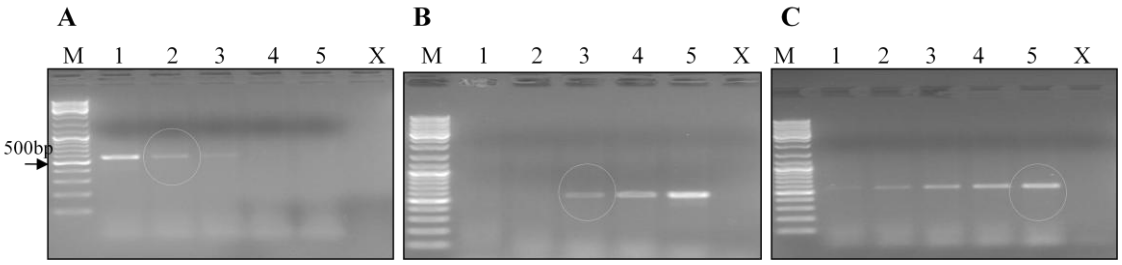


Figure 5-1: Representative ethidium bromide-stained 0.8 % gel pictures showing *Cryptosporidium* diagnostic PCR products (~550 bp) as a result of three optimization experiments. **(A)** Optimisation of Cry-9/Cry-15 primers annealing temperature (T_a); Lane-1, T_a of 56°C; lane-2, T_a of 57°C; Lane-3, T_a of 58°C; Lane 4, T_a of 59°C; Lane 5, T_a of 60°C. **(B)** Optimisation of $MgCl_2$ final concentration; Lane 1, 0.5 mM; lane 2, 1 mM; Lane 3, 1.5 mM; Lane 4, 2 mM; Lane 5, 2.5 mM. **(C)** Optimisation of Cry-9/Cry-15 primers concentration; Lane 1, 7.5 µM; lane 2, 10 µM; Lane 3, 12.5 µM; Lane 4, 15 µM; Lane 5, 20 µM. **M**, GeneRuler™ 100 bp DNA marker; the final parameters selected in the multiplex PCR protocol are circled.

5.3.2 *G. lamblia* diagnostic uniplex PCR

Following the optimization experiments (**Figure 5-2**), all subsequent PCR amplifications were done using 10 – 20 µM of the GDHeF and GDHiR primers, 1 µl of the template DNA, 1X Go Taq® green buffer, 0.2 mM of each dNTP, 1.5 – 2.5 mM $MgCl_2$, 1 % DMSO, and 1Uof GoTaq® HotStart polymerase as final concentrations. PCR reaction volumes of 20 µl were set up and reactions were completed using a touchdown PCR protocol as follows:

The protocol was started with initial denaturation at 94°C for 4 min, followed by 10 cycles of touchdown stage with denaturation at 94°C for 30 seconds, annealing temperature used was 66 – 70°C for 30 seconds. Then, the annealing temperature was decreased to 56 – 60°C by 1°C per cycle for the first 10 cycles. Extension temperature used was 72°C for 30 seconds and the subsequent 30 cycles were as follows; denaturation at 94°C for 30 sec, annealing at 56 – 60°C for 30 sec and extension at 72°C for 30 sec. A final extension step at 72°C for 7 – 10 min was included.

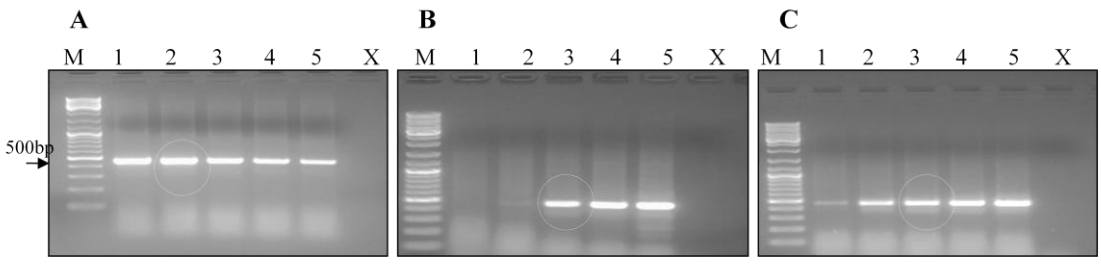


Figure 5-2: Representative ethidium bromide-stained 1 % gel pictures showing *G. lamblia* diagnostic PCR products (~450 bp) as a result of three optimization experiments. **(A)** Optimisation of primers annealing temperature; Lane-1, T_a of 56°C; lane-2, T_a of 57°C; Lane-3, T_a of 58°C; Lane-4, T_a of 59°C; Lane-5, T_a of 60°C. **(B)** Optimisation of $MgCl_2$ final concentration; Lane-1, 0.5 mM; lane-2, 1 mM; Lane-3, 1.5 mM; Lane-4, 2 mM; Lane-5, 2.5 mM. **(C)** Optimisation of primers concentration; Lane-1, 7.5 µM; lane-2, 10 µM; Lane-3, 12.5 µM; Lane-4, 15 µM; Lane-5, 20 µM. **M**, GeneRuler™ 100 bp DNA marker. The final parameters chosen for multiplex PCR are circled.

5.3.3 *E. histolytica* diagnostic uniplex PCR

As a consequence of optimisation experiments **(Figure 5-3)**, all the following PCR reactions were set up in a 20 µl-reaction volume included 1 µl of template DNA, 7.5 – 20 µM of EntaF and EhR primers, 1X Go Taq green buffer, 0.2 mM of each dNTP, 1 – 2.5 mM $MgCl_2$ and 1Uof GoTaq® HotStart polymerase (Promega). Then, PCR reactions were subjected to the following cycling conditions; the protocol initiated by an initial denaturation step for 4 min at 94°C, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 56 – 60°C for 30 sec and extension at 72°C for 30 sec. A final extension period of 7 – 10 min at 72°C was included.

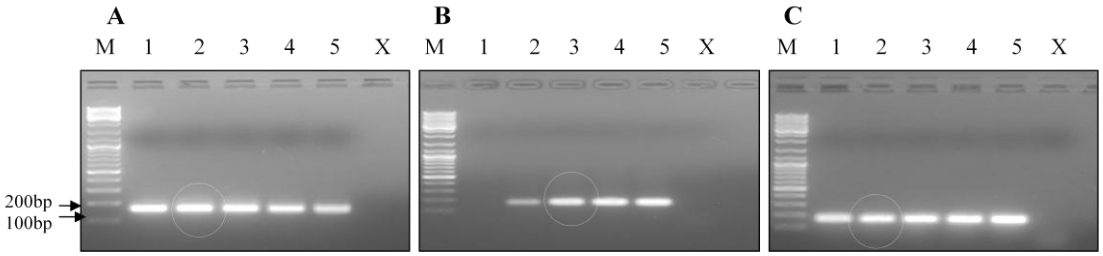


Figure 5-3: Representative ethidium bromide-stained 2% agarose gel pictures showing *E. histolytica* diagnostic PCR products (~170 bp) as a result of optimization experiments. **(A)** optimisation of primers (EntaF/EhR) annealing temperature; Lane-1, T_a of 56°C; lane-2, T_a of 57°C; Lane-3, T_a of 58°C; Lane-4, T_a of 59°C; Lane-5, T_a of 60°C. **(B)** $MgCl_2$ final concentration; Lane-1, 0.5 mM; lane-2, 1 mM; Lane-3, 1.5 mM; Lane-4, 2 mM; Lane-5, 2.5 mM. **(C)** optimisation of EntaF/EhR primers concentration; Lane-1, 7.5 µM; lane-2, 10 µM; Lane-3, 12.5 µM; Lane-4, 15 µM; Lane-5, 20 µM. **M**, GeneRuler™ 100bp DNA marker. The final parameters chosen for multiplex PCR are circled.

5.3.4 The three-protozoa-diagnostic multiplex PCR

Inclusion of the three primer pairs into one multiplex PCR reaction was achieved through a series of experiments. Initially, a single common annealing temperature

(T_a) for the three primer pairs was selected. Then, two primer pairs were included in the PCR mix for amplification of the two matching target genes in two separate PCR tubes using shared cycling conditions. The next stage was to amplify the two targets in one PCR tube (a duplex PCR). Finally, all the three primer pairs were included in a multiplex reaction. Primer concentrations were balanced to allow for equal unbiased amplification of all three target sequences (**Figure 5-4**). The fully optimised concentrations of various PCR components used in all subsequent multiplex PCR assays were outlined in **Table 5-1**.

Table 5-1: The multiplex PCR master mix preparation

Reagent	Stock concentration	Final concentration	volume
Template DNA	-	-	1 µl
dH_2O	-	-	Up to 20 µl
Green buffer	5X	1X	4 µl
$MgCl_2$	25 mM/µl	1.5 mM	1.2 µl
DMSO	100 %	5 %	1 µl
dNTP (mix)	10 pmol/µl (each)	0.2 mM(each)	0.4 µl
GDHeF/iR	10 pmol/µl (each)	12.5 pmol (each)	1.25 µl (each)
Cry-9/Cry-15	10 pmol/µl (each)	20 pmol (each)	2 µl (each)
EntaF/EhR	10 pmol/µl (each)	10 pmol (each)	1 µl (each)
HotStart Taq	5U/µl	1 u	0.2 µl

Subsequently, the PCR tubes were subjected to the following cycling conditions: A denaturation temperature of 94°C for 4 min was initially employed followed by a touchdown protocol. This protocol was initiated by denaturation at 94°C for 30 sec, annealing at 68°C for 30 sec and extension at 72°C for 30 sec for the first cycle. The

annealing temperature was then decreased to 57°C, at a rate of 1°C per cycle, for the first 10 cycles, whilst denaturation and extension criteria were maintained as for the first cycle. The remaining 30 cycles were completed as follows; denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 30 sec. A final extension at 72°C for 7 min was included in the protocol. PCR amplicons were visualized by gel electrophoresis and ethidium bromide staining of the total reaction mix (~20 µl) using 2 % agarose gel.

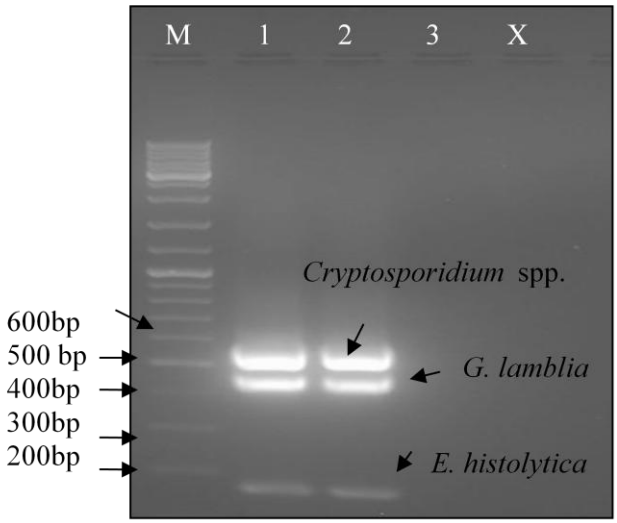


Figure 5-4: Representative ethidium bromide-stained 2 % agarose gel picture showing the amplification products of the multiplex PCR following the optimization experiments. 1 µl of recombinant plasmid DNA samples were initially amplified in the presence of equimolar primer mixture. *Cryptosporidium* Cry-9 and Cry-15 primers were scaled up and *E. histolytica* EntaF/EhR primers were scaled down until the three amplicons were detected on gel. **M**, GeneRuler™ 100 bp DNA marker; Lane-1 & 2, the expected amplicons separated on agarose gel; Lane-3, no-template master mix (PCR negative control).

5.4 Validation of the diagnostic primers

Initially, each primer pair was validated as a uniplex PCR for each target with the available control DNA samples (see **Materials and Methods**, section 2.2.1). The

Cryptosporidium diagnostic primer pair (Cry-9/Cry-15) successfully amplified the target gene sequence in the commonly reported human-derived *Cryptosporidium* isolates namely *C. parvum*, *C. hominis* and *C. meleagridis* genomic DNA samples. The *Cryptosporidium* reference PCR with primer pairs (XF1/XR1 and XF2/XR2) successfully amplified the 18S rDNA target gene sequence of the *Cryptosporidium* genomic DNA samples namely *C. parvum*, *C. hominis*, *C. meleagridis*, *C. felis*, *C. andersoni* and *C.* cervine genotype.

The *G. lamblia* diagnostic primers (GDHeF/GDHiR) and reference primers (RH4/RH11 and YH1) successfully amplified the *gdh* target gene sequence from assemblage A and B representative laboratory strains (WB and GS strains). Finally, the *E. histolytica* diagnostic PCR primer pair (EntaF/EhR) and the reference PCR primers (E-1/E-2 and EH-1/EH-2) successfully amplified the target gene sequence of *E. histolytica* representative laboratory strain (HM-1: IMSS).

The next stage was to validate the three diagnostic primer pairs as multiplex PCR using mock samples representative of triple, double and individual infections. A parasite-free stool sample was initially identified by microscopy and copro-antigen detection kits (see **Material and Methods**, Section 2.3.3). This sample was then subjected to DNA extraction through the modified QIAamp® Stool Mini Kit DNA extraction protocol described previously (see **Chapter-3**). The faecally-derived DNA sample was confirmed to be negative for the three target DNA sequences by the reference nested and standard PCR assays prior to spiking for future use. Finally, this parasite-free nucleic acid sample (50 µl) was divided into ten aliquots of 5 µl each. Seven aliquots were spiked with 1µl supplements containing ~10 pg of genomic DNA corresponding to one, two, or three of the specific target protozoa. The remaining three aliquots were

left unseeded. All spiked samples were thoroughly mixed and 1 µl of each aliquot was subjected to amplification by the multiplex PCR. All mock samples were successfully identified on agarose gel as can be seen in **Figure 5-5**.

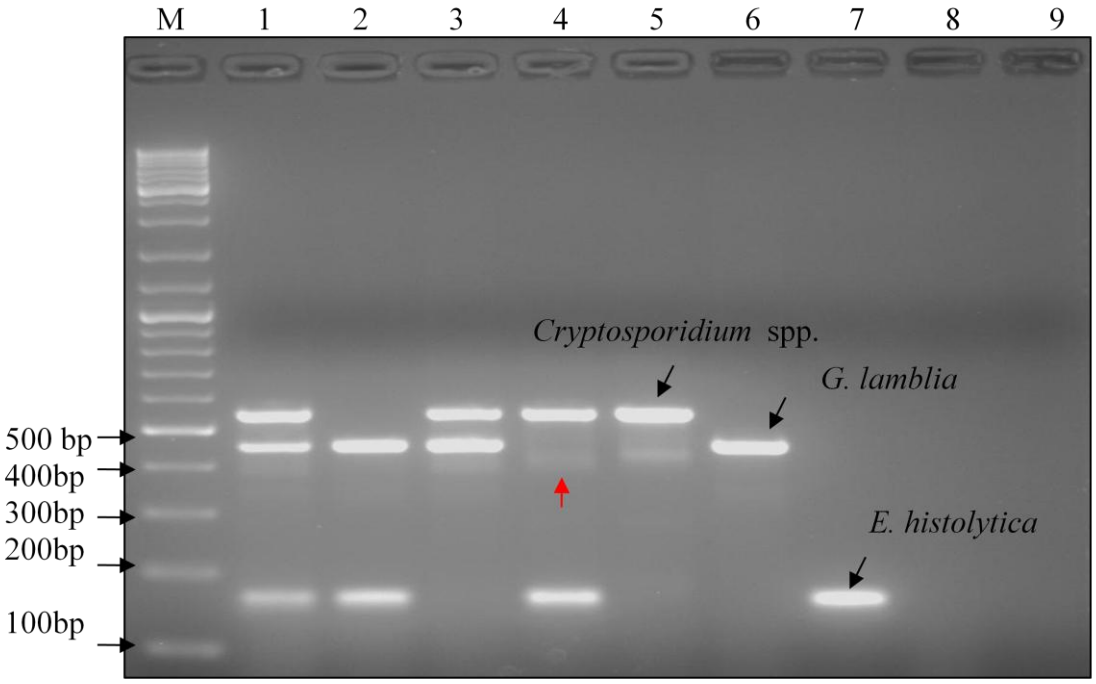


Figure 5-5: Representative ethidium bromide-stained 2 % agarose gel pictures showing various products of the multiplex PCR applied on mock clinical samples representative to individual, double and triple infections. Expected amplicons sizes was ~170, ~450 and ~550 bp of *E. histolytica* 18S rDNA, *G. lamblia gdh* and *Cryptosporidium cowp* target genes, respectively. **M**, GeneRuler™ 100 bp DNA marker; Lane-1, negative stool sample spiked with the three protozoal genomic DNA; lane-2, negative stool sample spiked with *G. lamblia* and *E. histolytica* genomic DNA; Lane-3, negative stool sample spiked with *Cryptosporidium* and *G. lamblia* genomic DNA; Lane-4, negative stool sample spiked with *Cryptosporidium* and *E. histolytica* genomic DNA; Lane-5, negative stool sample spiked with *Cryptosporidium* genomic DNA alone; Lane-6, negative stool sample spiked with *G. lamblia* genomic DNA alone; Lane-7, negative stool sample spiked with *E. histolytica* genomic DNA alone; Lane-8, negative stool sample left unseeded; Lane-9, no-template master mix (PCR negative control); Red arrow is pointing to non-specific amplification products. The size of these non-specific bands was smaller than the *G. lamblia* PCR product.

5.5 Estimation of the analytical sensitivities in absence of faecal extracts

The lower detection limit of each PCR assay was estimated using serial dilutions of the target-matching individual positive control plasmid DNA and genomic DNA (see **Table 5-2**).

5.5.1 *Cryptosporidium* spp. diagnostic PCR assay

1 ng of the positive control plasmid DNA (pGEM®-T Easy:: EC553) used as a positive control (see **Chapter-4**) and was serially diluted down to concentration of 0.04 fg per µl. Then, 1 µl of each dilution was subjected to PCR amplification. The uniplex PCR assay detected a minimum concentration of 0.4 fg, corresponding to ~ 100 copies of the target gene.

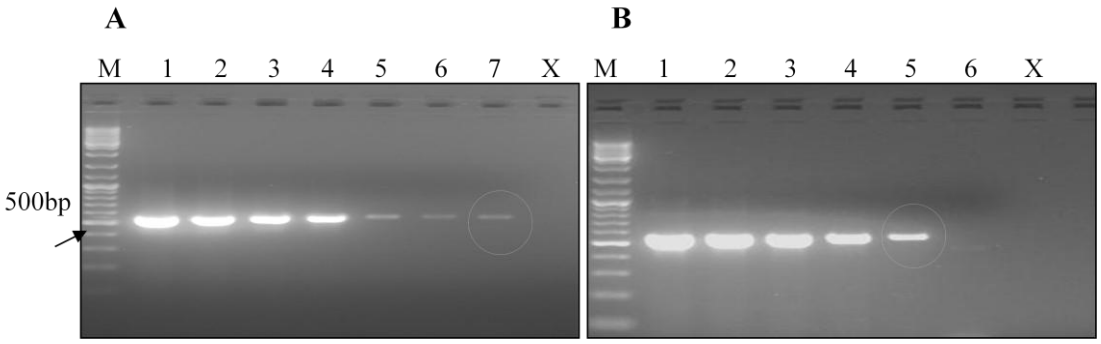


Figure 5-6: Representative ethidium bromide-stained 1 % agarose gel pictures showing the lower detection limits of the *Cryptosporidium* diagnostic PCR. **(A)** Application of the diagnostic PCR on decimal serial dilutions of positive control plasmid recombinant DNA sample; Lane-1, 0.4 ng/reaction; Lane-2, 40pg; Lane-3, 4pg; Lane-4, 0.4 pg; Lane-5, 40fg; Lane-6, 4fg; Lane-7, 0.4fg/reaction. **(B)** Application of the diagnostic PCR on decimal serial dilutions of partially purified genomic DNA sample; Lane-1, 0.5 ng/reaction; Lane-2, 50 pg; Lane-3, 5pg; Lane-4, 0.5 pg; Lane-5, 50 fg; Lane-6, 5 fg/reaction; **M**, GeneRuler™ 100 bp DNA marker

Table 5-2: The lower detection limits of the individual PCR assays.

Method used for estimation	The *Cryptosporidium* spp. diagnostic PCR	The *G. lamblia* diagnostic PCR	The *E. histolytica* diagnostic PCR
Serial dilutions of a positive control plasmid DNA down to 0.04 fg/ µl[1]	The PCR assay was able to detect 0.4 fg per reaction, corresponding to ~100 copies of the target gene	The PCR assay was able to detect 0.5 fg per reaction, corresponding to ~100 copies of the target gene.	The PCR assay was able to detect 0.3 fg per reaction, corresponding to ~78 copies of the target gene.
Serial dilutions of a positive control genomic DNA down to 5 fg/µl[2]	The PCR assay was able to detect 0.5 pg per reaction, corresponding to ~12 copies of the *Cryptosporidium* genome	The PCR assay was able to detect 2 pg per reaction, corresponding to ~12 copies of the *G. lamblia* genome	The PCR assay was able to detect 2.5 pg per reaction, corresponding to ~ 4 copies of the *E. histolytica* genome
Crude DNA extracted from aliquots of parasite-free stool sample spiked with variable oocysts/cysts counts	The PCR assay was able to detect DNA extracted from ~ 100 *Cryptosporidium* oocysts per 200 µl stool, corresponding to 500 oocysts per gram of stool or ~ 2 oocysts per reaction	The PCR assay was able to detect DNA extracted from ~ 100 *G. lamblia* cysts per 200 µl stool, corresponding to 500 cysts per gram of stool or ~ 2 cysts per reaction	The PCR assay was able to detect DNA extracted from ~ 100 *E. histolytica* cysts per 200 µl stool, corresponding to 500 cysts per gram of stool or ~ 2 cysts per reaction

[1]; the copy number of the target gene was counted following the previously mentioned equation (see **Chapter-4**, footnote of **Table 4-1**).

[2]; the copy number of the target genome was based on previous estimates mentioned **in the discussion part** of this chapter.

Similarly, a genomic DNA sample was serially diluted down to the concentration of 5 fg/µl. This was followed by application of the diagnostic uniplex PCR on 1 µl of each dilution. The assay successfully detected a minimum of 0.5 pg of the genomic DNA per reaction, corresponding to corresponding to ~12 copies of the *Cryptosporidium* genome.

5.5.2 *G. lamblia* diagnostic PCR assay

1 ng of the positive control plasmid DNA (pCR4®-TOPO:: EC455) previously processed as a positive control (see **Chapter-4**) was serially diluted down to concentration of 0.05 fg/µl. Then, 1 µl of each dilution was subjected to PCR amplification. The uniplex PCR assay detected a lower limit of 0.5 fg of the plasmid DNA which corresponds to ~100 copies of the target gene.

Similarly, two genomic DNA samples belonged to WB and GS strains were serially diluted down to the concentration of 16 fg and 20 fg per microliter, respectively. This was followed by application of the diagnostic uniplex PCR on 1 µl of each dilution. The *G. lamblia* uniplex PCR assay successfully detected a minimum of 1.6 pg of the WB strain genomic DNA and 2 pg of the GS strain genomic DNA **(Figure 5-7)**. These lower detection limits corresponds to ~12 copies of the *G. lamblia* genome.

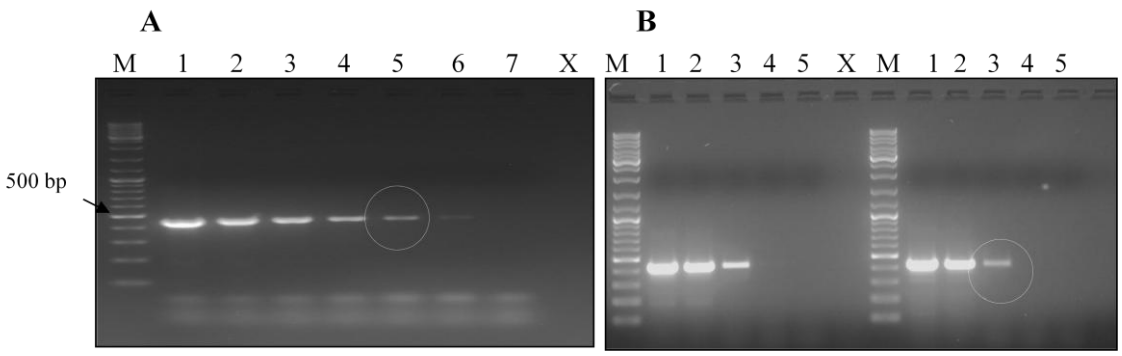


Figure 5-7: Representative ethidium bromide-stained 1 % agarose gel pictures showing the lower detection limits of *G. lamblia* diagnostic PCR. **(A)** Application of the diagnostic PCR on decimal serial dilutions of plasmid DNA sample; Lane-1, 50 pg/reaction; Lane-2, 0.5 pg; Lane-3, 50 fg; Lane-4, 5 fg; Lane-5, 0.5 fg; Lane-6, 0.05 fg; Lane-7, 0.005 fg/reaction. **(B)** Application of the diagnostic PCR on decimal serial dilutions of two genomic DNA samples; the left part of the picture serial dilutions of genomic DNA sample belonged to WB strain; Lane-1, 160 pg/reaction; Lane-2, 16 pg; Lane-3, 1.6 pg; Lane-4, 0.16 pg; Lane-5, 16 fg; the right part of the picture a serial dilutions of genomic DNA sample belonged to GS strain was prepared as follow; Lane-1, 200 pg/reaction; Lane-2, 20 pg; Lane-3, 2 pg; Lane-4, 0.2 pg; Lane-5, 20 fg; **M**, GeneRuler™ 100 bp DNA marker.

5.5.3 *E. histolytica* diagnostic PCR assay

Initially, 1 ng of the positive control plasmid DNA (pCR4®-TOPO:: EC167) processed as a positive control (see **Chapter-4**) was serially diluted down to a concentration of 0.03 fg /µl. Then, 1 µl of each dilution was subjected to PCR amplification. The lower detection limit of the diagnostic uniplex PCR was 0.3 fg of the recombinant DNA per reaction, corresponding to ~ 78 copies of the target gene.

Similarly, decimal serial dilutions of genomic DNA control sample were prepared and 1µl of each dilution was subjected to PCR amplification. The PCR successfully detected a minimum of 2.5 pg of *E. histolytica* genomic DNA per reaction corresponding to ~ 4 copies of the *E. histolytica* genome **(Figure 5-8)**.

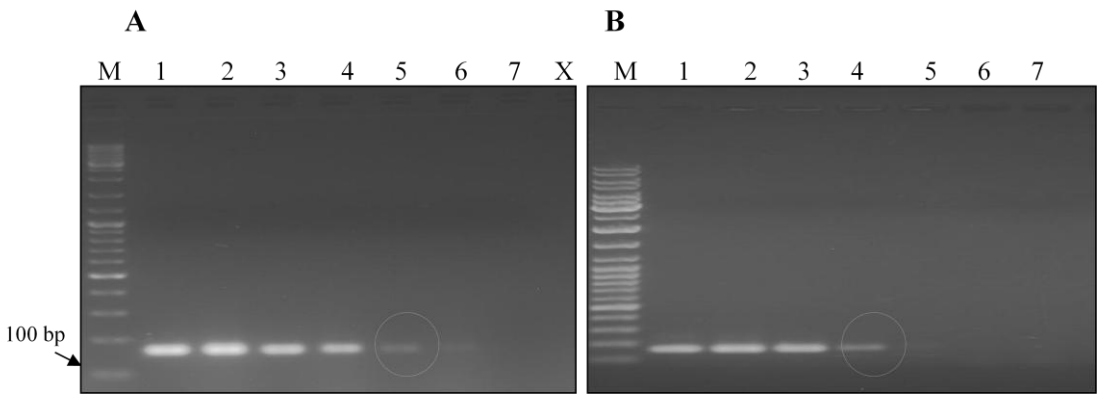


Figure 5-8: Representative ethidium bromide-stained 2% agarose gel pictures showing the lower detection limits of *E. histolytica* diagnostic PCR. **(A)** Application of the diagnostic PCR on decimal serial dilutions of plasmid DNA sample; Lane-1, 0.3 pg/reaction; Lane-2, 0.03 pg; Lane-3, 30 fg; Lane-4, 3 fg; Lane-5, 0.3 fg; Lane-6, 0.03 fg; Lane-7, 0.003 fg/reaction. **(B)** Application of the diagnostic PCR on decimal serial dilutions of genomic DNA sample; Lane-1, 2.5 ng/reaction; Lane-2, 250 pg; Lane-3, 25 pg; Lane-4, 2.5 pg; Lane-5, 250 fg; Lane-6, 25 fg; Lane-7, 2.5 fg/reaction; **M**, GeneRuler™ 100 bp DNA marker

5.5.4 The diagnostic multiplex PCR assay

The same dilutions of the three positive control plasmid DNA samples that were previously used for estimation of the lower detection limits of the three uniplex PCRs individually were also used for estimating the analytical sensitivity of the multiplex PCR. 3 µl of plasmid DNA, one for each target, were subjected to amplification by the multiplex PCR. The lower detection limits of the multiplex PCR were 0.4 fg, 0.5 fg and 0.3 fg of the *Cryptosporidium* spp., *G. lamblia* and *E. histolytica* positive control recombinant plasmid DNA, respectively. These lower detection limits correspond to ~100, 100 and 78 copies of the *Cryptosporidium* spp., *G. lamblia* and *E. histolytica* target gene, respectively (See **Figure 5-9**).

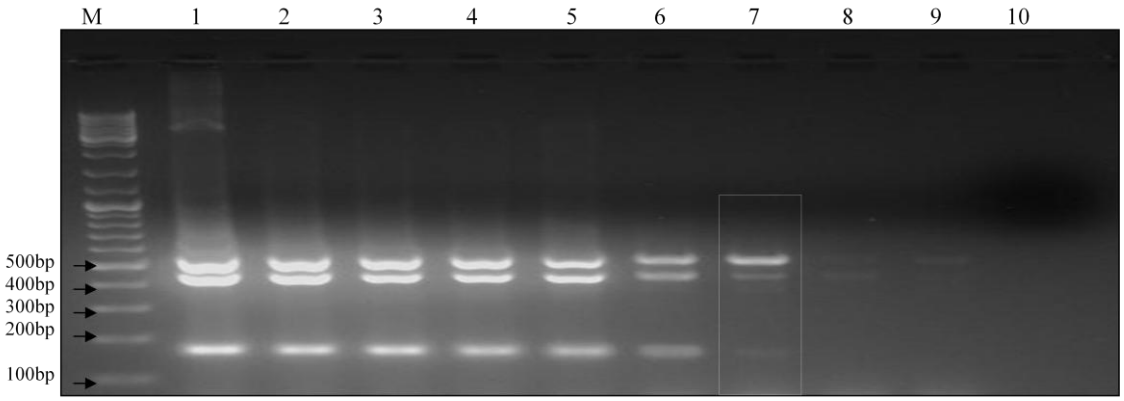


Figure 5-9: Representative ethidium bromide-stained 2 % agarose gel picture showing the lower detection limits of the diagnostic multiplex PCR using decimal serial dilutions of three plasmid DNA samples representative to the three target sequences. Lane-1 to 9, Application of the diagnostic multiplex PCR on decimal serial dilutions of 4, 5, 3 ng of *Cryptosporidium*, *G. lamblia* and *E. histolytica* plasmid DNA samples respectively; Lane-7, 0.4, 0.5 and 0.3 fg/reaction (The same lower detection limits that were previously estimated for the individual PCRs); Lane-10, no-template master mix sample (PCR negative control); **M**, GeneRuler™ 100 bp DNA marker.

5.6 Estimation of the analytical sensitivities in the presence of faecal extracts

To estimate the lower number of oocysts/cysts that can be detected using the full set of PCR assay (DNA extraction, amplification and analysis procedures), protozoa-free stool aliquots, spiked with oocysts/cysts suspension of known counts were subjected to DNA extraction and subsequent amplification with the multiplex and the individual target-uniplex PCR assays.

5.6.1 Preparation and counting of oocysts/cysts suspension

The *Cryptosporidium* oocyst suspension was prepared from microscopically-positive stool samples subjected to salt flotationconcentration technique (**Materials and Methods**, Section 2.3.4). The average number of oocysts per microscopic field was around nine oocysts. Accordingly, the number of oocysts per microscopic slide (per 20 µl) was estimated to be around 180 oocysts. In principal, the number of oocysts

present in 1 ml of the semi-purified suspension was estimated to be around 9×10^3 oocysts.

The *G. lamblia* cysts suspension was prepared from microscopically-positive stool sample concentrated and purified initially with the modified formol-ether concentration technique. Then, concentrated samples were purified further by the sucrose density gradient centrifugation technique (see **Materials and Methods**, Section 2.3.5). 2 µl of the purified cyst suspension was diluted in 18 µl dH_2O (1 : 10 dilution). A 17 µl-aliquot of the diluted cyst suspension was injected into the chamber of the Modified Fuchs Rosenthal haemocytometer slide and examined with x20 objective lens of the bright field microscope. Approximately six cysts were counted in one large square. Accordingly, around 30 cysts were present in five large squares (i.e., 1 µl). Following the previously mentioned equation (see **Materials and Methods**, 2.3.5**)**, the number of *G. lamblia* cysts present in one ml was estimated to be ~3×10^5 cysts.

The *E. histolytica* cysts suspension was prepared from stool samples diagnosed as *E. histolytica* positive samples by the *Entamoeba histolytica* II test (TechLab). Two positive stool samples were polled together and adequately homogenized. Similar to *Giardia*, the *E. histolytica* positive stool sample was concentrated, purified and counted using the same procedures. ~10 cysts were counted in five large squares of the chamber (i.e., 1 µl). The number of *E. histolytica* cysts present in one ml was calculated to be around one million cysts.

5.6.2 Preparation of oocysts/cysts-spiked faecal samples

Seven stool aliquots, 200 µl each, containing approximately 1700, 1500, 1000, 500, 100, 50 and 10 of the *Cryptosporidium* oocysts were prepared as follows; aliquots of

parasite-free stool sample of 23, 35, 88, 144.4, 188, 194.5 and 199 µl were seeded with 177, 165, 112, 55.5, 12, 5.5 and 1 µl of the previously prepared and counted *Cryptosporidium* oocysts suspension (~ 9 oocysts/µl) respectively. Following the same principal, another two sets of faecal aliquots spiked with approximately 1700, 1500, 1000, 500, 100, 50 and 10 *G. lamblia* or *E. histolytica* cysts per 200 µl of stool were prepared.

5.6.3 The lower detectable number of oocysts/cysts per PCR assay

Stool samples spiked with oocysts/cysts (200 µl each) were subjected to DNA extraction using the modified QIAamp® Stool Mini kit DNA extraction protocol and the subsequent PCR amplification using the multiplex and the individual target-uniplex diagnostic PCR assays. The target DNA sequence of each PCR assay was successfully extracted and subsequently amplified from stool samples seeded with oocysts/cysts down to ~100 oocysts/cysts, corresponding to ~500 oocysts per gram of stool and to ~2 oocysts/cysts per PCR reaction (**Figure 5-10**).

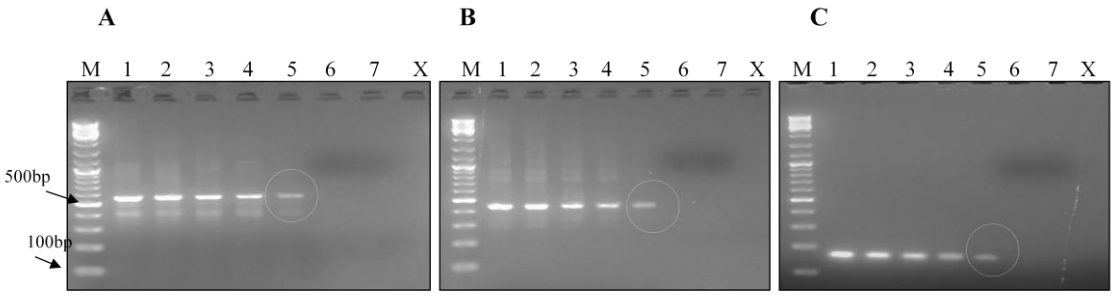


Figure 5-10: Representative ethidium bromide-stained agarose gel pictures showing the lower detection limits of the full set of the diagnostic uniplex PCR assays. **(A)** The lower detection limit of the *Cryptosporidium* diagnostic PCR assay using negative faecal samples spiked with definite oocysts counts. **(B)** The lower detection limit of the *G. lamblia* diagnostic PCR assay using negative faecal samples spiked with definite cysts counts. **(C)** The lower detection limit of the *E. histolytica* diagnostic PCR assay using negative faecal samples spiked with definite cysts counts. Lane-1, amplification product of DNA samples retrieved from stool extract spiked with ~1,700 oocysts/cysts; Lane-2, amplification product of DNA samples retrieved from stool extract spiked with ~1,500 oocysts/cysts; Lane-3, amplification product of DNA samples retrieved from stool extract spiked with ~1000 oocysts/cysts; Lane-4, amplification product of DNA samples retrieved from stool extract spiked with ~500 oocysts/cysts; Lane-5, The amplification product of DNA samples retrieved from stool extract spiked with ~100 oocysts/cysts; Lane-6, amplification product of DNA samples retrieved from stool extract spiked with ~50 oocysts/cysts per extract; Lane-7, amplification product of DNA samples retrieved from stool extract spiked with ~10 oocysts/cysts per extract; **M**, GeneRuler™ 100 bp DNA marker

5.7 Discussion

In this study, a simple and relatively cheap gel-based multiplex PCR for simultaneous detection of the three common diarrhea-causing enteric protozoa namely *Cryptosporidium* spp., *G. intestinalis* and *E. histolytica* was developed.

In developing this multiplex PCR, three sets of primers that had been previously published were selected due to a number of reasons: Firstly, these primers amplify the specific target gene sequence from the commonly reported human infecting species and strains namely *C. parvum, C. hominis, C. meleagridis, E. histolytica, G. lamblia*

assemblage A and *G. lamblia* assemblage B. Secondly, the primers selected have physical properties suitable for multiplexing. In particular the primers have close melting temperatures (T_m) and targeted DNA sequences of relatively short lengths which can be easily differentiated by the standard agarose gel electrophoresis. Finally, the DNA sequence targeted by the primer sets, particularly *Cryptosporidium* spp. and *G. lamblia* diagnostic primers, contain specific restriction enzymes sites allowing further identification of the protozoal species and/or isolate through the restriction profile of the PCR products as an alternative tool to DNA sequencing. Read and her colleagues have described the restriction profile of the *G. lamblia gdh* PCR product by restriction enzymes *Nla*IV and *Rsa*I (Read *et al.,* 2004). Similarly, Spano and his colleagues reported the restriction profile for the *Cryptosporidium* spp. *cowp* PCR product by restriction enzyme *Rsa*I on the standard agarose gel (Spano *et al.,* 1997).

Selection of the Go Taq® Hot start polymerase (Promega) to drive the diagnostic multiplex PCR assay was proved advantageous in this PCR assay. Firstly, it is a relatively cheap polymerase compared to other commercially available hot start polymerases. Secondly, it does not need an initial long heating step for its activation as required by other hot start polymerases. Thirdly, its green buffer has a tracking dye which helps loading the PCR products directly onto an agarose gel saving significant time. Importantly, the use of this green buffer in setting up PCR did not affect the downstream application of the PCR products such as cloning, restriction digestion and sequencing.

In developing the multiplex PCR, a major effort was initially directed towards minimizing the preferential amplification of one DNA target over another as reported previously (Polz *et al.,* 1998). The proper selection of the annealing temperature, final

concentration of $MgCl_2$, and careful balancing of the primer concentrations minimized preferential amplification. In addition, application of hot start polymerase, the touchdown protocol and inclusion of 1 % DMSO (Sigma) in the reaction proved to be very effective measures towards increasing the sensitivity and the specificity of the assay. This was in line with previous studies that had used a touchdown protocol to improve the specificity of PCR amplification (e.g., Hecker and Roux, 1996 and Larsen *et al.*, 2002).

An examination of the analytical sensitivity of each diagnostic PCR was made in view of what had been previously published. The *Cryptosporidium cowp* gene is expressed as single-copy within the nucleus of sporozoites. The *Cryptosporidium* oocyst contains four nucleated sporozoites. As a result of this, there are four copies of the *cowp* gene per oocyst. Accordingly, the lower detection limit of the *Cryptosporidium* diagnostic PCR using positive control plasmid DNA samples was ~100 copies of the target gene sequence present in ~ 25 *Cryptosporidium* oocysts. The DNA content of one sporozoite has been estimated at 40 fg (Guy *et al.*, 2003). Based on this estimate, the lower detection limit of the *Cryptosporidium* uniplex PCR, in absence of PCR- stool inhibitors, was ~12 trophozoites per reaction.

Similarly, the *G. lamblia gdh* gene is expressed as single-copy gene within the nucleus of *Giardia* trophozoite and each *Giardia* cyst contains genetic information for two trophozoites and each nucleous is tetraploidy (has four genome copies). In principal, the tetra-nucleated *Giardia* cyst has 16 copies of *gdh* gene (Bernander *et al.*, 2001). Accordingly, the lower detection limit of the diagnostic PCR assay was ~100 copies of the target gene sequence present in approximately six cysts. The DNA content of the *Giardia* trophozoite has been estimated at 0.144 pg and the DNA content of the

Giardia cyst at 0.313 pg (Erlandsen and Rasch, 1994). Thus in principal, the lower detection limit of the *Giardia lamblia* uniplex PCR, using pure DNA extract, was ~12 trophozoites or around five cysts per reaction.

Unlike the *Cryptosporidium cowp* and the *G. lamblia gdh* target genes, the *E. histolytica* 18S rDNA gene was present in more than 100 copies per trophozoite (Gonin and Trudel, 2002). In principal, the *E. histolytica* diagnostic PCR was theoretically able to detect less than one trophozoite per reaction. Furthermore, the DNA content of one *E. histolytica* trophozoite was previously estimated to be ~0.7 pg (Mackenstedt *et al.*, 1989). Accordingly, the lower detection limit of the diagnostic PCR was able to detect pure DNA equivalent to four trophozoites per reaction. The analytical sensitivity of the multiplex PCR matched closely that achieved by the individual uniplex assays. However, this analytical estimate was based on the efficiency of the PCR reaction alone and did not represent the whole assay. For this reason, a second estimate which reflected, to some extent, the efficiency of the DNA extraction and the subsequent PCR amplification was undertaken.

Assuming that the oocysts/cysts count in all spiked stool aliquots was precise and the DNA extraction was carried out from all the seeded oocysts/cysts with equal efficiency, which was ruled out earlier for few *Cryptosporidium*- and *Giardia*-positive stool samples (see **Chapter-3**, Section 3.2.2), the lower detection limit of the diagnostic PCR assays was ≤100 oocysts/cysts per 200 µl stool extract which corresponds to ~500 oocysts/cysts per gram of stool. Because the purified DNA was eluted in 50 µl of the elution buffer and only 1µl of the faecally derived DNA was subjected to PCR amplification, the lower detection limit of each PCR assay corresponded to ~2 oocysts/cysts per reaction. This lower detection limit of each PCR is substantially

below the previously estimated faecal burdens and parasite excretion rates in humans (Bushen *et al.,* 2007 and kohli *et al.,* 2008). The mean number of oocysts shed by a *Cryptosporidium* infected person was reported to be ~3.3×10^6 oocysts per ml of stool during symptomatic infection and ~3×10^5 oocysts per gram of stool for asymptomatic cases (Bushen *et al.,* 2007). Similarly, the mean number of cysts shed by a *Giardia*-infected individual was ~8.7×10^4 cysts per gram of stool in a symptomatic infection and ~6.9×10^5 cysts per ml of diarrheal stool (kohli *et al.,* 2008). In addition, the lower detection limit of the diagnostic assays was close to the infectious dose that has been previously reported to be ~10 *Giardia* cysts or ~30 *Cryptosporidium* oocysts or even one *E. histolytica* cyst (Rendtorff, 1954; DuPont *et al.,* 1995 and Ravdin, 1995). In spite of these promising results, the methods used for estimation of the lower detection limit of the diagnostic assay suffered from one major disadvantage. The faecally-derived oocysts /cysts suspensions used for the seeding experiments were not completely purified. As a result, accurate counting of oocysts/cysts under bright field microscopic examination was particularly challenging.

Direct comparison between the analytical sensitivity of the various species specific primers described previously was not possible because of the methods adopted to estimate the lower detection limits were different among studies. Primers like Cry-9/Cry-15 were frequently used in PCR assays developed for *Cryptosporidium* characterization purposes and the analytical sensitivity of these PCR assays was not estimated in most of the previously reported studies (Spano *et al*., 1997; Xiao *et al*., 2000; McLauchlin *et al*., 2000 and Leoni *et al*., 2000). Read and her colleagues have developed a semi-nested PCR assay using primers GDHeF/GDHiF/GDHiR targeting ~430 bp of *G. lamblia gdh* locus with analytical sensitivity of 2 pg of genomic DNA using DNA purified from the cultured *G. lamblia* strain p1c10 (*G. lamblia,*

assemblage A). These authors also reported success with amplification of the target DNA from a single cultured p1c10 trophozoite (Read *et al*., 2004). By comparison, our analytical sensitivity for the detection of *G. lamblia* by single-round uniplex and multiplex PCR assays targeting the same gene locus was 2 pg of genomic DNA based on DNA isolated from the WB cultured strain (*G. lamblia*, assemblage A) and the GS cultured strain (*G. lamblia*, assemblage B). The analytical sensitivity of EntaF/EhR primers, targeting the same DNA sequence of *E. histolytica* 18S rDNA gene, has been previously reported to be ~20 pg of genomic DNA sample extracted from the HM-1: IMSS strain (Hamzah *et al*., 2006). By comparison, our analytical sensitivity for the detection of *E. histolytica* uniplex and multiplex PCR assays targeting the same gene locus was 2.5 pg of genomic DNA based on DNA isolated from the same laboratory strain.

The analytical specificity of PCR assays was not examined further in this study as this had been previously investigated in many studies using different genomic DNA samples extracted from related and unrelated enteric pathogens (Read *et al.*, 2004; Spano *et al*., 1997 and Hamzah *et al*., 2006). Nevertheless, in this study, PCR amplification was always target-specific when the assays were applied to faecally-derived stools that contained DNA from various defined sources (see **Chapter-6**).

In summary, a single-round multiplex PCR was developed for simultaneous detection of the three common enteric protozoa affecting humans. This multiplex PCR was based on three previously reported primer pairs, each targeting one of the pathogens as the basis of highly specific and sensitive PCR assays. The adoption of the hot start and touchdown protocols proved to be useful in further increasing the sensitivity and

specificity of the primer pairs used in the multiplex PCR. The multiplex PCR showed an adequate analytical sensitivity equivalent to those were demonstrated with the individual PCR assays. The analytical sensitivities demonstrated by this study were comparable to, if not better than those of the previous studies. Finally, the multiplex PCR assay was developed, fully optimised, and rigorously tested to ensure performance characteristics compatible with a routine microbiology laboratory test prior to its subsequent clinical evaluation.

6 Validation of the multiplex PCR assay on clinical samples from Leicester

6.1 Introduction

As stated previously, PCR detection of enteric protozoa is largely dependent upon the method used for DNA extraction from the stool specimens. The majority of the previously developed PCR assays have reported high levels of sensitivity and specificity using pure genomic DNA samples. This analytical sensitivity is actually estimating the performance of PCR amplification step and not the overall diagnostic process.

In this chapter, a relatively large number of clinical samples were used to evaluate the performance of the multiplex PCR that was developed as part of this study. For this evaluation, two sets of clinical samples were used. The first set included positive and negative stool samples appropriately selected to estimate the diagnostic performance of the assay. The second set was a fairly large group of random stool samples with blinded conventional test results to estimate the performance characteristics of the multiplex PCR assay as compared to current routine testing procedures underway at the Clinical Microbiology laboratory, University Hospitals of Leicester (see **Materials and Methods**, Section 2.3.3 for more details).

For further evaluation, the concordance of results obtained with the multiplex PCR assay, the individual diagnostic PCR assays and the copro-antigen based commercial kits was assessed.

6.2 Application of the multiplex PCR assay on selected stool samples

6.2.1 Selection criteria for faecal samples

A large group of stool samples (*n* = 170) were subjected to microscopic examination and copro-antigen detection for *Cryptosporidium* and *Giardia*. Samples that diagnosed negatives by the former tests were subjected to DNA extraction by the modified QIAamp® Stool Mini Kit DNA extraction protocol and the subsequent DNA amplification by the three individual nested or un-nested reference PCR assays described previously (see **Chapter-5**). Based on the three tests-results, three positive control groups were assigned as shown in **Table 6-1**. In addition, 70 samples diagnosed negatives for the three protozoa by the three tests were designated as a protozoal negative control group (Group-4).

Stool samples diagnosed positives by microscopy (see **Figure 6-1**) and the copro-antigen detection kits were selected as a positive control group (Group-1). This group included 33 *Cryptosporidium* and 25 *Giardia* positive stool samples.

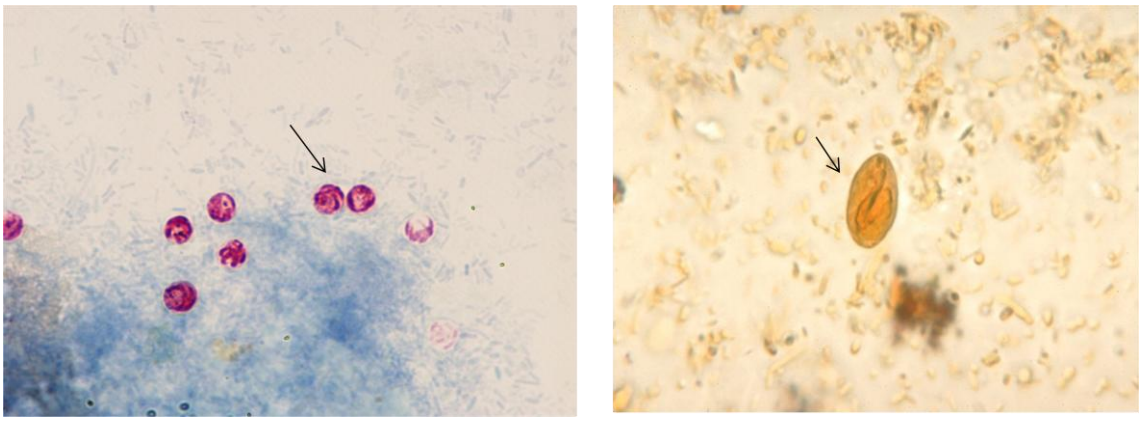


Figure 6-1: Representative bright field microscopic pictures for the *Cryptosporidium* oocysts stained with Modified Ziehl-Neelsen dye (left) and the *Giardia lamblia* cysts stained with iodine (right) with x200 magnification.

Stool samples that tested positive for protozoal copro-antigen(s) and protozoal specific copro-DNA were designated as a protozoal positive control group (Group-2). This group contained 10 *Cryptosporidium* and 15 *Giardia* positive stool samples (see **Figure 6-2 A** and **C**, respectively).

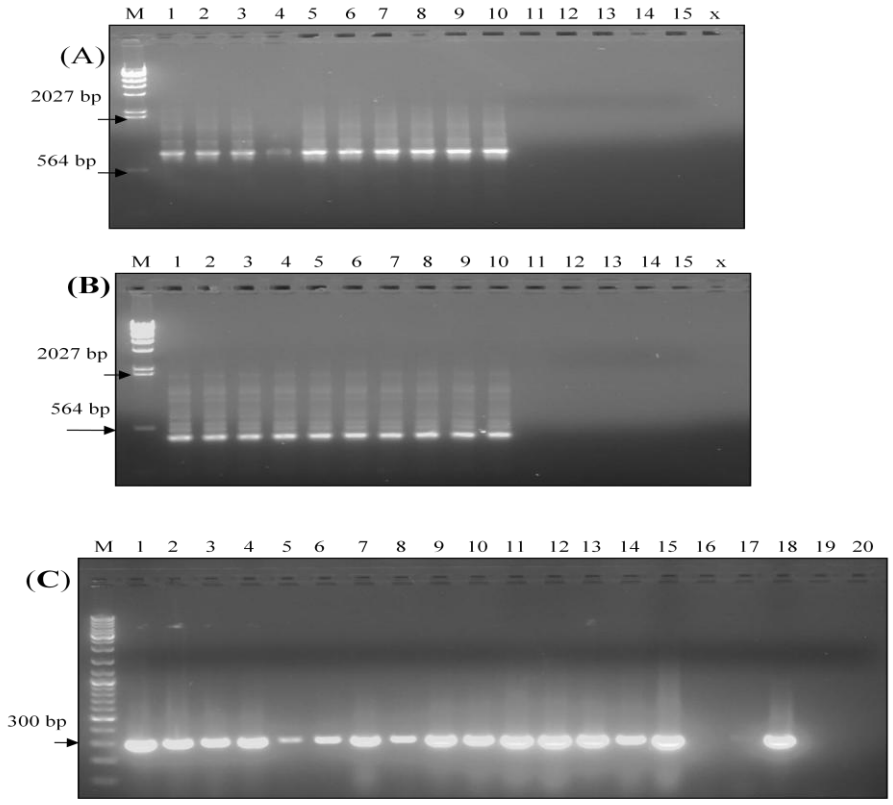


Figure 6-2: Representative ethidium bromide-stained 1 % agarose gel pictures showing the three reference PCRs amplification products **(A)** Amplification products (~825 bp) of *Cryptosporidium* spp. reference nested PCR. Lane 1 to 10, *Cryptosporidium* positive faecally-derived DNA samples; Lane 11 to 15, *Cryptosporidium* negative faecally-derived DNA samples; **M**, λ-*Hin*dIII DNA molecular marker. **(B)** Amplification products (~440 bp) of *E. histolytica* reference nested PCR; Lane 1 to 10, *E. histolytica* positive stool samples; Lane 11–15, *E. histolytica* negative samples; **M**, λ-*Hin*dIII DNA marker **(C)** Amplification products (~290 bp)of *G. lamblia* reference PCR. Lane 1–15, *G. lamblia* positive clinical samples; Lane 16–17, *G. lamblia* negative stool samples; Lane 18, positive control plasmid DNA sample (PCR positive control); Lane 19, no-template master mix sample (PCR negative control); **M**, GeneRuler™ 100 bp DNA marker.

Stool samples that were diagnosed as positives by the reference PCR but were negative by microscopy and copro-antigens detection assays were considered as a third

protozoal positive control group (Group-3). This group included seven *Cryptosporidium* and 10 *Giardia* positive stool samples. *E. histolytica* could not be identified by microscopy or the nested reference PCR in any of the 170 test samples obtained from diarrheic stool samples submitted to the Clinical Microbiology laboratory, UHL. Accordingly, *E. histolytica* EIA positive clinical faecal samples collected in Egypt (n = 15) were confirmed by the nested reference *E. histolytica* PCR and included in this study as part of the positive control Group-2 (see **Figure 6-2B**). In addition, no dual infections were identified in any of the 170 test samples.

Table 6-1: Selection criteria for the positive control samples

Protozoon	Microscopy + copro-antigen(s) detection test[1] (Group-1)	copro-antigen(s) detection test + PCR (Group-2)	PCR[2] (Group-3)	Total[3]
Cryptosporidium spp.	33	10	7	50
G. lamblia	25	15	10	50
E. histolytica	0	15	0	15
Total	58	40	17	115

[1]It is three-kits based immunoassay algorithm as the routine diagnostic test adopted at the Clinical Microbiology laboratory, UHL. This algorithm relies on two-step immunoassay test; *Giardia*/*Cryptosporidium* Check™ EIA screening test (TechLab) as a first step and two other commercially available lateral flow immunochromatographic tests (LF) Rida® Quick *Giardia* and Rida® Quick *Cryptosporidium* (R-Biopharm) as discriminatory kits for the positive samples only.
[2] The corresponding individual target-uniplex reference PCR assay.
[3] A fourth group included 70 samples that were negatives for the three protozoa by the three tests, was designated as Group-4 (negative control group).

All faecal control samples were subjected to DNA extraction with the modified QIAamp DNA Stool Mini Kit DNA extraction protocol (see **Chapter-3**). To compare the diagnostic performances between the uniplex PCRs and the multiplex PCR, the

same faecally-derived DNA samples were subjected to sequential amplification by both PCR sets

6.2.2 The diagnostic performance of the uniplex assays

The faecally-derived DNA samples were subjected to DNA amplification using the individual target-uniplex PCR assays and the following results were shown; The *Cryptosporidium* diagnostic PCR successfully amplified the target DNA sequence from 49 out of 50 *Cryptosporidium* positive control samples (see **Figure 6-3**). The only false-negative sample belonged to Group-3. Importantly, no amplification was detected for the *Cryptosporidium* spp. specific target DNA in all 70 negative control samples (Group-4). Equally important, no amplification products were identified when 25 *G. lamblia* and 15 *E. histolytica* positive faecally-derived DNA samples were subjected to amplification by the *Cryptosporidium* diagnostic PCR as another set of negative controls. Based on these results, The *Cryptosporidium* uniplex diagnostic PCR assay was found to exhibit sensitivity, specificity, negative predictive value and positive predictive value of 98 %, 100 %, 98.5 % and 100 %, respectively (see **Table 6-2**).

Table 6-2: Diagnostic performance of the uniplex and the multiplex PCR assays

Assay	Sensitivity (95 % C.I.)	Specificity (95 % C.I.)	PPV (95 % C.I.)	NPV (95 % C.I.)	Total positive controls (*n*)	Total negative controls (*n*)	Nominated 'gold standards'
Cryptosporidium **uniplex PCR**	98 % (0.8- 0.9)	100 % (0.9-1.0)	100 % (0.9-1.0)	98.5 % (0-94-0.99)	50	110[a]	18S rDNA nested PCR
G. lamblia **uniplex PCR**	96 % (0.85-0.99)	100 % (0.9-1.0)	100 % (0.9-1.0)	97.2 % (0.93-0.99)	50	110[b]	18S rDNA semi-nested PCR
E. histolytica **uniplex PCR**	100 % (0.7-1.0)	100 % (0.9-1.0)	100 % (0.7-1.0)	100 % (0.9-1.0)	15	135[c]	18S rDNA nested PCR
Multiplex PCR	97 % (0.91-0.99)	100 % (0.9-1.0)	100 % (0.9-1.0)	95 % (0.87-0.98)	115	70	Nested and semi-nested PCRs

(C.I.) stands for 95% confidence intervals

(PPV) stands for positive predictive value (Definition; the probability that patient with positive test result is truly infected).

(NPV) stands for negative predictive value (Definition; the probability that patient with negative test result is truly uninfected).

[a]; (70 prtozoa-free + 25 *Cryptosporidium*-negative but *Giardia*-positive + 15 *Cryptosporidium*-negative but *Entamoeba*-positive) stool samples

[b]; (70 prtozoa-free + 25 *Giardia*-negative but *Cryptosporidium*-positive + 15 *Giardia*-negative but *Entamoeba*-positive) stool samples

[c]; (70 prtozoa-free + 30 *Entamoeba*-negative but *Cryptosporidium*-positive + 35 *Entamoeba*- negative but *Giardia*-positive) stool samples

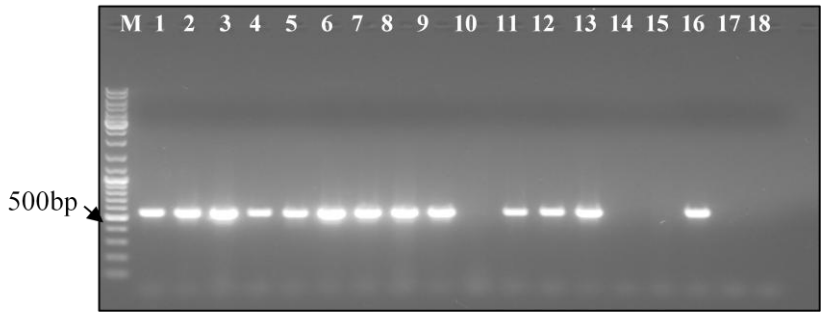


Figure 6-3: Representative ethidium bromide-stained 1 % agarose gel picture showing the amplification products (~550 bp) of the *Cryptosporidium* single-round diagnostic PCR assay when applied on the *Cryptosporidium* positive faecally-derived DNA samples. **M**, GeneRuler™ 100 bp DNA marker; Lane –1 to 13, *Cryptosporidium* positive faecally-extracted DNA control samples; Lane –14 and 15, *Cryptosporidium* negative faecally-extracted DNA control sample (extraction method negative control); Lane-16, positive control plasmid DNA sample (PCR positive control); Lane-17 and 18, no-template master mix samples (PCR negative controls). Lane– 10, The *Cryptosporidium* spp. specific DNA could not be identified in a *Cryptosporidium* positive control sample (false negative result).

The *G. lamblia* diagnostic PCR successfully amplified the target DNA sequence from all *Giardia* positive samples apart from two samples (see **Figure 6-4**). These two samples belonged to Group-3. By running an additional PCR round with 1 µl of the PCR product as a template, using a nested primer (GDHiF) and the primary reverse primer (GDHiR), the target DNA was identified. No amplification was detected for the *G. lamblia* specific DNA in any of the 70 negative control samples (Group-4) and in 25 *Cryptosporidium* and 15 *E. histolytica* positive faecally-extracted DNA samples when used as a second set of negative controls. Based on these results, the *G. lamblia* uniplex PCR assay was found to exhibit sensitivity, specificity, negative predictive value and positive predictive value of 96 %, 100 %, 97.2 % and 100 %, respectively.

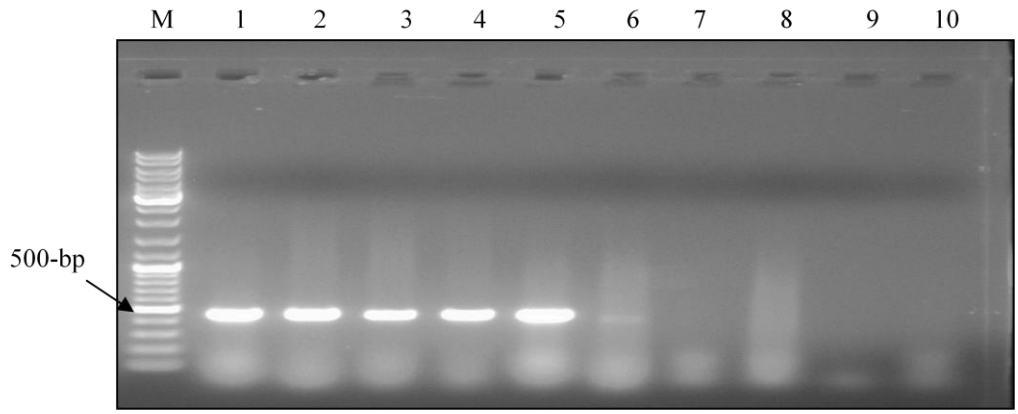


Figure 6-4: Representative ethidium bromide-stained 1.5 % agarose gel picture showing the amplification products of the *G. lamblia* single-round diagnostic PCR (~450 bp) when applied on faecally-derived *Giardia* positive and negative DNA control samples. **M**, GeneRuler™ 100 bp DNA marker; Lane –1 to 6, *G. lamblia* positive faecally-extracted DNA control samples; Lane –7 to 10, *G. lamblia* negative faecally-extracted DNA control samples.

The *E. histolytica* diagnostic PCR successfully amplified the target DNA from all 15 *E. histolytica* positive control samples (see **Figure 6-5**). No amplification product was detected when the assay was applied on the 70 negative control samples (Group-4). No amplification of the *E. histolytica* target DNA was detected when 30 *Cryptosporidium* positive and 35 *G. lamblia* positive faecally-derived DNA samples were used as a second set of negative controls for the *E. histolytica* uniplex PCR assay. Based on this small set of test samples, the diagnostic sensitivity, specificity, negative predictive value and the positive predictive value were all 100 %.

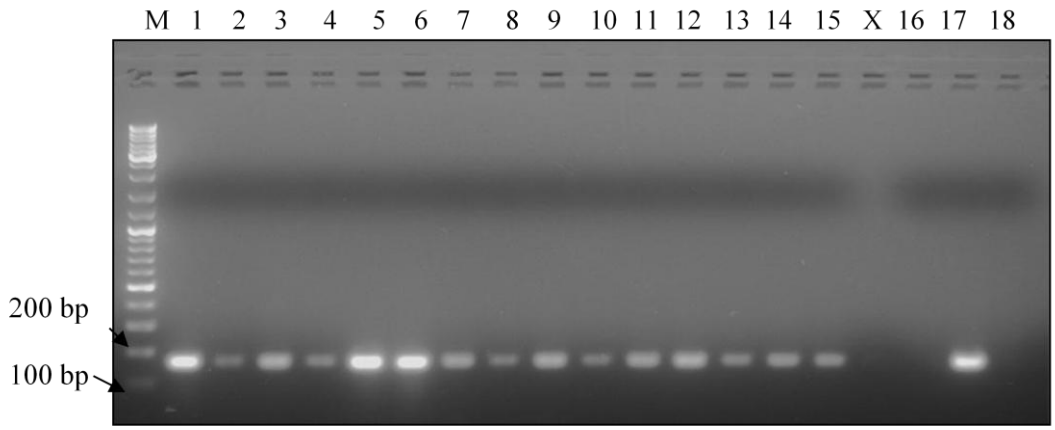


Figure 6-5: Representative ethidium bromide-stained 2 % agarose gel picture showing the amplification products of the *E. histolytica* diagnostic PCR (~170 bp) when applied on faecally-derived control samples; **M**, GeneRuler™ 100bp DNA marker; Lane-1-15, *E. histolytica* positive faecally-extracted DNA control samples; Lane-16, *G. lamblia* negative faecally-extracted DNA control sample (DNA extraction method positive control); Lane-17, positive control plasmid DNA sample (PCR positive control); Lane-18, no-template master mix sample (PCR negative control).

6.2.3 The diagnostic performance of the multiplex PCR assays

All faecally-derived positive control DNA samples (*n* = 115) were successfully amplified by the multiplex PCR assay apart from a single *Cryptosporidium* and two *G. lamblia* positive control samples. All three samples producing multiplex PCR false negative results belonged to Group-3 had also tested negative by the corresponding individual target-uniplex PCR assays. No positive amplicons were detected from all 70 negative samples when subjected to the multiplex PCR. In view of these results, the multiplex PCR showed sensitivity, specificity, negative predictive value and positive predictive value of 97 %, 100 %, 95 % and 100 %, respectively (see **Table 6-2**).

6.3 Application of the multiplex PCR assay on random diarrheal stool samples

The further clinical evaluation of the multiplex PCR assay was accomplished by analysing 212 random stool specimens obtained from diarrheal stool samples from 212 distinct patients submitted to the Clinical Microbiology laboratory, UHL between June and October, 2009. Fresh unpreserved stool samples were tested for *Cryptosporidium* spp. and *Giardia* copro-antigens by three-kits based immunoassay algorithm.

Stool samples stored unpreserved at 4°C for up to 2 weeks until used. All stool samples were subjected to DNA extraction with the modified QIAamp® Stool Mini kit DNA extraction protocol as mentioned previously. The faecally-derived DNA samples were subjected to amplification using the uniplex and the multiplex single-round diagnostic PCR assays. The results of copro-antigen, uniplex PCR, multiplex PCR assays are collectively summarized in the clinical data table at **Appendix A**. These PCR tests were performed in a blinded fashion with respect to prior combined copro-antigen detection test results and the concordance of results was addressed. Samples that showed discordant results were subjected to PCR amplification with the nested reference PCR.

6.3.1 Multiplex PCR assay results

Fifty-seven of the 212 random blinded samples tested positive for either *Cryptosporidium* spp. or *G. lamblia* specific DNA by the multiplex PCR, while four specimens were positive for both targets (see **Figure 6-7**). One or both of these protozoa was identified in 39 % (9/23) of specimens from children with sporadic gastroenteritis who were under five years of age, 59 % (10/17) of patients aged 5 – 19 years, 44 %

(12/27) of patients aged 20 – 39 years, 46 % (22/48) of patients aged 40 – 59 years and 8 % (8/95) of patients over 60 years old (see **Figure 6-6**)

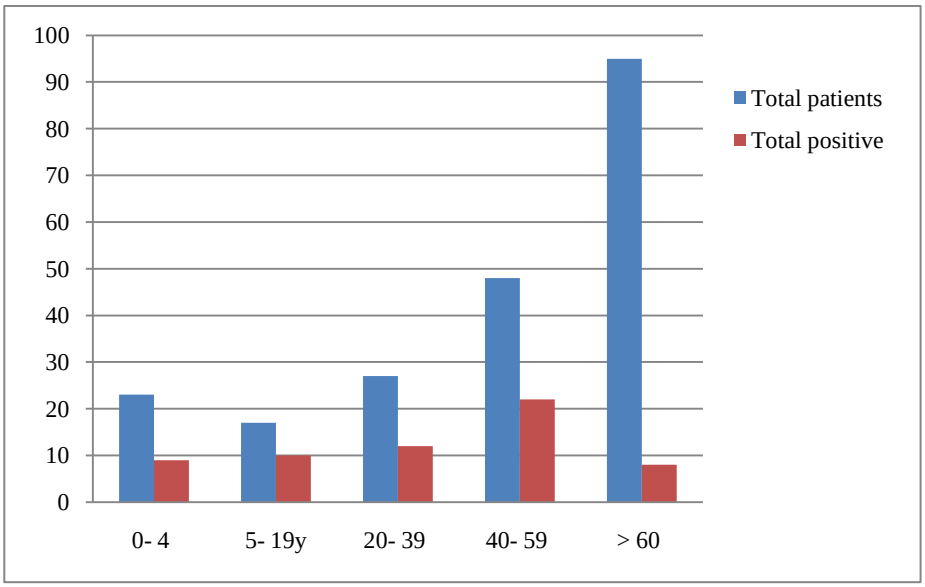


Figure 6-6: Age distribution (x-axis) of total number of patients and number of patients with positive results (y-axis).

The *Cryptosporidium* specific copro-DNA target was detected in 24 samples (~11 %), *G. lamblia* specific copro-DNA target was identified in 33 samples (~15 %) and four samples (~2 %) were positives for both *Cryptosporidium* and *G. lamblia* DNA targets. The majority of *Cryptosporidium* positive samples (*n* = 17) were for children below 10 years and the remaining seven samples were for patients aged from 10 to 45 years. Most of the *G. lamblia* positive samples (n = 28) were collected from middle aged patients and the remaining five samples were for children below 10 years. These data yielded a collective percentage positivity of 28.7 % (61/212) from diarrheal stool specimens submitted to a large UK teaching hospital for these two major enteric

parasites. *E. histolytica* specific DNA was not detected in any of the 212 clinical samples collected in the UK.

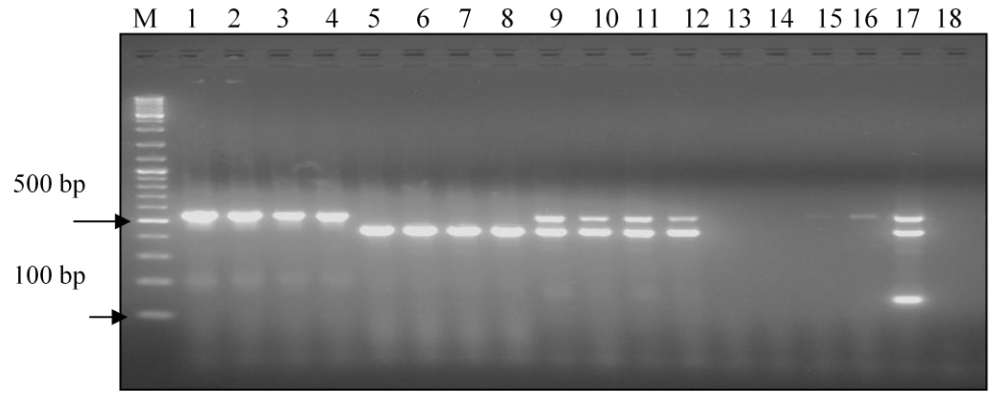


Figure 6-7: Representative ethidium bromide-stained 2 % agarose gel picture showing the amplification products of the multiplex diagnostic PCR assay when applied on faecally-derived DNA samples. **M**, GeneRuler™ 100 bp DNA marker; Lane 1–4, *Cryptosporidium* spp. specific DNA products of ~550 bp; Lane 5–8, the *G. lamblia* specific DNA of ~450 bp; Lane 9–13, both *Cryptosporidium* spp. and *G. lamblia* specific DNA; Lane 13–14, *Cryptosporidium*, *G. lamblia* and *E. histolytica* negative samples; Lane 15–16, two weak amplicons of *Cryptosporidium* spp. specific DNA; Lane 17, plasmid DNA sample mix (multiplex PCR positive control); Lane 18, no-template master mix sample (PCR negative control).

6.3.2 Concomitant infections

Very few infective enteric pathogens other than protozoa were identified by additional review of the laboratory records (see **Appendix A**). *Campylobacter* spp. was found in 10 samples, two of these samples were also *Giardia* positives. *Salmonella* spp. was identified in four samples, one as a concomitant infection with *Giardia,* two with *Cryptosporidium* and the last clinical sample as a sole *Salmonella* spp. infection. In addition, *Clostridium defficile* was identified in five samples by *Clostridium difficile* toxin test.

6.3.3 Concordance of results with the uniplex PCR assays

The same faecally-derived DNA samples (*n* = 212) were subjected to PCR amplification by the three diagnostic uniplex PCR assays and the results were compared to the multiplex PCR results. The *Cryptosporidium* spp. specific target DNA was identified by the multiplex PCR assay in 28 uniplex positive samples (i.e., including four samples that diagnosed as *C*/*G* dual infections), resulting in perfect agreement (212/212) between the assays. The *G. lamblia* specific target DNA was identified in 40 samples by the uniplex PCR assay versus 37 samples by the multiplex PCR assay (33 samples diagnosed as *G. lamblia* solo infections and four samples diagnosed as *C*/*G* dual infections). Three *G. lamblia* uniplex positive samples were missed by the multiplex PCR assay. One of these three samples was also *Cryptosporidium* positive by the uniplex and multiplex PCR assays. When a second semi-nested round was applied on 1 µl of the first round PCR product, the *G. lamblia* specific DNA was successfully detected in all three samples. The percent of agreement between the two PCR assays was 98.5 % (209/212). No amplification product was detected in the remaining 148 samples **(Table 6-3).** Samples with discordant results were proved to be *Giardia* positive by the reference semi-nested PCR. In addition, the *E. histolytica* specific DNA could not be detected in all 212 samples by either the uniplex or multiplex PCR assays.

6.3.4 Inhibition control-experiments

Twenty-two representative multiplex PCR-negative samples were randomly selected for inhibition control experiments to exclude the possibility that a negative PCR result was due to failure of amplification. The target-matching IAC, with the previously

estimated working concentration, was incorporated in *Cryptosporidium*, *G. lamblia* and *E. histolytica* diagnostic PCRs as duplex reactions. IAC DNA was successfully detected in all samples tested, confirming a very low, to negligible rate of PCR inhibition based on the DNA extraction method used.

Table 6-3: The percent of agreement between the multiplex and the uniplex PCR assays

Target protozoan	$uPCR^{+}/mPCR^{+}$	$uPCR^{-}/mPCR^{-}$	$uPCR^{+}/mPCR^{-}$	$uPCR^{-}/mPCR^{+}$	agreement%
Cryptosporidium spp.	28	184	0	0	100 % (212/212)
G. lamblia	37	172	3	0	98.5 % (209/212)
E. histolytica	0	212	0	0	100 % (212/212)

uPCR and mPCR stand for uniplex and multiplex PCR, respectively.

6.3.5 Concordance of results with the *Giardia*/*Cryptosporidium* Check™ test

The 212 clinical stool samples were also tested in parallel under real-time constrains using the routine *Giardia*/*Cryptosporidium Check*™ test (TechLab). *Giardia* and/or *Cryptosporidium* copro-antigens were identified in 76 samples. The multiplex PCR amplified the *G. lamblia* specific DNA in 33 samples. Of these positive samples, there were two samples picked by the multiplex PCR that were negative by the primary EIA copro-antigen screening test. *Cryptosporidium* target DNA was detected by the multiplex PCR in 24 samples and both *G. lamblia* and *Cryptosporidium* DNA targets were found in four samples. The overall agreement between the two tests was 91.9 %

(195/212). Fifteen samples which were negative by multiplex PCR but positive by the *Giardia*/*Cryptosporidium Check*™ screening test also proved to be negative by the nested PCR and the secondary routine discriminatory and confirmatory copro-antigen detection assay. Two discordant samples were EIA screening test negative but tested positive for *G. lamblia* by the multiplex PCR assay. Both samples were confirmed as *Giardia* positive by Rida® Quick *Giardia* assay and the nested PCR test. Interestingly, the optical density readings at 450 nm for the 15 false-positive TechLab screening test results were very close to the manufacturer-defined assay cutoff value.

Effectively, multiplex PCR picked 100 % of all EIA screening test-identified specimens that were subsequently confirmed by the discriminatory lateral flow tests and/or reference PCR assays.

Table 6-4: The percent of agreement between the multiplex PCR and the EIA[1] test

EIA^+/ $mPCR^+$	EIA^-/ $mPCR^-$	EIA^+/ $mPCR^-$	EIA^-/ $mPCR^+$	% agreement
59	136	15[*]	2[†]	91.9 % (195/212)

[1]; EIA stands for enzyme immunoassay based test (i.e. *Giardia*/*Cryptosporidium* Check™)
[*]; 15 samples proved to be negative by RIDA®Quick *Cryptosporidium* and RIDA®Quick *Giardia* kits and nested PCRs
[†]; *G. lamblia* specific DNA target was successfully amplified in these two samples by the *G. lamblia* diagnostic and the semi-nested reference PCR

6.3.6 Concordance of results with the three-kits based immunoassay test

As shown in **Figure 6-8**, out of 212 clinical stool samples, the *Cryptosporidium* copro-antigen was identified in 24 stool samples by copro-antigen combined

immunoassay test that included *Giardia*/*Cryptosporidium Check*™ EIA test and Rida® Quick *Cryptosporidium* lateral flow (LF) test. The multiplex PCR detected the *Cryptosporidium* target DNA in all these 24 samples. *Giardia* copro-antigen was identified in 31 samples by the combined immunoassay tests namely *Giardia*/*Cryptosporidium Check*™ EIA test and Rida® Quick *Giardia* LF test. The multiplex PCR identified the *G. lamblia* DNA in all of these samples plus two samples that were missed by the EIA screening test and would hence have not normally been tested by the discriminatory LF assay.

Cryptosporidium/*Giardia* dual infection was reported in three samples with combined copro-antigen detection test. The multiplex PCR detected DNA targets to both *Cryptosporidium* spp. and *G. lamblia* in the three combined test-identified dual infection samples plus one further sample that was identified as *Cryptosporidium* positive only by the combined copro-antigen detection test protocol. The overall agreement between both diagnostic tests was 98.5 % (209/212). A final three discordant samples that were multiplex PCR positive but copro-antigens negative by the three-kit based immunoassay were subjected to PCR amplification using the uniplex diagnostic and reference PCR assays. *G. lamblia* DNA was identified in the three samples and *Cryptosporidium* DNA was also detected in one of these three samples.

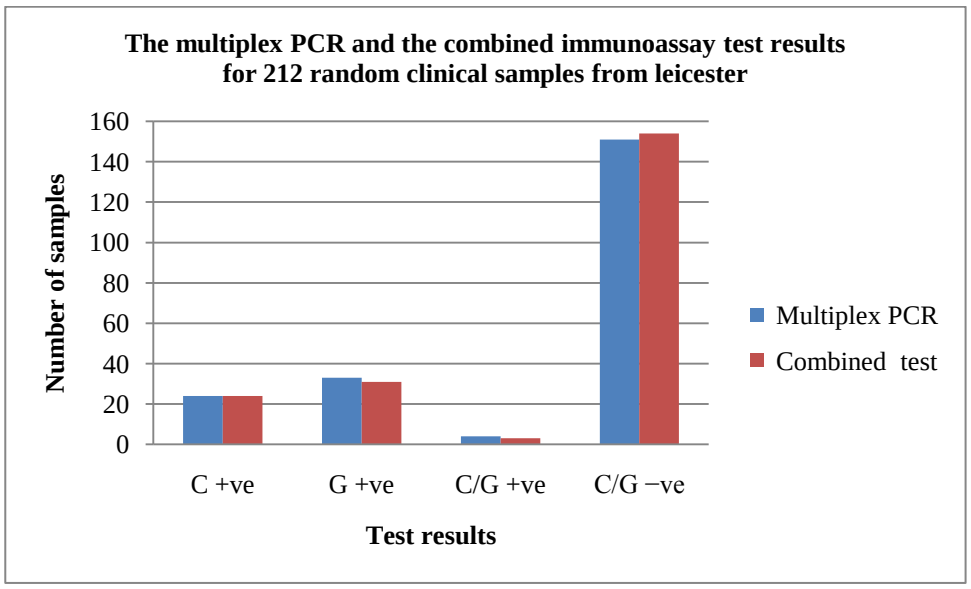


Figure 6-8: A bar chart showing the multiplex PCR and the combined immunoassay test results for 212 random stool samples. The multiplex PCR picked two *G. lamblia* and one *Cryptosporidium*/*Giardia* (*C*/*G*) dual infection from those previously diagnosed negative samples by the combined immunoassay test. These three samples proved to be positives by the nested reference PCRs and confirmed further by sending the target amplicons for DNA sequencing. The overall agreement between both tests was 98.5 %.

6.3.7 Preliminary sequencing results

PCR amplification products of 24 samples including samples that showed discordant results among uniplex PCR, multiplex PCR and the combined copro-antigen detection test were purified from the agarose gel and sent for DNA sequencing. The retrieved target DNA sequences were compared to those available in the GenBank database with the BLASTN program run on the National Center for Biotechnology information Server (http://www.ncbi.nlm.nih.gov/BLAST). All 24 amplicon-derived sequences showed >99 % homology with sequences stored in the GenBank, six sequences for *C. hominis*, five for *C. parvum*, six for *G. lamblia* assemblage A and seven for *G. lamblia* assemblage B.

6.3.8 Diagnostic performance of the *Giardia*/*Cryptosporidium* Check™ test

Based on the multiplex PCR test results, the diagnostic sensitivity, specificity, negative predictive value and positive predictive value of the *Giardia*/*Cryptosporidium Check*™ EIA test was 95 % (58/61), 90 % (136/151), 97.8 % and 79.4 %, respectively. On the other hand, the diagnostic sensitivity, specificity, negative predictive value and positive predictive value of the three-kits based immunoassay test were ~95 % (58/61), 100 % (151/151), 98 % and 79 %, respectively.

6.3.9 Operational characteristics of the multiplex PCR assay

Extraction of the random clinical samples was carried out in 24-samples batches. Samples were processed in 90 – 120 minutes by the modified QIAamp® Stool Mini Kit DNA extraction protocol. PCR reaction preparation and subsequent amplification took around 150 min. PCR amplification products were analysed in batches of 24 by ethidium bromide-stained gel electrophoresis, a procedure requiring ~60 min. A simple table microfuge, heating block, gradient block-based thermocycler and gel electrophoresis apparatus were the basic equipment used in performing the tests. The price of testing each clinical sample, including the necessary supporting controls and estimated 20 % re-testing or wastage likely to be incurred during routine use, was US$6 per specimen (**Table 6-5**). This compared favorably with the consumable costs associated with the current commercial three-kit immunoassay strategy, with a cost per specimen of about US$6 as well based on approximately a third of the specimens requiring both screening and discriminatory testing.

Table 6-5: The Cost-effectiveness and turnaround time for the multiplex PCR assay.

Step	Price per sample[2]	Batch size[1]	Hands on time	Time to complete
DNA extraction	~£2.8	24	35 – 60 min	90 – 120 min
PCR amplification	~£1	26	~30 min	~150 min
Gel electrophoresis	~£1	27	15 – 30 min	60 – 90 min
Total	~£4 (~US$6)	24	80 – 120 min	4 – 6 hours

[1]; including positive and negative controls
[2]; Price was roughly estimated excluding the consumables

6.3.10 Operational characteristics of the *Giardia*/*Cryptosporidium* Check™ test

Approximately 96 stool samples can be screened by *Giardia*/*Cryptosporidium Check*™ EIA test in 2 – 4 hours through a fully automated system. Pre-screened positive samples have to be subjected to two other discriminatory tests. Each test sample required additional 5 min per sample. Sample screened negative cost ~£2.5 while the positive one required ~£4 more (see **Table 6-6)**.

Table 6-6: The Cost-effectiveness and turnaround time for *Giardia*/*Cryptosporidium Check*™ test

Parameter	*Giardia*/*Cryptosporidium Check*™ test[1]
Batch size	96 samples
Hands on time	~2 hours
Time to completion	2 – 4 hours
Price per sample	£2.5 – 7*
Supplementary tests for positive samples†	RIDA®Quick *Cryptosporidium* and RIDA®Quick *Giardia* lateral flow tests

[1]This screening test was done through a fully automated system
*sample screened as negative costs ~£2.5 while that screened as positive costs ~£5 more.
†samples screened negatives require no additional test.

6.4 Discussion

The multiplex PCR assay was evaluated using a fairly large number of clinical stool samples and proved to be a simple and an economical screening tool in diagnostic laboratories.

To address the performance of the multiplex PCR assay, a composite reference standard test was adopted in this study to select the control clinical samples. This composite test was based on three different diagnostic methods with variable sensitivities and specificities. The apparently complex method adopted in selection of the control samples was used for the following reasons: Firstly, the target protozoa particularly *Cryptosporidium* spp. cannot be easily cultured. It therefore appeared that, relying on protozoal culture results as a gold standard method was not possible. Secondly, in spite of the high sensitivity and specificity reported for the majority of the previously developed PCR assays, none of these assays has been standardized sufficiently to be considered as a sole reference test. Furthermore, although the commercially available copro-antigen detection kits have shown sensitivities and specificities higher than microscopic based methods, non-of these kits has been widely accepted as a gold standard. Finally, adoption of more than one method for selection of the control samples increases the quality of the control sets defined and the potential use of the criteria used to define these sets as future gold standards.

On the other hands, the use of a composite reference test in selection of the control samples carried two major disadvantages. Firstly, selection of the control sample could lead to selection bias. Secondly, application of a reference nested PCR, copro-antigen detection kit as well as microscopic diagnosis on a large number of clinical samples

would have been expensive, laborious and time consuming. For these two reasons, a modified composite reference test was used instead. Based on the previously reported sensitivities of the three diagnostic methods and with some assumption taken, the result of the composite reference test was interpreted as follows; firstly, the stool sample that tested positive by microscopy was assumed to contain high parasite loads. Secondly, samples that were negative by microscopy but diagnosed as positive by one or both of the other tests were likely to have only moderate numbers of the target protozoon. Finally, samples that tested positive by the nested PCR only and were negative by the copro-antigen detection assay and microscopy was considered to have low parasite loads.

The diagnostic sensitivity and specificity of the multiplex PCR assay appeared equivalent to that of the uniplex PCR assays. This was probably due to the adequate optimisation of the PCR components and the low frequency of dual infections among the control samples used for the initial multiplex PCR assay validation. Three faecally-derived protozoal DNA were amplified by the nested reference PCR and could not be identified by the diagnostic uniplex and the multiplex PCR assays. This is probably due to the lower sensitivity of the single-round PCR compared to the nested PCR assays. The diagnostic sensitivity of the *G. lamblia* uniplex PCR was slightly lower than that achieved by *Cryptosporidium* and *E. histolytica* uniplex PCR. This may be related to the presence of three degenerate nucleotide bases present in the sequences of the two primers.

The multiplex PCR assay was subjected to further evaluation using 212 random stool samples. The *G. lamblia* and *Cryptosporidium* spp. specific DNA was identified in 37 (17 %) and 28 (13 %) samples respectively. The relatively high rate of infection does

not reflect the true prevalence of these infections and may be related to two factors: Firstly, these samples were collected within a short period between June and October, 2009 and these protozoa, from data analysis of the year preceeding the study, have shown high prevalence rates during this period of the year (see **section 2.1)**. This goes with other studies that reported the high prevalence of *Cryptosporidium* in UK within these months (Baxby and Hart, 1986). Secondly, this clinical study was applied on diarrheal stool samples only increasing the possibility for protozoal detection. The overall agreement between the multiplex PCR and the uniplex PCR assays was around 98 % (208/212). Three *Giardia* positive samples were picked by the uniplex single-round and the nested reference PCR assays. Although the results were comparable, it is clear that the sensitivity of *G. lamblia* primers were slightly reduced with the presence of the other primers in the same reaction.

Concomitant infections with *Campylobacter* and *Salmonella* species were identified in five samples diagnosed as *G. lamblia* or *Cryptosporidium* positives. These results agree with several previous studies that have reported apparently high incidence of co-infection for *Campylobacter* or *Salmonella* infections in patients with enteric protozoal infection (Casemore *et al*., 1994). The apparently higher rates of co-infection than would be expected by pure chance probably relate to common faecal-oral routes of infection and similar breakdowns in personal and public hygiene protocols. Furthermore, these concomitant infections highlight the need for inclusion of the targets specific to these and other common enteric pathogens in the multiplex PCR assay of the future.

The overall agreement between the multiplex PCR assay results and the combined three-kit based copro-antigen test was ~98.5 %. In spite of this high percentage of

agreement, the copro-antigen based test resulted in many false positive results (n = 15) at a false positive rate of ~20 %, a recognized and previously reported outcome for the *Giardia*/*Cryptosporidium* copro-antigen detection rapid immunoassay tests (CDR, 2004). Samples with false positive results were picked by the secondary discriminatory kits. Although the final results were not affected as the samples were ultimately reported as negatives, these samples required unnecessary re-testing. Each sample that screened positive initially required re-testing with RIDA®Quick *Cryptosporidium* and RIDA®Quick *Giardia* immunochromatographic kits at a consumable-only additional cost per specimen of about £5.

When the multiplex PCR test results were taken as a gold standard, the *Giardia*/*Cryptosporidium Check*™ EIA test (TechLab) showed sensitivity and specificity of ~95 % (58/61) and ~90 % (136/151), respectively. This disagreed with a recent study has reported a sensitivity and specificity of 98.4 % and 100 %, respectively for the above kit (Youn *et al.*, 2008). The discordance between the two studies probably relates to the gold standard test adopted in each study. Youn and colleagues had employed the alternative commercially available copro-antigen detection EIA kits *Giardia* II and *Cryptosporidium* II (TechLab) as gold standards; whereas in our study, the multiplex PCR assay developed was the gold standard for the above comparison.

The combined copro-antigen detection test showed a sensitivity of ~95 % (58/61) and specificity of 100 % (151/151). In view of these figures, it is clear that the multiplex PCR and the combined copro-antigen test gave comparable results. The choice between both assays should depend upon the available laboratory resources, the number of specimens likely to be tested, the intended turn-around time and the extent of the infection in each population. The combined copro-antigen test can be very useful in

population with low prevalence of *Cryptosporidium* and *Giardia* infections. A large number of samples can be screened with much ease of use and less hand-on time than the multiplex PCR. On the other hand, the multiplex PCR assay may be more useful in a population with high prevalence of these protozoal infections. Furthermore, the multiplex PCR assay allows identification of parasite species/genotypes whenever genotyping is proposed as in infection outbreak management. The *Cryptosporidium* spp. and *G. lamblia* targeted DNA sequences can be used to define the species and/or the genotypes of the protozoa either by the restriction digestion patterns on gel as previously reported (Spano *et al*., 1997 and Read *et al*., 2004) or by sending the PCR products for DNA sequencing. In this study, although the number of samples sent for sequencing was relatively small, the preliminary results were informative. *C. hominis* and *C. parvum*, the two widely accepted *Cryptosporidium* spp. as a cause of most of human infections (Morgan *et al.*, 1999a and Coupe *et al*., 2004), were also the only two species detected in the 11 samples tested. This finding is in line with previous studies (Morgan *et al.,* 1999a). Similarly, *G lamblia* assemblage A and B, the two major genotypes that have been reported in human infections (Amar *et al*., 2002; Guy *et al*., 2004), were ientified in 13 samples tested.

Further improvements for the multiplex PCR assay can be done through automation of the assay. The simple DNA extraction method and the single-round PCR amplification make the assay amenable for automation. Finally, the block-based DNA amplification and the use of a direct DNA extraction method allow for ready inclusion of targets specific for several other bacterial and viral enteric pathogens that are likely to be detected in this same specimen type, diarrheal stool samples.

In summary, the multiplex PCR assay performed well with the faecally-derived DNA samples extracted with the modified QIAamp® Stool Mini Kit DNA extraction protocol. The DNA extraction protocol proved to be effective in extracting and purifying the protozoal DNA directly from the same stool specimen. The multiplex PCR assay showed a comparable diagnostic sensitivity and specificity with the uniplex PCR assays. In addition, the multiplex PCR assay developed was marginally superior in absolute numbers, though not to the point of statistical significance, as compared to the routine three-kit combined copro-antigen detection test strategy currently utilized for routine enteric parasite diagnostics at the Clinical Microbiology laboratory, UHL. Baring cost and personnel training issues, the three-protozoon multiplex PCR assay developed has real potential to be adopted as routine test in diagnostic microbiology laboratories, especially those present in high prevalence countries.

7 Conclusion and future work

7.1 Conclusion

A multiplex PCR-based assay was developed for the simultaneous identification of *Cryptosporidium*, *G. lamblia* and *E. histolytica*, the three predominant enteric protozoa in human infections. Under the test conditions previously prescribed (sample submission, sample storage, DNA extraction, target DNA amplification, detection and analysis), the multiplex PCR assay proved to be simple, rapid, cost-effective, showed an adequate sensitivity and high specificity features that enable its use as a screening test in the clinical laboratories. This assay is based on the followings; first, DNA extraction directly from 200µl faecal specimen using QIAamp Stool Mini Kit with minor modifications introduced over the manufacturer's protocol. The faecally-derived DNA was sufficiently purified and proved to be very compatible with PCR technique. Accordingly, no PCR inhibition was detected in all the clinical samples subjected to amplification. Together with the subsequent PCR protocol, it was able to directly extract and amplify the target-specific DNA from as low as 500 (oo)cysts/gram of stool. Secondly, the faecally-derived protozoal DNA was successfully amplified in Techne-TC4000 thermal cycler using 35-40 amplification cycles. Based on previously published oligonucleotide primers, three PCR assays were developed and validated as uniplex PCR assays for identification of the corresponding protozoon in stool specimens before multiplexing. Internal and external standard controls were also constructed and validated in this study, and were included in the corresponding PCR assays to monitor the amplification procedure. The Go-Taq Hot Start polymerase, 1% DMSO and the touch-down profile adopted in PCR protocol proved to be very effective

in increasing the specificity and sensitivity of the assay. The in-house constructed standard amplification controls proved to be very helpful in monitoring the amplification procedure, and in ruling out any detectable PCR inhibition. The Go Taq green buffer used in the assay allowed loading the products of amplification directly on gels saving considerable time for the whole assay without any affecting downstream application of the amplification products such as restriction digestion and DNA sequence analysis.

The analytical sensitivity demonstrated by single-round uniplex PCR assays were comparable to, if not better, than those of the previous studies. Subsequently, the three uniplex PCR assays were combined into a single-round multiplex PCR assay that demonstrated an adequate analytical sensitivity equivalent to that of the individual PCR assays.

The clinical performance of PCR assays was addressed initially using a large group of control clinical stool samples. The uniplex and multiplex PCR assays both exhibited adequate and comparable diagnostic sensitivities and specificities as follows: Firstly, the multiplex PCR as well as *Cryptosporidium* uniplex PCR failed to identify just one out of 50 *Cryptosporidium*-positives control samples with sensitivity reaching 98% and NPV of 98.5%. Secondly, the multiplex PCR as well as *Giardia* uniplex PCR failed to identify two out of 50 *Giardia*- positives samples, with sensitivity reaching 96% and NPV of 97%. Thirdly, the multiplex PCR as well as *Entamoeba* uniplex have successfully identified all 15 *E. histolytica*-positive samples demonstrating a sensitivity and NPV reaching 100%. Finally, the multiplex PCR as well as the three uniplex assays showed no amplifications in the 70 samples selected as negative controls with specificity and PPV reaching 100%.

The multiplex PCR assay was further validated on a large group of random stool samples with blinded conventional test results as generated by routine testing procedures at the Clinical Microbiology laboratory, Leicester Royal Infirmary, University Hospitals of Leicester. The multiplex PCR identified 61 positives out of 212 test samples (24 *Cryptosporidium* and 33 *G. Lamblia*, 4 *Cryptosporidium*/*Giardia* double infections and 0 *E. histolytica).* The multiplex PCR showed perfect agreement with *Cryptosporidium* and *Entamoeba* uniplex PCR assays. However, the multiplex PCR missed identification of 3 *Giardia* DNA that were diagnosed by the uniplex PCR and confirmed by the nested reference PCR, as well as DNA sequence analysis with an overall agreement of 98.5%.

Further evaluation of the multiplex PCR assay was carried out through estimating the percent agreement between the assay and the commercial copro-antigens detection kits, individually and combined, using 212 randomly-selected clinical samples-results. The screening EIA kit failed to identify the copro-antigens for two *Giardia*-positive samples which were successfully diagnosed by the multiplex PCR and confirmed by the lateral flow discriminatory kits demonstrating sensitivity of 95% and NPV of 97.8%. Furthermore, the screening EIA kit falsely diagnosed 15 samples as *Cryptosporidium* and/or *Giardia* copro-antigens positive that were found to be negative by the multiplex PCR and confirmed as negative by the discriminatory kits achieving specificity of 90% and PPV of 79%. The overall agreement of the multiplex PCR-results and those of the screening kit were 91%. However, with inclusion of secondary discriminatory tests-results, the overall agreement was raised to 98.5%. Importantly, no false positive results were reported in the multiplex PCR assay in any of the 212 test samples. Equally important, no PCR amplification inhibition was demonstrated for the DNA

samples retrieved directly from faecal specimens with the modified QIAamp® Stool Mini Kit DNA extraction protocol.

The operational characteristics of the multiplex PCR versus the combined copro-antigens detection assays were put in preliminary comparison. Twenty-four samples were prepared and screened by the multiplex PCR in 4 – 6hs, versus 96 samples screened for parasite copro-antigens with the combined immunoassay in 2 – 4hs. The price per sample tested by the multiplex PCR was roughly estimated to be ~ US$6 versus US$2.5 –7 for the immunoassay test.

In view of these results, although the multiplex PCR demonstrated better performance in absolute numbers, the overall performance with the combined immunoassay test was comparable. Therefore, the selection between the two assays has to be subjected to factors such as the laboratory facilities, aims, and prevalence of protozoal infections in the population. In other words, the copro-antigen detection assay will do better in populations with low burdens of protozoal infections, while the multiplex PCR will perform better in populations with higher prevalence rates and in the presence of well-trained PCR-operators. The multiplex PCR assay will be of great benefit when the genotyping of the protozoa is needed as a part of infection control, or in studying the epidemiology of infection outbreaks.

In view of the results of this study, the multiplex PCR assay should be suitable for co-implementation, and in some cases, possibly replacement of existing diagnostic procedures and assays, after larger scale comparison studies to the existing diagnostic methods have been carried out.

7.2 Future work

The multiplex PCR, developed in the study with standardized protocol directed towards the detection of protozoal pathogens in such a difficult material as faeces, proved to be more sensitive and more specific than the commercial copro-antigens kit- and the microscopically-based conventional diagnostic methods used for identification of the three target protozoa since it is based on stable genotypic characteristics, rather than relying on less-sensitive and less-specific morphological or biochemical traits.

The economic burden of managing the false-negative or false-positive results that commonly happen with adoption of less sensitive and less specific conventional diagnostic assays has to be considered while selecting a diagnostic laboratory test. Patient with false-negative test results require returning to the health services, re-testing and prolonged absence from school or work. Furthermore, patient with false-negative test results very often take wrongly-prescribed non-effective medications which increases the potential for greater spreading of the infection to the environment. Similarly, false-positive results not only have a worse impact on patient management, but can also over-estimate the epidemiological picture of the infection in the community as well.

Conversely, the multiplex PCR under the conditions recommended has a number of advantages: The assay requires just 200µl of stool for identification of the three target protozoa. In principal, no large-volume samples, or multiple samples, are required for a correct diagnosis. By the high performance rates achieved, and simple protocols adopted in the assay, the multiplex PCR assay provides consistant results within the same day of sampling, improving the clinical output, time to diagnosis directly providing help in patient management.

In addition to the high performance rates achieved from the application of the in-house multiplex PCR as a screening tool in the clinical laboratories, archiving the faecally-derived nucleic acid samples, as a part of the diagnostic procedures, will also be of great value when identification of the protozoal genotype, or isolate populations is an aim such as might occur in infections-control, or the managing of infection outbreaks. By giving the aforementioned cost-benefit outputs a greater consideration, the three-protozoon multiplex PCR developed in this study offers the promise of a cost- and time-efficient tool for screening a large number of diarrheal stool samples for these enteric infections especially in resource-poor countries where the infections are prevalent.

It remains, however, to investigate further the utility, ease of routine implementation, costs and performance characteristics of the multiplex assay reported in this study by conducting a larger prospective clinical study. This would include stool samples representative of more than one human population from various geographic locations covering both endemic and non-endemic regions. There are many reasons for conducting such study as follows; Firstly, the multiplex PCR developed in the study was evaluated using clinical samples from a limited population (the UK), a group which also exhibited negligible rates of *E. histolytica* infection. Deficent clinical details to correlate test-results to the clinical state of the patients were also an issue. Secondly, the variability of the constituents of the feacal materials among populations can cause differences in DNA extraction, and have to be tested in the field. Genetic mutations could also happen for a clinical isolate of one population, which could affect the hybridization of the primers with the genomic DNA of an isolate. Moreover, the laboratory infra-structure, availability of reagents and equipments, the experience of PCR-operator, all these elements could alter reproducibility of the PCR assay and has

to be evaluated. Finally, cost-benefits studies have to be investigated and interpreted individually for each population in view of the available conventional diagnostic methods and the prevalence of the protozoal infections.

Additionally, many improvements could be investigated to further develop the multiplex PCR assay. Given the aforementioned advantages, the multiplex PCR could be improved by inclusion of more targets of clinically-related and epidemiologically relevant diarrhea-causing enteric pathogens (viruses, bacteria, and/or parasites) for detection. Many of the diarrhea-causing enteric pathogen have similar presentation, and cannot always be differentiated on the bases of the clinical pictures presented. Tests based on multiple target pathogens detection may improve the outcome of patients with diarrhea, by shortening the time to a positive result, reducing the overall costs of diagnostics and offering early prescription of the optimal treatment. Accordingly, such a test would be well-accepted as a wide spectrum screening and diagnostic tool for 'enteric infections'. However, the modifications introduced over the DNA extraction kit protocol have to be investigated to confirm no impact on an extraction of DNA for the new bacterial or viral pathogen individually and in multiplex format. Moreover, the simple kit-based extraction protocol and the single-round PCR amplification adopted in the multiplex PCR assay make the assay highly amenable for automation. Partial or complete automation of the multiplex PCR assay should be investigated to decrease turn-around time, reduce human error,77 and increase reproducibility of the assay.

Finally, like any new diagnostic assay, evaluation of the multiplex PCR has to be carried out continuously and further improvements have to be searched for in view of the rapidely evolving PCR technology with the subsequent reduction in prices of the excisting PCR reagents and equipments.

8 References

Anonymous. 1997. WHO/PAHO/UNESCO report. A consultation with experts on amoebiasis. Epidemiological Bulletin PAHO 18: 13-14.

Abbaszadegan, M.R., Velayati, A., Tavasoli, A. and Dadkhah, E., 2007. Rapid DNA extraction protocol from stool, suitable for molecular genetic diagnosis of colon cancer. *Iran.Biomed.J.*, **11,** 203-208.

Abdulmawjood, A., Roth, S. and Bulte, M., 2002. Two methods for construction of internal amplification controls for the detection of *Escherichia coli* O157 by polymerase chain reaction. *Mol.Cell.Probes,* **16,** 335-339.

Abrahamsen, M.S., Templeton, T.J., Enomoto, S., Abrahante, J.E., Zhu, G., Lancto, C.A., Deng, M., Liu, C., Widmer, G., Tzipori, S., Buck, G.A., Xu, P., Bankier, A.T., Dear, P.H., Konfortov, B.A., Spriggs, H.F., Iyer, L., Anantharaman, V., Aravind, L., Kapur, V., 2004. Complete Genome Sequence of the Apicomplexan, *Cryptosporidium* parvum. *Science.* **304,** 441-445.

Acuna-Soto, R., Samuelson, J., De Girolami, P., Zarate, L., Millan-Velasco, F., Schoolnick, G. and Wirth, D., 1993. Application of the polymerase chain reaction to the epidemiology of pathogenic and nonpathogenic *Entamoeba histolytica*. *Am.J.Trop.Med.Hyg.,* **48,** 58-70.

Adam, A.A., Hassan, H.S., Shears, P. and Elshibly, E., 1994. *Cryptosporidium* in Khartoum, Sudan. *East Afr.Med.J.,* **71,** 745-746.

Adam, R.D., 2001. Biology of *Giardia lamblia*. *Clin.Microbiol.Rev.,* **14,** 447-475.

Ajjampur, S.S.R., Gladstone, B.P., Selvapandian, D., Muliyil, J.P., Ward, H., Kang, G., 2007. Molecular and spatial epidemiology of cryptosporidiosis in children in a semiurban community in South India. Journal of Clinical Microbiology 45, 915 – 920.

Albaugh, G.P., Iyengar, V., Lohani, A., Malayeri, M., Bala, S. and Nair, P.P., 1992. Isolation of exfoliated colonic epithelial cells, a novel, non-invasive approach to the study of cellular markers. *Int.J.Cancer,* **52,** 347-350.

Ali, S.A. & Hill, D.R., 2003. *Giardia intestinalis*. *Curr.Opin.Infect.Dis.,* **16,** 453-460.

Amar, C., Pedraza-Díaz, S. and McLauchlin, J., 2001. Extraction and genotyping of *Cryptosporidium parvum* DNA from fecal smears on glass slides stained conventionally for direct microscope examination. *J.Clin.Microbiol.,* **39,** 401-403.

Amar, C.F., Dear, P.H., Pedraza-Diaz, S., Looker, N., Linnane, E. and McLauchlin, J., 2002. Sensitive PCR-restriction fragment length polymorphism assay for detection and genotyping of *Giardia duodenalis* in human feces. *J.Clin.Microbiol.,* **40,** 446-452.

Amar, C.F.L., Chalmers, R.M., Elwin, K., Tynan, P. and McLauchlin, J., 2002. Blinded evaluation of DNA extraction and genotyping of stained *Cryptosporidium* on glass slides. *Lett.Appl.Microbiol.,* **35,** 486-488.

Amar, C.F.L., Dear, P.H. and McLauchlin, J., 2003. Detection and genotyping by real-time PCR/RFLP analyses of *Giardia duodenalis* from human faeces. *J.Med.Microbiol.,* **52,** 681-683.

Aronson, N.E., Cheney, C., Rholl, V., Burris, D. and Hadro, N., 2001. Biliary giardiasis in a patient with human immunodeficiency virus. *J.Clin.Gastroenterol.,* **33,** 167-170.

Arrowood, M.J., 2002. *In vitro* cultivation of *Cryptosporidium* species. *Clin.Microbiol.Rev.,* **15,** 390-400.

Baxby, D. & Hart, C.A., 1986. The incidence of cryptosporidiosis: a two-year prospective survey in a children's hospital. *J.Hyg.(Lond),* **96,** 107-111.

Bernander, R., Palm, J.E. and Svard, S.G., 2001. Genome ploidy in different stages of the *Giardia lamblia* life cycle. *Cell.Microbiol.,* **3,** 55-62.

Berrilli, F., Di Cave, D., D'Orazi, C., Orecchia, P., Xhelilaj, L., Bejko, D., Caca, P., Bebeci, D., Cenko, F., Donia, D. and Divizia, M., 2006. Prevalence and genotyping of human isolates of *Giardia duodenalis* from Albania. *Parasitol.Int.,* **55,** 295-297.

Bertrand, I., Albertini, L. and Schwartzbrod, J., 2005. Comparison of two target genes for detection and genotyping of *Giardia lamblia* in human feces by PCR and PCR-restriction fragment length polymorphism. *J.Clin.Microbiol.,* **43,** 5940-5944.

Bhatia, V.N. & Warhurst, D.C., 1981. Hatching and subsequent cultivation of cysts of *Giardia intestinalis* in Diamond's medium. *J.Trop.Med.Hyg.,* **84,** 45.

Birnboim, H.C. & Doly, J., 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.,* **7,** 1513-1523.

Blessmann, J., Buss, H., Nu, P.A.T., Dinh, B.T., Ngo, Q.T.V., Van, A.L., Alla, M.D.A., Jackson, T.F.H.G., Ravdin, J.I. and Tannich, E., 2002. Real-time PCR for detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* in fecal samples. *J.Clin.Microbiol.,* **40,** 4413-4417.

Blessmann, J. & Tannich, E., 2002. Treatment of asymptomatic intestinal *Entamoeba histolytica* infection [7]. *N.Engl.J.Med.,* **347,** 1384.

Boehmer, T.K., Alden, N.B., Ghosh, T.S. and Vogt, R.L., 2009. Cryptosporidiosis from a community swimming pool: outbreak investigation and follow-up study. *Epidemiol.Infect.,* **137,** 1651-1654.

Brodsky, R.E., Spencer, H.C.,Jr and Schultz, M.G., 1974. Giardiasis in American travelers to the Soviet Union. *J.Infect.Dis.,* **130,** 319-323.

Burkardt, H.J., 2000. Standardization and quality control of PCR analyses. *Clin.Chem.Lab.Med.,* **38,** 87-91.

Bushen, O.Y., Kohli, A., Pinkerton, R.C., Dupnik, K., Newman, R.D., Sears, C.L., Fayer, R., Lima, A.A. and Guerrant, R.L., 2007. Heavy cryptosporidial

infections in children in northeast Brazil: comparison of *Cryptosporidium hominis* and *Cryptosporidium parvum* . *Trans.R.Soc.Trop.Med.Hyg.,* **101,** 378-384.

Caccio, S.M., 2003. Molecular techniques to detect and identify protozoan parasites in the environment. *Acta Microbiol.Pol.,* **52 Suppl,** 23-34.

Cacciò, S.M., De Giacomo, M. and Pozio, E., 2002. Sequence analysis of the β-giardin gene and development of a polymerase chain reaction-restriction fragment length polymorphism assay to genotype *Giardia duodenalis* cysts from human faecal samples. *Int.J.Parasitol.,* **32,** 1023-1030.

Caccio, S.M. & Pozio, E., 2006. Advances in the epidemiology, diagnosis and treatment of cryptosporidiosis. *Expert Rev.Anti Infect.Ther.,* **4,** 429-443.

Cacciò, S.M., Thompson, R.C.A., McLauchlin, J. and Smith, H.V., 2005. Unravelling *Cryptosporidium* and *Giardia* epidemiology. *Trends Parasitol.,* **21,** 430-437.

Calderaro, A., Gorrini, C., Bommezzadri, S., Piccolo, G., Dettori, G. and Chezzi, C., 2006. *Entamoeba histolytica* and *Entamoeba dispar*: Comparison of two PCR assays for diagnosis in a non-endemic setting. *Trans.R.Soc.Trop.Med.Hyg.,* **100,** 450-457.

Casemore, D.P., 1990. Epidemiological aspects of human Cryptosporidiosis. *Epidemiol.Infect.,* **104,** 1-28.

Casemore, D.P., Armstrong, M. and Sands, R.L., 1985. Laboratory diagnosis of cryptosporidiosis. *J.Clin.Pathol.,* **38,** 1337-1341.

Casemore, D.P., Gardner, C.A. and O'Mahony, C., 1994. Cryptosporidial infection, with special reference to nosocomial transmission of *Cryptosporidium parvum*: a review. *Folia.Parasitol.(Praha),* **41,** 17-21.

CDR 2001 Surveillance of waterborne disease and water quality: January to June 2001, and summary of 2000, *CDR Wkly* **11** (45), 8 November 2001.

CDR 2002 Surveillance of waterborne disease and water quality: January to June 2002, and summary of 2001, *CDR Wkly* **12** (37), 12 September 2002.

CDR 2003 Surveillance of waterborne disease and water quality: January to June 2003, and summary of 2002, *CDR Wkly* **13** (41), 9 October 2003.

Centers for Disease Control and Prevention (CDC) (2004) Manufacturer's recall of rapid cartridge assay kits on the basis of false-positive *Cryptosporidium* antigen tests—Colorado, 2004. MMWR 53:198

Chalmers, R.M., Hadfield, S.J., Jackson, C.J., Elwin, K., Xiao, L. and Hunter, P., 2008. Geographic linkage and variation in *Cryptosporidium hominis*. *Emerging Infectious Diseases,* **14,** 496-498.

Chalmers, R.M., Robinson, G., Elwin, K., Hadfield, S.J., Xiao, L., Ryan, U., Modha, D. and Mallaghan, C., 2009. *Cryptosporidium* sp. rabbit genotype, a newly identified human pathogen. *Emerg.Infect.Dis.,* **15,** 829-830.

Chen, K.T., Chen, C.J. and Chiu, J.P., 2001. A school waterborne outbreak involving both *Shigella sonnei* and *Entamoeba histolytica*. *J.Environ.Health,* **64,** 9-13, 26.

Cheun, H.-., Choi, T.-., Chung, G.-., Cho, S.-., Lee, Y.-., Kimata, I. and Kim, T.-., 2007. Genotypic characterization of *Cryptosporidium* oocysts isolated from healthy people in three different counties of Korea. *Journal of Veterinary Medical Science,* **69,** 1099-1101.

Church, D., Miller, K., Lichtenfeld, A., Semeniuk, H., Kirkham, B., Laupland, K. and Elsayed, S., 2005. Screening for *Giardia*/*Cryptosporidium* infections using an enzyme immunoassay in a centralized regional microbiology laboratory. *Arch.Pathol.Lab.Med.,* **129,** 754-759.

Clark, C.G. & Diamond, L.S., 1991. Ribosomal RNA genes of 'pathogenic' and 'nonpathogenic' *Entamoeba histolytica* are distinct. *Mol.Biochem.Parasitol.,* **49,** 297-302.

Clark, C.G., Kaffashian, F., Tawari, B., Windsor, J.J., Twigg-Flesner, A., Davies-Morel, M.C.G., Blessmann, J., Ebert, F., Peschel, B., Le Van, A., Jackson, C.J., Macfarlane, L. and Tannich, E., 2006. New insights into the phylogeny of *Entamoeba* species provided by analysis of four new small-subunit rRNA genes. *Int.J.Syst.Evol.Microbiol.,* **56,** 2235-2239.

Clark, D.P., 1999. New insights into human Cryptosporidiosis. *Clin.Microbiol.Rev.,* **12,** 554-563.

Cedillo-Rivera, R., Leal, Y.A., Yépez-Mulia, L., Gómez-Delgado, A., Ortega-Pierres, G., Tapia-Conyer, R. and Muñoz, O., 2009. Seroepidemiology of giardiasis in Mexico. Am.J.Trop.Med.Hyg., 80, 6-10.

Cooper, M.A., Adam, R.D., Worobey, M. and Sterling, C.R., 2007. Population Genetics Provides Evidence for Recombination in *Giardia*. *Current Biology,* **17,** 1984-1988.

Coupe, S., Sarfati, C., Hamane, S. and Derouin, F., 2005. Detection of *Cryptosporidium* and identification to the species level by nested PCR and restriction fragment length polymorphism. *J.Clin.Microbiol.,* **43,** 1017-1023.

Cubero, J., van der Wolf, J., van Beckhoven, J. and Lopez, M.M., 2002. An internal control for the diagnosis of crown gall by PCR. *J.Microbiol.Methods*, **51,** 387-392.

Current, W.L. & Garcia, L.S., 1991. Cryptosporidiosis. *Clin.Lab.Med.,* **11,** 873-895.

Current, W.L., Reese, N.C. and Ernst, J.V., 1983. Human cryptosporidiosis in immunocompetent and immunodeficient persons. Studies of an outbreak and experimental transmission. *N.Engl.J.Med.,* **308,** 1252-1257.

Current, W.L., Upton, S.J. and Haynes, T.B., 1986. The life cycle of *Cryptosporidium baileyi* n. sp. (Apicomplexa, Cryptosporidiidae) infecting chickens. *J.Protozool.,* **33,** 289-296.

da Silva, A.J., Bornay-Llinares, F.J., Moura, I.N., Slemenda, S.B., Tuttle, J.L. and Pieniazek, N.J., 1999. Fast and reliable extraction of protozoan parasite DNA from fecal specimens. *Mol.Diagn.,* **4,** 57-64.

Dalle, F., Roz, P., Dautin, G., Di-Palma, M., Kohli, E., Sire-Bidault, C., Fleischmann, M.G., Gallay, A., Carbonel, S., Bon, F., Tillier, C., Beaudeau, P. and Bonnin, A., 2003. Molecular characterization of isolates of waterborne *Cryptosporidium* spp. collected during an outbreak of gastroenteritis in South Burgundy, France. *J.Clin.Microbiol.,* **41,** 2690-2693.

DALY, E.R., ROY, S.J., BLANEY, D.D., MANNING, J.S., HILL, V.R., XIAO, L. and STULL, J.W., 2009. Outbreak of giardiasis associated with a community drinking-water source. *Epidemiol.Infect.,* 1-10.

Diamond, L.S., 1982. A new liquid medium for xenic cultivation of *Entamoeba histolytica* and other lumen-dwelling protozoa. *J.Parasitol.,* **68,** 958-959.

Diamond, L.S. & Clark, C.G., 1993. A redescription of *Entamoeba histolytica* Schaudinn, 1903 (Emended Walker, 1911) separating it from *Entamoeba dispar* Brumpt, 1925. *J.Eukaryot.Microbiol.,* **40,** 340-344.

Diamond, L.S., Harlow, D.R. and Cunnick, C.C., 1978. A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. *Trans.R.Soc.Trop.Med.Hyg.,* **72,** 431-432.

Ditrich, O., Palkovic, L., Sterba, J., Prokopic, J., Loudova, J. and Giboda, M., 1991. The first finding of *Cryptosporidium baileyi* in man. *Parasitol.Res.*, **77,** 44-47.

DuPont, H.L., Chappell, C.L., Sterling, C.R., Okhuysen, P.C., Rose, J.B. and Jakubowski, W., 1995. The infectivity of *Cryptosporidium parvum* in healthy volunteers. *N.Engl.J.Med.,* **332,** 855-859.

Edwards, U., Rogall, T., Blocker, H., Emde, M. and Bottger, E.C., 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res.,* **17,** 7843-7853.

Eisenstein, L., Bodager, D. and Ginzl, D., 2008. Outbreak of giardiasis and cryptosporidiosis associated with a neighborhood interactive water fountain-Florida, 2006. *J.Environ.Health,* **71,** 18-22.

Elwin, K., Chalmers, R.M., Hadfield, S.J., Hughes, S., Hesketh, L.M., Rothburn, M.M., Muller, T. and Hunter, P.R., 2007. Serological responses to *Cryptosporidium* in human populations living in areas reporting high and low incidences of symptomatic cryptosporidiosis. *Clin.Microbiol.Infect.,* **13,** 1179-1185.

Erlandsen, S.L. & Rasch, E.M., 1994. The DNA content of trophozoites and cysts of *Giardia lamblia* by microdensitometric quantitation of Feulgen staining and examination by laser scanning confocal microscopy. *J.Histochem.Cytochem.,* **42,** 1413-1416.

Erlich, H.A., 1989. Polymerase chain reaction. *J.Clin.Immunol.,* **9,** 437-447.

Escobedo, A.A., Almirall, P., Alfonso, M., Cimerman, S., Rey, S. and Terry, S.L., 2009. Treatment of intestinal protozoan infections in children. *Arch.Dis.Child.,* **94,** 478-482.

Ethelberg, S., Lisby, M., Vestergaard, L.S., Enemark, H.I., Olsen, K.E.P., Stensvold, C.R., Nielsen, H.V., Porsbo, L.J., Plesner, A.A.-. and Mølbak, K., 2009. A foodborne outbreak of *Cryptosporidium hominis* infection. *Epidemiol.Infect.,* **137,** 348-356.

Ey, P.L., Mansouri, M., Kulda, J., Nohynkova, E., Monis, P.T., Andrews, R.H. and Mayrhofer, G., 1997. Genetic analysis of *Giardia* from hoofed farm animals reveals artiodactyl-specific and potentially zoonotic genotypes. *J.Eukaryot.Microbiol.,* **44,** 626-635.

Fayer, R., Farley, C.A., Lewis, E.J., Trout, J.M. and Graczyk, T.K., 1997. Potential Role of the Eastern Oyster, Crassostrea virginica, in the Epidemiology of *Cryptosporidium parvum* . *Appl.Environ.Microbiol.,* **63,** 2086-2088.

Fayer, R., Morgan, U. and Upton, S.J., 2000. Epidemiology of *Cryptosporidium*: transmission, detection and identification. *Int.J.Parasitol.,* **30,** 1305-1322.

Fayer, R., Santin, M. and Trout, J.M., 2008. *Cryptosporidium ryanae* n. sp. (Apicomplexa: Cryptosporidiidae) in cattle (Bos taurus). *Vet.Parasitol.,* **156,** 191-198.

Fayer, R., Santin, M. and Xiao, L., 2005. *Cryptosporidium bovis* n. sp. (Apicomplexa: Cryptosporidiidae) in cattle (Bos taurus). *J.Parasitol.,* **91,** 624-629.

Fayer, R., Trout, J.M., Xiao, L., Morgant, U.M., Lal, A.A. and Dubey, J.P., 2001. *Cryptosporidium canis* n. sp. from domestic dogs. *J.Parasitol.,* **87,** 1415-1422.

Fayer, R. & Xiao, L., 2007. *Cryptosporidium* and cryptosporidiosis. 2nd ed. Boca Raton : London: IWA Publishing/CRC Press.

Filice, FP. Studies on the cytology and life history of a *Giardia* from the laboratory rat: *Univ California Publ Zool* 1952; 57: 53-146.

Fotedar, R., Stark, D., Beebe, N., Marriott, D., Ellis, J. and Harkness, J., 2007. PCR detection of *Entamoeba histolytica*, *Entamoeba dispar*, and *Entamoeba moshkovskii* in stool samples from Sydney, Australia. *J.Clin.Microbiol.,* **45,** 1035-1037.

Fotedar, R., Stark, D., Marriott, D., Ellis, J. and Harkness, J., 2008. *Entamoeba moshkovskii* infections in Sydney, Australia. *Eur.J.Clin.Microbiol.Infect.Dis.,* **27,** 133-137.

Freed, D.L., 2002. False-negative food challenges. *Lancet,* **359,** 980-981.

Furrows, S.J., Moody, A.H. and Chiodini, P.L., 2004. Comparison of PCR and antigen detection methods for diagnosis of *Entamoeba histolytica* infection. *J.Clin.Pathol.,* **57,** 1264-1266.

Galen, R.S., Gambino, S.R., 1975. Beyond Normality: The Predictive Value and Efficiency of Medical Diagnosis. John Wiley, New York, NY.

Garcia, L.S. & Shimizu, R.Y., 1997. Evaluation of nine immunoassay kits (enzyme immunoassay and direct fluorescence) for detection of *Giardia lamblia* and *Cryptosporidium parvum* in human fecal specimens. *J.Clin.Microbiol.,* **35,** 1526-1529.

Garcia, L.S., Shimizu, R.Y. and Bernard, C.N., 2000. Detection of *Giardia lamblia*, *Entamoeba histolytica*/*Entamoeba dispar*, and *Cryptosporidium parvum* antigens in human fecal specimens using the triage parasite panel enzyme immunoassay. *J.Clin.Microbiol.,* **38,** 3337-3340.

Gardner, A.L., Roche, J.K., Weikel, C.S. and Guerrant, R.L., 1991. Intestinal cryptosporidiosis: pathophysiologic alterations and specific cellular and humoral immune responses in rnu/+ and rnu/rnu (athymic) rats. *Am.J.Trop.Med.Hyg.,* **44,** 49-62.

Ghosh, S., Debnath, A., Sil, A., De, S., Chattopadhyay, D.J. and Das, P., 2000. PCR detection of *Giardia lamblia* in stool: Targeting intergenic spacer region of multicopy rRNA gene. *Mol.Cell.Probes,* **14,** 181-189.

Gillin, F.D., Reiner, D.S. and Boucher, S.E., 1988. Small-intestinal factors promote encystation of *Giardia lamblia* in vitro. *Infect.Immun.,* **56,** 705-707.

Glaberman, S., Moore, J.E., Lowery, C.J., Chalmers, R.M., Sulaiman, I., Elwin, K., Rooney, P.J., Millar, B.C., Dooley, J.S., Lal, A.A. and Xiao, L., 2002. Three drinking-water-associated cryptosporidiosis outbreaks, Northern Ireland. *Emerg.Infect.Dis.,* **8,** 631-633.

Gomez Morales, M.A., Ausiello, C.M., Guarino, A., Urbani, F., Spagnuolo, M.I., Pignata, C. and Pozio, E., 1996. Severe, protracted intestinal cryptosporidiosis associated with interferon γ deficiency: Pediatric case report. *Clinical Infectious Diseases,* **22,** 848-850.

Gonin, P. & Trudel, L., 2003. Detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* isolates in clinical samples by PCR and enzyme-linked immunosorbent assay. *J.Clin.Microbiol.,* **41,** 237-241.

Guy, R.A., Payment, P., Krull, U.J. and Horgen, P.A., 2003. Real-time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. *Appl.Environ.Microbiol.,* **69,** 5178-5185.

Guy, R.A., Xiao, C. and Horgen, P.A., 2004. Real-time PCR assay for detection and genotype differentiation of *Giardia lamblia* in stool specimens. *J.Clin.Microbiol.,* **42,** 3317-3320.

Hamzah, Z., Petmitr, S., Mungthin, M., Leelayoova, S. and Chavalitshewinkoon-Petmitr, P., 2006. Differential detection of *Entamoeba histolytica*, *Entamoeba dispar*, and *Entamoeba moshkovskii* by a single-round PCR assay. *J.Clin.Microbiol.,* **44,** 3196-3200.

Haque, R., Huston, C.D., Hughes, M., Houpt, E. and Petri, W.A.,Jr, 2003. Amebiasis. *N.Engl.J.Med.,* **348,** 1565-1573.

Haque, R., Mondal, D., Duggal, P., Kabir, M., Roy, S., Farr, B.M., Sack, R.B. and Petri, W.A.,Jr, 2006. *Entamoeba histolytica* infection in children and protection from subsequent amebiasis. *Infect.Immun.,* **74,** 904-909.

Haque, R., Neville, L.M., Hahn, P. and Petri, W.A.,Jr, 1995. Rapid diagnosis of *Entamoeba* infection by using *Entamoeba* and *Entamoeba histolytica* stool antigen detection kits. *J.Clin.Microbiol.,* **33,** 2558-2561.

Haque, R., Roy, S., Siddique, A., Mondal, U., Rahman, S.M., Mondal, D., Houpt, E. and Petri, W.A.,Jr, 2007. Multiplex real-time PCR assay for detection of *Entamoeba histolytica*, *Giardia intestinalis*, and *Cryptosporidium* spp. *Am.J.Trop.Med.Hyg.,* **76,** 713-717.

Hecker, K.H. & Roux, K.H., 1996. High and low annealing temperatures increase both specificity and yield in touchdown and stepdown PCR. *BioTechniques,* **20,** 478-485.

Heyman, M.B., Shigekuni, L.K. and Ammann, A.J., 1986. Separation of *Cryptosporidium* oocysts from fecal debris by density gradient centrifugation and glass bead columns. *J.Clin.Microbiol.,* **23,** 789-791.

Hill, D.R., 1993. Giardiasis. Issues in diagnosis and management. *Infect.Dis.Clin.North Am.,* **7,** 503-525.

Homan, W.L., Gilsing, M., Bentala, H., Limper, L. and van Knapen, F., 1998. Characterization of *Giardia duodenalis* by polymerase-chain-reaction fingerprinting. *Parasitol.Res.,* **84,** 707-714.

Hoorfar, J., Wolffs, P. and Radstrom, P., 2004. Diagnostic PCR: validation and sample preparation are two sides of the same coin. *APMIS,* **112,** 808-814.

Hooshyar, H., Rezaian, M., Kazemi, B., Jeddi-Tehrani, M. and Solaymani-Mohammadi, S., 2004. The distribution of *Entamoeba histolytica* and *Entamoeba dispar* in northern, central, and southern Iran. *Parasitol.Res.,* **94,** 96-100.

Hopkins, R.M., Meloni, B.P., Groth, D.M., Wetherall, J.D., Reynoldson, J.A. and Thompson, R.C., 1997. Ribosomal RNA sequencing reveals differences between the genotypes of *Giardia* isolates recovered from humans and dogs living in the same locality. *J.Parasitol.,* **83,** 44-51.

Hoque, M.E., Hope, V.T. and Scragg, R., 2002. *Giardia* infection in Auckland and New Zealand: Trends and international comparison. *N.Z.Med.J.,* **115,** 121-123.

Huang, D.B. & White, A.C., 2006. An updated review on *Cryptosporidium* and *Giardia*. *Gastroenterol.Clin.North Am.,* **35,** 291-314, viii.

Hung, C.-., Deng, H.-., Hsiao, W.-., Hsieh, S.-., Hsiao, C.-., Chen, M.-., Chang, S.-. and Su, K.-., 2005. Invasive amebiasis as an emerging parasitic disease in patients with human immunodeficiency virus type 1 infection in Taiwan. *Arch.Intern.Med.,* **165,** 409-415.

Hunter, P.R., Hadfield, S.J., Wilkinson, D., Lake, I.R., Harrison, F.C.D. and Chalmers, R.M., 2007. Subtypes of *Cryptosporidium parvum* in humans and disease risk. *Emerging Infectious Diseases,* **13,** 82-88.

Hunter, P.R., Hughes, S., Woodhouse, S., Syed, Q., Verlander, N.Q., Chalmers, R.M., Morgan, K., Nichols, G., Beeching, N. and Osborn, K., 2004. Sporadic cryptosporidiosis case-control study with genotyping. *Emerg.Infect.Dis.,* **10,** 1241-1249.

Iseki, M., Maekawa, T., Moriya, K., Uni, S. and Takada, S., 1989. Infectivity of *Cryptosporidium muris* (strain RN 66) in various laboratory animals. *Parasitol.Res.,* **75,** 218-222.

Jarroll, E.L., Bingham, A.K. and Meyer, E.A., 1981. Effect of chlorine on *Giardia lamblia* cyst viability. *Appl.Environ.Microbiol.,* **41,** 483-487.

Jokipii, L. & Jokipii, A.M., 1986. Timing of symptoms and oocyst excretion in human cryptosporidiosis. *N.Engl.J.Med.,* **315,** 1643-1647.

Jung, V., Pestka, S.B. and Pestka, S., 1990. Efficient cloning of PCR generated DNA containing terminal restriction endonuclease recognition sites. *Nucleic Acids Res.,* **18,** 6156.

Karanis, P., Kourenti, C. and Smith, H., 2007. Waterborne transmission of protozoan parasites: a worldwide review of outbreaks and lessons learnt. *J.Water.Health.,* **5,** 1-38.

Kaushik, K., Khurana, S., Wanchu, A. and Malla, N., 2008. Evaluation of staining techniques, antigen detection and nested PCR for the diagnosis of cryptosporidiosis in HIV seropositive and seronegative patients. *Acta Trop.,* **107,** 1-7.

Kebede, A., Verweij, J.J., Endeshaw, T., Messele, T., Tasew, G., Petros, B. and Polderman, A.M., 2004. The use of real-time PCR to identify *Entamoeba histolytica* and *E. dispar* infections in prisoners and primary-school children in Ethiopia. *Ann.Trop.Med.Parasitol.,* **98,** 43-48.

Khairnar, K. & Parija, S.C., 2007. A novel nested multiplex polymerase chain reaction (PCR) assay for differential detection of *Entamoeba histolytica*, *E. moshkovskii* and *E. dispar* DNA in stool samples. *BMC Microbiology*, **7,** .

Kohli, A., Bushen, O.Y., Pinkerton, R.C., Houpt, E., Newman, R.D., Sears, C.L., Lima, A.A. and Guerrant, R.L., 2008. *Giardia duodenalis* assemblage, clinical presentation and markers of intestinal inflammation in Brazilian children. *Trans.R.Soc.Trop.Med.Hyg.,* **102,** 718-725.

Kosek, M., Alcantara, C., Lima, A.A. and Guerrant, R.L., 2001. Cryptosporidiosis: An update. *Lancet Infectious Diseases,* **1,** 262-269.

Kramer, M.H., Herwaldt, B.L., Craun, G.F., Calderon, R.L. and Juranek, D.D., 1996. Surveillance for waterborne-disease outbreaks--United States, 1993-1994. *MMWR CDC Surveill.Summ.,* **45,** 1-33.

LaGier, M.J., Joseph, L.A., Passaretti, T.V., Musser, K.A. and Cirino, N.M., 2004. A real-time multiplexed PCR assay for rapid detection and differentiation of *Campylobacter jejuni* and *Campylobacter coli*. *Mol.Cell.Probes,* **18,** 275-282.

Lane, D.J., Pace, B., Olsen, G.J., Stahl, D.A., Sogin, M.L. and Pace, N.R., 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc.Natl.Acad.Sci.U.S.A.,* **82,** 6955-6959.

Larsen, H.H., Masur, H., Kovacs, J.A., Gill, V.J., Silcott, V.A., Kogulan, P., Maenza, J., Smith, M., Lucey, D.R. and Fischer, S.H., 2002. Development and evaluation of a quantitative, touchdown, real-time PCR assay for diagnosing *Pneumocystis carinii* pneumonia. *J.Clin.Microbiol.,* **40,** 490-494.

Lebbad, M. & Svard, S.G., 2005. PCR differentiation of *Entamoeba histolytica* and *Entamoeba dispar* from patients with amoeba infection initially diagnosed by microscopy. *Scand.J.Infect.Dis.,* **37,** 680-685.

Lee, J.H., Lee, J., Park, S.J., Yong, T.S. and Hwang, U.W., 2006. Detection and genotyping of *Giardia intestinalis* isolates using intergenic spacers(IGS)-based PCR. *Korean J.Parasitol.,* **44,** 343-353.

Lee, S.H., Levy, D.A., Craun, G.F., Beach, M.J. and Calderon, R.L., 2002. Surveillance for waterborne-disease outbreaks--United States, 1999-2000. *MMWR Surveill.Summ.,* **51,** 1-47.

Leoni, F., Amar, C., Nichols, G., Pedraza-Diaz, S. and McLauchlin, J., 2006. Genetic analysis of *Cryptosporidium* from 2414 humans with diarrhea in England between 1985 and 2000. *J.Med.Microbiol.,* **55,** 703-707.

Leoni, F., Mallon, M.E., Smith, H.V., Tait, A. and McLauchlin, J., 2007. Multilocus analysis of *Cryptosporidium hominis* and *Cryptosporidium parvum* isolates from sporadic and outbreak-related human cases and *C. parvum* isolates from sporadic livestock cases in the United Kingdom. *J.Clin.Microbiol.,* **45,** 3286-3294.

Levine, N.D., 1980. Some corrections of coccidian (Apicomplexa: Protozoa) nomenclature. *J.Parasitol.,* **66,** 830-834.

Li, D., Craik, S.A., Smith, D.W. and Belosevic, M., 2009. Infectivity of *Giardia lamblia* cysts obtained from wastewater treated with ultraviolet light. *Water Res.,* **43,** 3037-3046.

Li, F., Liu, S.L. and Mullins, J.I., 1999. Site-directed mutagenesis using uracil-containing double-stranded DNA templates and *Dpn*I digestion. *BioTechniques,* **27,** 734-738.

Liang, S.Y., Chan, Y.H., Hsia, K.T., Lee, J.L., Kuo, M.C., Hwa, K.Y., Chan, C.W., Chiang, T.Y., Chen, J.S., Wu, F.T. and Ji, D.D., 2009. Development of loop-mediated isothermal amplification assay for detection of *Entamoeba histolytica*. *J.Clin.Microbiol.,* **47,** 1892-1895.

Libman, M.D., Gyorkos, T.W., Kokoskin, E. and Maclean, J.D., 2008. Detection of pathogenic protozoa in the diagnostic laboratory: result reproducibility, specimen pooling, and competency assessment. *J.Clin.Microbiol.,* **46,** 2200-2205.

Limor, J.R., Lal, A.A. and Xiao, L., 2002. Detection and differentiation of *Cryptosporidium* parasites that are pathogenic for humans by real-time PCR. *J.Clin.Microbiol.,* **40,** 2335-2338.

Lindsay, D.S., Upton, S.J., Owens, D.S., Morgan, U.M., Mead, J.R. and Blagburn, B.L., 2000. *Cryptosporidium andersoni* n. sp. (Apicomplexa: Cryptosporiidae) from cattle, Bos taurus. *J.Eukaryot.Microbiol.,* **47,** 91-95.

Llorente, M.T., Clavel, A., Goñi, M.P., Varea, M., Seral, C., Becerril, R., Suarez, L. and Gómez-Lus, R., 2007. Genetic characterization of *Cryptosporidium* species from humans in Spain. *Parasitol.Int.,* **56,** 201-205.

Loftus, B., Anderson, I., Davies, R., Alsmark, U.C., Samuelson, J., Amedeo, P., Roncaglia, P., Berriman, M., Hirt, R.P., Mann, B.J., Nozaki, T., Suh, B., Pop, M., Duchene, M., Ackers, J., Tannich, E., Leippe, M., Hofer, M., Bruchhaus, I., Willhoeft, U., Bhattacharya, A., Chillingworth, T., Churcher, C., Hance, Z., Harris, B., Harris, D., Jagels, K., Moule, S., Mungall, K., Ormond, D., Squares, R., Whitehead, S., Quail, M.A., Rabbinowitsch, E., Norbertczak, H., Price, C., Wang, Z., Guillen, N., Gilchrist, C., Stroup, S.E., Bhattacharya, S., Lohia, A., Foster, P.G., Sicheritz-Ponten, T., Weber, C., Singh, U., Mukherjee, C., El-Sayed, N.M., Petri, W.A.,Jr, Clark, C.G., Embley, T.M., Barrell, B., Fraser, C.M. and Hall, N., 2005. The genome of the protist parasite *Entamoeba histolytica*. *Nature,* **433,** 865-868.

Logsdon, G.S., 2000. Effective management and operation of coagulation and filtration. *Water Air Soil Pollut.,* **123,** 159-166.

Loughlin EH, Spitz SH (1949). Diagnosis of helminthiasis. JAMA 139:997–1000

Louie, M., Louie, L. and Simor, A.E., 2000. The role of DNA amplification technology in the diagnosis of infectious diseases. *CMAJ,* **163,** 301-309.

Mackenstedt, U., Schmidt, M., Raether, W., Mehlhorn, H. and Uphoff, M., 1990. Increase of DNA content in tissue stages of *Entamoeba histolytica* strain SFL 3. *Parasitol.Res.,* **76,** 373-378.

Magi, B., Canocchi, V., Tordini, G., Cellesi, C. and Barberi, A., 2006. *Cryptosporidium* infection: Diagnostic techniques. *Parasitol.Res.,* **98,** 150-152.

Makioka, A., Kumagai, M., Kobayashi, S. and Takeuchi, T., 2006. Effect of artificial gastrointestinal fluids on the excystation and metacystic development of *Entamoeba* invadens. *Parasitol.Res.*, **98,** 443-446.

Mallon, M.E., MacLeod, A., Wastling, J.M., Smith, H. and Tait, A., 2003. Multilocus genotyping of *Cryptosporidium parvum* Type 2: population genetics and sub-structuring. *Infect.Genet.Evol.*, **3,** 207-218.

Mank, T.G., Zaat, J.O.M., Deelder, A.M., Van Eijk, J.T.M. and Polderman, A.M., 1997. Sensitivity of microscopy versus enzyme immunoassay in the laboratory diagnosis of giardiasis. *European Journal of Clinical Microbiology and Infectious Diseases,* **16,** 615-619.

Mayrhofer, G., Andrews, R.H., Ey, P.L. and Chilton, N.B., 1995. Division of *Giardia* isolates from humans into two genetically distinct assemblages by electrophoretic analysis of enzymes encoded at 27 loci and comparison with *Giardia muris*. *Parasitology,* **111,** 11-17.

McDonald, V., Stables, R., Warhurst, D.C., Barer, M.R., Blewett, D.A., Chapman, H.D., Connolly, G.M., Chiodini, P.L. and McAdam, K.P., 1990. In vitro cultivation of *Cryptosporidium parvum* and screening for anticryptosporidial drugs. *Antimicrob.Agents Chemother.*, **34,** 1498-1500.

McLauchlin, J., Amar, C., Pedraza-Diaz, S. and Nichols, G.L., 2000. Molecular epidemiological analysis of *Cryptosporidium* spp. in the United Kingdom: results of genotyping *Cryptosporidium* spp. in 1,705 fecal samples from humans and 105 fecal samples from livestock animals. *J.Clin.Microbiol.*, **38,** 3984-3990.

McLauchlin, J., Amar, C.F., Pedraza-Diaz, S., Mieli-Vergani, G., Hadzic, N. and Davies, E.G., 2003. Polymerase chain reaction-based diagnosis of infection with *Cryptosporidium* in children with primary immunodeficiencies. *Pediatr.Infect.Dis.J.*, **22,** 329-335.

McLauchlin, J., Pedraza-Díaz, S., Amar-Hoetzeneder, C. and Nichols, G.L., 1999. Genetic characterization of *Cryptosporidium* strains from 218 patients with

diarrhea diagnosed as having sporadic cryptosporidiosis. *J.Clin.Microbiol.,* **37,** 3153-3158.

Meamar, A.R., Guyot, K., Certad, G., Dei-Cas, E., Mohraz, M., Mohebali, M., Mohammad, K., Mehbod, A.A., Rezaie, S. and Rezaian, M., 2007. Molecular characterization of *Cryptosporidium* isolates from humans and animals in Iran. *Appl.Environ.Microbiol.,* **73,** 1033-1035.

Meisel, J.L., Perera, D.R., Meligro, C. and Rubin, C.E., 1976. Overwhelming watery diarrhea associated with a *Cryptosporidium* in an immunosuppressed patient. *Gastroenterology,* **70,** 1156-1160.

Meloni, B.P., Lymbery, A.J. and Thompson, R.C., 1988. Isoenzyme electrophoresis of 30 isolates of *Giardia* from humans and felines. *Am.J.Trop.Med.Hyg.,* **38,** 65-73.

Millar, C., Moore, J., Lowery, C., McCorry, K. and Dooley, J., 2001. Successful PCR amplification of genomic DNA from *Cryptosporidium parvum* oocysts extracted from a human faecal sample: A rapid and simple method suited for outbreak analysis. *Int.J.Hyg.Environ.Health,* **204,** 191-194.

Millard, P.S., Gensheimer, K.F., Addiss, D.G., Sosin, D.M., Beckett, G.A., Houck-Jankoski, A. and Hudson, A., 1994. An outbreak of cryptosporidiosis from fresh-pressed apple cider. *JAMA,* **272,** 1592-1596.

Miller, K.M. & Sterling, C.R., 2007. Sensitivity of nested PCR in the detection of low numbers of *Giardia lamblia* cysts. *Appl.Environ.Microbiol.,* **73,** 5949-5950.

Modry, D., Slapeta, J.R. and Koudela, B., 2000. Six new species of coccidia (Apicomplexa: Eimeriidae) from East African chameleons (Sauria: Chamaeleonidae). *J.Parasitol.,* **86,** 373-379.

Monis, P.T., Andrews, R.H., Mayrhofer, G. and Ey, P.L., 2003. Genetic diversity within the morphological species *Giardia intestinalis* and its relationship to host origin. *Infection, Genetics and Evolution,* **3,** 29-38.

Monis, P.T., Andrews, R.H., Mayrhofer, G. and Ey, P.L., 1999. Molecular systematics of the parasitic protozoan *Giardia intestinalis*. *Mol.Biol.Evol.*, **16,** 1135-1144.

Monis, P.T., Andrews, R.H., Mayrhofer, G., Mackrill, J., Kulda, J., Isaac-Renton, J.L. and Ey, P.L., 1998. Novel lineages of *Giardia intestinalis* identified by genetic analysis of organisms isolated from dogs in Australia. *Parasitology,* **116 (Pt 1),** 7-19.

Monis, P.T. & Thompson, R.C., 2003. *Cryptosporidium* and *Giardia*-zoonoses: fact or fiction? *Infect.Genet.Evol.*, **3,** 233-244.

Morgan, U., Xiao, L., Sulaiman, I., Weber, R., Lal, A.A., Thompson, R.C. and Deplazes, P., 1999. Which genotypes/species of *Cryptosporidium* are humans susceptible to? *J.Eukaryot.Microbiol.*, **46,** 42S-43S.

Morgan-Ryan, U.M., Fall, A., Ward, L.A., Hijjawi, N., Sulaiman, I., Fayer, R., Andrew Thompson, R.C., Olson, M., Lal, A. and Xiao, L., 2002. *Cryptosporidium hominis* n. sp. (Apicomplexa: Cryptosporidiidae) from Homo sapiens. *J.Eukaryot.Microbiol.*, **49,** 433-440.

Morrison, H.G., McArthur, A.G., Gillin, F.D., Aley, S.B., Adam, R.D., Olsen, G.J., Best, A.A., Cande, W.Z., Chen, F., Cipriano, M.J., Davids, B.J., Dawson, S.C., Elmendorf, H.G., Hehl, A.B., Holder, M.E., Huse, S.M., Kim, U.U., Lasek-Nesselquist, E., Manning, G., Nigam, A., Nixon, J.E., Palm, D., Passamaneck, N.E., Prabhu, A., Reich, C.I., Reiner, D.S., Samuelson, J., Svard, S.G. and Sogin, M.L., 2007. Genomic minimalism in the early diverging intestinal parasite *Giardia lamblia*. *Science,* **317,** 1921-1926.

Nantavisai, K., Mungthin, M., Tan-ariya, P., Rangsin, R., Naaglor, T. and Leelayoova, S., 2007. Evaluation of the sensitivities of DNA extraction and PCR methods for detection of *Giardia duodenalis* in stool specimens. *J.Clin.Microbiol.,* **45,** 581-583.

Nash, T.E., McCutchan, T., Keister, D., Dame, J.B., Conrad, J.D. and Gillin, F.D., 1985. Restriction-endonuclease analysis of DNA from 15 *Giardia* isolates obtained from humans and animals. *J.Infect.Dis.,* **152,** 64-73.

Navarro-I-Martinez, L., Da Silva, A.J., Botero Garces, J.H., Montoya Palacio, M.N., Del Aguila, C. and Bornay-Llinares, F.J., 2006. Cryptosporidiosis in HIV-positive patients from Medellín, Colombia. *J.Eukaryot.Microbiol.,* **53,** S37-S39.

Ng, C.T., Gilchrist, C.A., Lane, A., Roy, S., Haque, R. and Houpt, E.R., 2005. Multiplex real-time PCR assay using Scorpion probes and DNA capture for genotype-specific detection of *Giardia lamblia* on fecal samples. *J.Clin.Microbiol.,* **43,** 1256-1260.

Nichols, G.L., 2000. Food-borne protozoa. *Br.Med.Bull.,* **56,** 209-235.

Nichols, R.A.B., Moore, J.E. and Smith, H.V., 2006. A rapid method for extracting oocyst DNA from *Cryptosporidium*-positive human faeces for outbreak investigations. *J.Microbiol.Methods,* **65,** 512-524.

Nime, F.A., Burek, J.D. and Page, D.L., 1976. Acute enterocolitis in a human being infected with the protozoan *Cryptosporidium*. *Gastroenterology,* **70,** 592-598.

Nygård, K., Schimmer, B., Søbstad, Ø., Walde, A., Tveit, I., Langeland, N., Hausken, T. and Aavitsland, P., 2006. A large community outbreak of waterborne giardiasis- delayed detection in a non-endemic urban area. *BMC Public Health,* **6,** .

O'Donoghue, P.J., 1995. *Cryptosporidium* and cryptosporidiosis in man and animals. *Int.J.Parasitol.,* **25,** 139-195.

Oikonomou, I., Halatsi, K. and Kyriacou, A., 2008. Selective PCR: a novel internal amplification control strategy for enhanced sensitivity in *Salmonella* diagnosis. *Lett.Appl.Microbiol.,* **46,** 456-461.

Ortega, Y.R. & Adam, R.D., 1997. *Giardia*: overview and update. *Clinical Infectious Diseases,* **25,** 545-550.

Paglia, M.G. & Visca, P., 2004. An improved PCR-based method for detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* in formalin-fixed stools. *Acta Trop.,* **92,** 273-277.

Palmer, C.J., Xiao, L., Terashima, A., Guerra, H., Gotuzzo, E., Saldias, G., Bonilla, J.A., Zhou, L., Lindquist, A. and Upton, S.J., 2003. *Cryptosporidium muris*, a rodent pathogen, recovered from a human in Peru. *Emerg.Infect.Dis.,* **9,** 1174-1176.

Parija, S.C. & Khairnar, K., 2005. *Entamoeba moshkovskii* and *Entamoeba dispar*-associated infections in pondicherry, India. *J.Health Popul.Nutr.,* **23,** 292-295.

Patel, S., Pedraza-Díaz, S. and McLauchlin, J., 1999. The identification of *Cryptosporidium* species and *Cryptosporidium parvum* directly from whole faeces by analysis of a multiplex PCR of the 18S rRNA gene and by PCR/RFLP of the *Cryptosporidium* outer wall protein (COWP) gene. *Int.J.Parasitol.,* **29,** 1241-1247.

Pedraza-Diaz, S., Amar, C., Iversen, A.M., Stanley, P.J. and McLauchlin, J., 2001. Unusual *Cryptosporidium* species recovered from human faeces: first description of *Cryptosporidium* felis and *Cryptosporidium* 'dog type' from patients in England. *J.Med.Microbiol.,* **50,** 293-296.

Pedraza-Diaz, S., Amar, C.F., McLauchlin, J., Nichols, G.L., Cotton, K.M., Godwin, P., Iversen, A.M., Milne, L., Mulla, J.R., Nye, K., Panigrahl, H., Venn, S.R., Wiggins, R., Williams, M. and Youngs, E.R., 2001. *Cryptosporidium* meleagridis from humans: molecular analysis and description of affected patients. *J.Infect.,* **42,** 243-250.

Persson, S. & Olsen, K.E., 2005. Multiplex PCR for identification of Campylobacter coli and Campylobacter jejuni from pure cultures and directly on stool samples. *J.Med.Microbiol.,* **54,** 1043-1047.

Plutzer, J. & Karanis, P., 2009. Genetic polymorphism in *Cryptosporidium* species: an update. *Vet.Parasitol.,* **165,** 187-199.

Power, M.L. & Ryan, U.M., 2008. A new species of *Cryptosporidium* (Apicomplexa: Cryptosporidiidae) from eastern grey kangaroos (Macropus giganteus). *J.Parasitol.,* **94,** 1114-1117.

Poxleitner, M.K., Carpenter, M.L., Mancuso, J.J., Wang, C.-.R., Dawson, S.C. and Cande, W.Z., 2008. Evidence for karyogamy and exchange of genetic material in the binucleate intestinal parasite *Giardia intestinalis*. *Science,* **319,** 1530-1533.

Pritt, B.S. & Graham Clark, C., 2008. Amebiasis. *Mayo Clin.Proc.,* **83,** 1154-1160.

Radstrom, P., Knutsson, R., Wolffs, P., Dahlenborg, M. and Lofstrom, C., 2003. Pre-PCR processing of samples. *Methods Mol.Biol.,* **216,** 31-50.

Ravdin, J.I., 1995. Amebiasis. *Clin.Infect.Dis.,* **20,** 1453-64; quiz 1465-6.

Read, C., Walters, J., Robertson, I.D. and Thompson, R.C., 2002. Correlation between genotype of *Giardia duodenalis* and diarrhea. *Int.J.Parasitol.,* **32,** 229-231.

Read, C.M., Monis, P.T. and Thompson, R.C., 2004. Discrimination of all genotypes of *Giardia duodenalis* at the glutamate dehydrogenase locus using PCR-RFLP. *Infect.Genet.Evol.,* **4,** 125-130.

RENDTORFF, R.C. & HOLT, C.J., 1954. The experimental transmission of human intestinal protozoan parasites. IV. Attempts to transmit Endamoeba coli and *Giardia lamblia* cysts by water. *Am.J.Hyg.,* **60,** 327-338.

Robinson, G., Elwin, K. and Chalmers, R.M., 2008. Unusual *Cryptosporidium* genotypes in human cases of diarrhea. *Emerging Infectious Diseases,* **14,** 1800-1802.

Robinson, G.L., 1968. Laboratory cultivation of some human parasitic amoebae. *J.Gen.Microbiol.,* **53,** 69-79.

Rose, J.B., Huffman, D.E. and Gennaccaro, A., 2002. Risk and control of waterborne cryptosporidiosis. *FEMS Microbiol.Rev.*, **26,** 113-123.

Rose, J.B. & Slifko, T.R., 1999. *Giardia, Cryptosporidium,* and *Cyclospora* and their impact on foods: a review. *J.Food Prot.*, **62,** 1059-1070.

Rossignol, J., 2009. *Cryptosporidium* and *Giardia*: Treatment Options and Prospects for New Drugs, submitted for publication.

Roy, S., Kabir, M., Mondal, D., Ali, I.K.M., Petri Jr., W.A. and Haque, R., 2005. Real-time-PCR assay for diagnosis of *Entamoeba histolytica* infection. *J.Clin.Microbiol.*, **43,** 2168-2172.

Ryan, U.M., Monis, P., Enemark, H.L., Sulaiman, I., Samarasinghe, B., Read, C., Buddle, R., Robertson, I., Zhou, L., Thompson, R.C. and Xiao, L., 2004. *Cryptosporidium suis* n. sp. (Apicomplexa: Cryptosporidiidae) in pigs (Sus scrofa). *J.Parasitol.*, **90,** 769-773.

Ryan, U.M., Power, M. and Xiao, L., 2008. *Cryptosporidium fayeri* n. sp. (Apicomplexa: Cryptosporidiidae) from the Red Kangaroo (Macropus rufus). *J.Eukaryot.Microbiol.*, **55,** 22-26.

Ryan, U.M., Xiao, L., Read, C., Sulaiman, I.M., Monis, P., Lal, A.A., Fayer, R. and Pavlasek, I., 2003. A redescription of *Cryptosporidium galli* Pavlasek, 1999 (Apicomplexa: Cryptosporidiidae) from birds. *J.Parasitol.*, **89,** 809-813.

Sachadyn, P. & Kur, J., 1998. The construction and use of a PCR internal control. *Mol.Cell.Probes,* **12,** 259-262.

Santos, H.L.C., Peralta, R.H.S., de Macedo, H.W., Barreto, M.G.M. and Peralta, J.M., 2007. Comparison of multiplex-PCR and antigen detection for differential diagnosis of *Entamoeba histolytica*. *Brazilian Journal of Infectious Diseases,* **11,** 365-370.

Schuurman, T., Lankamp, P., van Belkum, A., Kooistra-Smid, M. and van Zwet, A., 2007. Comparison of microscopy, real-time PCR and a rapid immunoassay for

the detection of *Giardia lamblia* in human stool specimens. *Clinical Microbiology and Infection,* **13,** 1187-1191.

Sehgal, R., Abd-Alla, M., Moody, A.H., Chiodini, P.L. and Ackers, J.P., 1995. Comparison of two media for the isolation and short-term culture of *Entamoeba histolytica* and *E. dispar*. *Trans.R.Soc.Trop.Med.Hyg.*, **89,** 394.

Shrikhande, S.N., Chande, C.A., Shegokar, V.R. and Powar, R.M., 2009. Pulmonary cryptosporidiosis in HIV negative, immunocompromised host. *Indian Journal of Pathology and Microbiology,* **52,** 267-268.

Siebert, P.D. & Larrick, J.W., 1992. Competitive PCR. *Nature,* **359,** 557-558.

Siwila, J., Phiri, I.G.K., Enemark, H.L., Nchito, M. and Olsen, A., 2009. Intestinal helminths and protozoa in children in pre-schools in Kafue district, Zambia. *Trans.R.Soc.Trop.Med.Hyg.,* .

SLAVIN, D., 1955. *Cryptosporidium meleagridis* (sp. nov.). *J.Comp.Pathol.*, **65,** 262-266.

Soba, B., Petrovec, M., Mioc, V. and Logar, J., 2006. Molecular characterisation of *Cryptosporidium* isolates from humans in Slovenia. *Clin.Microbiol.Infect.,* **12,** 918-921.

Spano, F., Putignani, L., McLauchlin, J., Casemore, D.P. and Crisanti, A., 1997. PCR-RFLP analysis of the *Cryptosporidium* oocyst wall protein (COWP) gene discriminates between *C. wrairi* and *C. parvum*, and between *C. parvum* isolates of human and animal origin. *FEMS Microbiol.Lett.,* **150,** 209-217.

Stanley Jr., S.L., 2003. Amoebiasis. *Lancet,* **361,** 1025-1034.

Stanley, S.L.,Jr, Becker, A., Kunz-Jenkins, C., Foster, L. and Li, E., 1990. Cloning and expression of a membrane antigen of *Entamoeba histolytica* possessing multiple tandem repeats. *Proc.Natl.Acad.Sci.U.S.A.,* **87,** 4976-4980.

Stephen, A.M. & Cummings, J.H., 1980. The microbial contribution to human faecal mass. *J.Med.Microbiol.,* **13,** 45-56.

Stroup, S.E., Roy, S., Mchele, J., Maro, V., Ntabaguzi, S., Siddique, A., Kang, G., Guerrant, R.L., Kirkpatrick, B.D., Fayer, R., Herbein, J., Ward, H., Haque, R. and Houpt, E.R., 2006. Real-time PCR detection and speciation of *Cryptosporidium* infection using Scorpion probes. *J.Med.Microbiol.,* **55,** 1217-1222.

Subrungruang, I., Mungthin, M., Chavalitshewinkoon-Petmitr, P., Rangsin, R., Naaglor, T. and Leelayoova, S., 2004. Evaluation of DNA extraction and PCR methods for detection of *Enterocytozoon bienuesi* in stool specimens. *J.Clin.Microbiol.,* **42,** 3490-3494.

Sulaiman, I.M., Fayer, R., Bern, C., Gilman, R.H., Trout, J.M., Schantz, P.M., Das, P., Lal, A.A. and Xiao, L., 2003. Triosephosphate Isomerase Gene Characterization and Potential Zoonotic Transmission of *Giardia duodenalis*. *Emerging Infectious Diseases,* **9,** 1444-1452.

Tachibana, H., Kobayashi, S., Takekoshi, M. and Ihara, S., 1991. Distinguishing pathogenic isolates of *Entamoeba histolytica* by polymerase chain reaction. *J.Infect.Dis.,* **164,** 825-826.

Tanyuksel, M. & Petri, W.A.,Jr, 2003. Laboratory diagnosis of amebiasis. *Clin.Microbiol.Rev.,* **16,** 713-729.

ten Hove, R.J., van Esbroeck, M., Vervoort, T., van den Ende, J., van Lieshout, L. and Verweij, J.J., 2009. Molecular diagnostics of intestinal parasites in returning travellers. *Eur.J.Clin.Microbiol.Infect.Dis.,* **28,** 1045-1053.

Thielman, N.M. & Guerrant, R.L., 2004. Acute Infectious Diarrhea. *N.Engl.J.Med.,* **350,** 38-47.

Thompson, R.C., 2000. Giardiasis as a re-emerging infectious disease and its zoonotic potential. *Int.J.Parasitol.,* **30,** 1259-1267.

Thompson, R.C., Hopkins, R.M. and Homan, W.L., 2000. Nomenclature and genetic groupings of *Giardia* infecting mammals. *Parasitol.Today,* **16,** 210-213.

Tyzzer, E.E., 1910. An extracellular coccidium, *Cryptosporidium muris* (gen. et sp. nov.), of the gastric glands of the common mouse. *J.Med.Res.*, **23,** 487-509.

Tzipori, S. & Ward, H., 2002. Cryptosporidiosis: biology, pathogenesis and disease. *Microbes Infect.,* **4,** 1047-1058.

van der Giessen, J.W.B., de Vries, A., Roos, M., Wielinga, P., Kortbeek, L.M. and Mank, T.G., 2006. Genotyping of *Giardia* in Dutch patients and animals: A phylogenetic analysis of human and animal isolates. *Int.J.Parasitol.,* **36,** 849-858.

van Gool, T., Weijts, R., Lommerse, E. and Mank, T.G., 2003. Triple Faeces Test: an effective tool for detection of intestinal parasites in routine clinical practice. *Eur.J.Clin.Microbiol.Infect.Dis.,* **22,** 284-290.

Verweij, J.J., Blangé, R.A., Templeton, K., Schinkel, J., Brienen, E.A.T., Van Rooyen, M.A.A., Van Lieshout, L. and Polderman, A.M., 2004. Simultaneous Detection of *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum* in Fecal Samples by Using Multiplex Real-Time PCR. *J.Clin.Microbiol.,* **42,** 1220-1223.

Verweij, J.J., Oostvogel, F., Brienen, E.A.T., Nang-Beifubah, A., Ziem, J. and Polderman, A.M., 2003. Short communication: Prevalence of *Entamoeba histolytica* and *Entamoeba dispar* in northern Ghana. *Tropical Medicine and International Health,* **8,** 1153-1156.

Weinke, T., Friedrich-Janicke, B., Hopp, P. and Janitschke, K., 1990. Prevalence and clinical importance of *Entamoeba histolytica* in two high-risk groups: travelers returning from the tropics and male homosexuals. *J.Infect.Dis.,* **161,** 1029-1031.

Wolfe, M.S., 1978. Giardiasis. *N.Engl.J.Med.,* **298,** 319-321.

Xiao, L., Bern, C., Limor, J., Sulaiman, I., Roberts, J., Checkley, W., Cabrera, L., Gilman, R.H. and Lal, A.A., 2001. Identification of 5 types of

Cryptosporidium parasites in children in Lima, Peru. *J.Infect.Dis.,* **183,** 492-497.

Xiao, L., Fayer, R., Ryan, U. and Upton, S.J., 2004. *Cryptosporidium* taxonomy: recent advances and implications for public health. *Clin.Microbiol.Rev.,* **17,** 72-97.

Xiao, L., Ryan, U., 2008. Molecular Epidemiology. In: Fayer, R., Xiao, L. (Eds.), *Cryptosporidium* and Cryptosporidiosis. CRC Press, Boca Raton, pp. 119–163.

Youn, S., Kabir, M., Haque, R. and Petri, W.A.,Jr, 2009. Evaluation of a screening test for detection of *Giardia* and *Cryptosporidium* parasites. *J.Clin.Microbiol.,* **47,** 451-452.

Zaki, M., Verweij, J.J. and Clark, C.G., 2003. *Entamoeba histolytica*: direct PCR-based typing of strains using faecal DNA. *Exp.Parasitol.,* **104,** 77-80.

9 Appendices

Appendix A: Clinical specimens data table

This table represents data collected from a comparative study of the utility of the multiplex PCR versus several in-house diagnostics for 212 clinical samples submitted to the Clinical Microbiology laboratory, Leicester Royal Infirmary (LRI), University Hospital of Leicester between June and October, 2009.

ID	D.O.C. M/D/Y	D.O.E. M/D/Y	*Crypto*. spp. (*u*PCR)	*G. lamblia* (*u*PCR)	Screening kit[1]	Discriminatory kits[2]	*m*PCR	Sequencing	Age	Sex	Co- infection
123280E	6.7.2009	6.9.2009	(-)	(-)	(-)	NA	(-)	NA	82	F	none
122559	5.27.2009	" . ". "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	assemblage B	37	M	none
123289M	6.9.2009	" . ". "	(-)	(-)	(-)	NA	(-)	NA	42	M	none
123281H	6.8.2009	" . ". "	(-)	(-)	(-)	NA	(-)	NA	78	M	none
123207G	6.6.2009	" . ". "	(-)	(-)	(-)	NA	(-)	NA	62	M	none
123283P	6.8.2009	" . ". "	(-)	(-)	(-)	NA	(-)	NA	83	F	none
123276K	6.8.2009	" . ". "	(-)	(-)	(-)	NA	(-)	NA	65	M	*Campylobacter* spp.
123278	6.9.2009	" . ". "	(-)	(-)	(-)	NA	(-)	NA	0	M	none
122705E	4.3.2009	" . ". "	(-)	(-)	G &/or C	(-)	(-)	NA	6	F	none
122709P	5.29.2009	" . ". "	(-)	(-)	G &/or C	(-)	(-)	NA	97	F	none
122477T	5.22.2009	" . ". "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	assemblage B	43	F	none

ID	D.O.C. M/D/Y	D.O.E. M/D/Y	*Crypto*. spp. (*u*PCR)	*G. lamblia* (*u*PCR)	Screening kit[1]	Discriminatory kits[2]	*m*PCR	Sequencing	Age	Sex	Co- infection
123035L	6.4.2009	“ . ”. "	(-)	(-)	G &/or C	(-)	(-)	NA	23	F	none
122786B	6.1.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	(-)	(-)	NA	6	M	none
123033X	6.4.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	NA	23	F	none
122984H	6.3.2009	“ . ”. "	(-)	(-)	G &/or C	(-)	(-)	NA	62	F	none
122299D	5.22.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	assemblage B	81	F	none
122692V	5.8.2009	“ . ”. "	(-)	(-)	G &/or C	(-)	(-)	NA	84	F	none
122466K	5.26.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	NA	42	M	none
122074D	5.18.2009	“ . ”. "	(-)	(-)	G &/or C	(-)	(-)	NA	0	F	none
122445V	5.24.2009	“ . ”. "	(-)	(-)	G &/or C	(-)	(-)	NA	64	F	none
123044R	6.4.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	NA	41	M	none
123034	6.4.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	assemblage A	23	F	none
123286C	6.8.2009	“ . ”. "	(-)	(-)	(-)		(-)	NA	43	M	none
123288K	6.9.2009	“ . ”. "	(-)	(-)	(-)		(-)	NA	25	F	none
122396T	5.24.2009	“ . ”. "	(-)	(-)	G &/or C	(-)	(-)	NA	63	M	none
121774T	5.11.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	assemblage B	28	M	none
123287T	6.8.2009	“ . ”. "	(-)	(-)	(-)		(-)	NA	23	F	none
123285F	6.9.2009	“ . ”. "	(-)	(-)	(-)		(-)	NA	78	F	none
123279	6.8.2009	“ . ”. "	(-)	(-)	(-)		(-)	NA	43	M	none
122984H	6.3.2009	“ . ”. "	(-)	(-)	G &/or C	(-)	(-)	NA	62	F	none
123279V	6.3.2009	“ . ”. "	(-)	(-)	(-)		(-)	NA	42	M	none
123284A	6.3.2009	“ . ”. "	(-)	(-)	(-)		(-)	NA	29	M	none
116829	6.5.2009	“ . ”. "	*Crypto.*	(-)	G &/or C	*Crypto.*	*Crypto.*	*C. hominis*	61	F	none
123277M	6.8.2009	“ . ”. "	(-)	(-)	(-)		(-)	NA	48	F	none

ID	D.O.C. M/D/Y	D.O.E. M/D/Y	*Crypto*. spp. (*u*PCR)	*G. lamblia* (*u*PCR)	Screening kit[1]	Discriminatory kits[2]	*m*PCR	Sequencing	Age	Sex	Co- infection
123729	6.15.2009	6.16.2009	(-)	(-)	(-)		(-)	NA	64	F	none
123734	6.15.2009	“ . ”. "	(-)	(-)	(-)		(-)	NA	86	M	*Campylobacter* spp.
123725	6.15.2009	“ . ”. "	(-)	(-)	(-)		(-)	NA	79	F	none
123728	6.16.2009	“ . ”. "	(-)	(-)	(-)		(-)	NA	29	M	none
123324	6.8.2009	“ . ”. "	(-)	(-)	G &/or C	(-)	(-)	NA	0	F	*Salmonella* spp.
123441	6.10.2009	“ . ”. "	(-)	(-)	G &/or C	(-)	(-)	NA	75	M	none
123731	6.16.2009	“ . ”. "	(-)	(-)	(-)		(-)	NA	81	M	none
123724	6.16.2009	“ . ”. "	(-)	(-)	(-)		(-)	NA	88	F	*Campylobacter* spp.
123491	6.11.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	assemblage B	47	F	none
123504	6.8.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	assemblage A	47	M	*Campylobacter* spp.
123722	6.15.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA		F	*Campylobacter* spp.
123453	6.10.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	assemblage A	74	M	none
123736	6.16.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	61	M	none
123732	6.16.2009	“ . ”. "	(-)	*G. lamblia*	(-)	NA	(-)	NA	88	F	none
123730	6.16.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	89	F	none
123723	6.15.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	23	M	none
123518	6.11.2009	“ . ”. "	(-)	(-)	G &/or C	(-)	(-)	NA	71	M	none
123505	6.9.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	assemblage A	47	M	*Campylobacter* spp.
123726	6.16.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	78	F	none
123437	6.11.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	assemblage B	21	F	none
123717	6.16.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	0	F	none
123718	6.16.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	76	M	none
123733	6.16.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	78	F	none

ID	D.O.C. M/D/Y	D.O.E. M/D/Y	*Crypto*. spp. (*u*PCR)	*G. lamblia* (*u*PCR)	Screening kit[1]	Discriminatory kits[2]	*m*PCR	Sequencing	Age	Sex	Co- infection
123720	6.15.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	79	F	none
124488	6.11.2009	6.29.2009	(-)	(-)	(-)	NA	(-)	NA	83	M	none
124365	6.24.2009	“ . ”. "	(-)	(-)	G &/or C	(-)	(-)	NA	0	F	none
124496	6.29.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	76	F	none
124495	6.28.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	84	F	none
124205	6.23.2009	“ . ”. "	*Crypto.*	(-)	G &/or C	*Crypto.*	*Crypto.*	*C. hominis*	42	F	none
123709	6.14.2009	“ . ”. "	*Crypto.*	(-)	G &/or C	*Crypto.*	*Crypto.*	*C. parvum*	2	F	none
124257	6.23.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	74	F	none
124487	6.28.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	33	M	none
124197	6.23.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	88	F	none
123942	6.18.2009	“ . ”. "	(-)	(-)	G &/or C	(-)	(-)	NA	0	M	none
124247	6.23.2009	“ . ”. "	*Crypto.*	(-)	G &/or C	*Crypto.*	*Crypto.*	*C. parvum*	5	F	none
124494	6.28.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	85	M	none
124144	6.22.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	84	F	none
124489	6.28.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	NA	71	F	none
124205	6.23.2009	“ . ”. "	*Crypto.*	(-)	G &/or C	*Crypto.*	*Crypto.*	*C. hominis*	42	F	none
124497	6.28.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	66	M	none
124493	6.28.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	76	M	none
124484	6.28.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	NA	62	M	none
124238	6.22.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	61	M	none
124485	6.28.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	13	M	*Clostridium difficile*
124158	6.23.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	68	F	none
124490	6.28.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	58	F	none

ID	D.O.C. M/D/Y	D.O.E. M/D/Y	*Crypto*. spp. (*u*PCR)	*G. lamblia* (*u*PCR)	Screening kit[1]	Discriminatory kits[2]	*m*PCR	Sequencing	Age	Sex	Co- infection
124492	6.29.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	88	M	none
124486	6.29.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	73	F	none
124938	7.5.2009	7.7.2009	(-)	(-)	(-)	NA	(-)	NA	0	M	none
124949	7.4.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	77	M	none
124952	7.6.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	73	M	none
124930	7.5.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	41	F	none
124947	7.5.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	72	F	none
124959	7.5.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	83	F	none
124933	7.5.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	78	M	none
124937	6.30.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	12	F	none
124744	7.1.2009	“ . ”. "	(-)	(-)	G &/or C	(-)	(-)	NA	73	M	none
124958	7.5.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	89	F	*Clostridium difficile*
124939	7.6.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	85	F	none
124950	7.2.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	52	M	none
124928	7.4.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	55	M	none
124926	7.6.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	65	M	none
124953	7.5.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	53	M	none
124960	7.4.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	51	M	none
124929	7.5.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	80	F	none
124935	7.5.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	0	F	none
124980	7.6.2009	7.14.2009	*Crypto.*	(-)	G &/or C	*Crypto.*	*Crypto.*	*C. parvum*	41	F	none
125368	7.12.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	77	M	none
125366	7.12.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	23	F	none

ID	D.O.C. M/D/Y	D.O.E. M/D/Y	*Crypto*. spp. (*u*PCR)	*G. lamblia* (*u*PCR)	Screening kit[1]	Discriminatory kits[2]	*m*PCR	Sequencing	Age	Sex	Co- infection
125180	7.3.2009	“ . ”. "	*Crypto.*	(-)	G &/or C	*Crypto.*	*Crypto.*	*C. hominis*	2	F	none
125396	7.11.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	47	F	none
125384	7.2.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	80	M	none
125357	7.1.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	47	F	none
125351	7.12.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	82	F	none
125352	7.12.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	35	F	none
125359	7.1.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	43	M	none
125341	7.11.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	87	F	none
125083	07.07.2009	“ . ”. "	*Crypto.*	*G. lamblia*	G &/or C	*Crypto.*	C & G	*C. parvum*	10	F	none
125378	7.12.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	53	F	none
125354	7.12.2009	“ . ”. "	(-)	*G. lamblia*	(-)	NA	*G. lamblia*	NA	76	F	none
125383	7.12.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	60	M	none
124980	7.6.2009	“ . ”. "	*Crypto.*	(-)	G &/or C	*Crypto.*	*Crypto.*	*C. parvum*	41	F	none
125356	7.11.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	83	F	none
125033	7.3.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	assemblage A	33	F	none
125364	7.13.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	31	M	none
125355	7.11.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	65	M	none
125923	7.21.2009	7.22.2009	(-)	(-)	(-)	NA	(-)	NA	U	U	*Clostridium difficile*
125924	7.21.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	49	F	none
125907	7.20.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	89	M	none
125901	7.21.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	23	M	*Campylobacter* spp.
125882	7.20.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	65	M	*Campylobacter* spp.
125885	7.20.2009	“ . ”. "	(-)	*G. lamblia*	(-)	NA	*G. lamblia*	NA	46	M	none

ID	D.O.C. M/D/Y	D.O.E. M/D/Y	*Crypto*. spp. (*u*PCR)	*G. lamblia* (*u*PCR)	Screening kit[1]	Discriminatory kits[2]	*m*PCR	Sequencing	Age	Sex	Co- infection
125940	7.21.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	77	F	none
125939	7.21.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	48	F	none
125927	7.21.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	0	M	none
125916	7.21.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	80	M	*Clostridium difficile*
125918	7.21.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	78	M	none
125920	7.20.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	40	M	none
125953	7.21.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	8	M	*Campylobacter* spp.
125954	7.21.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	68	M	none
125942	7.21.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	72	M	none
125943	7.21.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	31	M	none
125957	7.21.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	45	F	none
125960	7.21.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	63	F	none
125969	7.21.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	50	F	none
125970	7.21.2009	“ . ”. "	(-)	*G. lamblia*	(-)	NA	*G. lamblia*	NA	58	F	none
125984	7.21.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	79	F	none
125986	7.21.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	33	F	none
125987	7.21.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	60	F	none
125989	7.21.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	74	M	none
127045	8.7.2009	8.10.2009	(-)	(-)	(-)	NA	(-)	NA	88	F	none
127043	8.9.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	48	F	none
127042	8.8.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	72	M	none
127038	8.8.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	60	F	*Campylobacter* spp.
127037	8.8.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	78	M	none

ID	D.O.C. M/D/Y	D.O.E. M/D/Y	*Crypto*. spp. (*u*PCR)	*G. lamblia* (*u*PCR)	Screening kit[1]	Discriminatory kits[2]	*m*PCR	Sequencing	Age	Sex	Co- infection
127033	8.7.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	79	F	none
127074	8.8.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	75	F	none
127068	8.8.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	86	M	none
127059	8.10.2009	“ . ”. "	(-)	*G. lamblia*	(-)	NA	*G. lamblia*	NA	68	M	none
127055	8.9.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	85	M	none
127050	8.8.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	16	F	none
127046	8.7.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	62	F	none
127065	8.9.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	78	M	none
127078	8.9.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	89	F	none
127075	8.9.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	48	F	none
126356	7.27.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	NA	42	F	none
126019	7.22.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	NA	38	F	none
126717	8.3.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	NA	64	F	none
126548	7.31.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	NA	58	M	none
126870	8.5.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	NA	53	M	none
126809	8.3.2009	“ . ”. "	*Crypto.*	(-)	G &/or C	*Crypto.*	*Crypto.*	NA	32	M	none
126955	8.7.2009	“ . ”. "	*Crypto.*	(-)	G &/or C	*Crypto.*	*Crypto.*	NA	39	F	none
126594	7.30.2009	“ . ”. "	*Crypto.*	(-)	G &/or C	*Crypto.*	*Crypto.*	NA	14	M	none
126695	8.3.2009	“ . ”. "	*Crypto.*	(-)	G &/or C	*Crypto.*	*Crypto.*	NA	8	M	*Salmonella* spp.
127977	8.25.2009	8.25.2009	(-)	(-)	(-)	NA	(-)	NA	85	F	none
127981	8.25.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	74	F	none
127972	8.24.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	0	F	none
127711	8.18.2009	“ . ”. "	*Crypto.*	(-)	G &/or C	*Crypto.*	*Crypto.*	NA	3	F	none

ID	D.O.C. M/D/Y	D.O.E. M/D/Y	*Crypto*. spp. (*u*PCR)	*G. lamblia* (*u*PCR)	Screening kit[1]	Discriminatory kits[2]	*m*PCR	Sequencing	Age	Sex	Co- infection
127701	8.17.2009	“ . ”. "	*Crypto*.	(-)	G &/or C	*Crypto*.	*Crypto*.	NA	6	F	none
127638	8.17.2009	“ . ”. "	*Crypto*.	(-)	G &/or C	*Crypto*.	*Crypto*.	NA	7	F	none
127832	8.18.2009	“ . ”. "	*Crypto*.	(-)	G &/or C	*Crypto*.	*Crypto*.	NA	1	M	none
127340	8.13.2009	“ . ”. "	*Crypto*.	(-)	G &/or C	*Crypto*.	*Crypto*.	NA	32	F	none
127434	8.14.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	0	M	none
127962	8.24.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	21	F	none
127971	8.24.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	69	F	none
127961	8.24.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	67	M	none
127767	8.20.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	NA	38	M	none
127975	8.24.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	75	F	*Clostridium difficile*
127815	8.21.2009	“ . ”. "	*Crypto*.	*G. lamblia*	G &/or C	G & C	C & G	NA	22	F	none
127963	8.24.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	67	F	none
127974	8.24.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	64	F	none
127966	8.25.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	55	F	none
127907	8.21.2009	9.15.2009	*Crypto*.	(-)	G &/or C	*Crypto*.	*Crypto*.	NA	7	F	none
128110	8.26.2009	“ . ”. "	*Crypto*.	(-)	G &/or C	*Crypto*.	*Crypto*.	NA	5	M	*Salmonella* spp.
128130	8.27.2009	“ . ”. "	*Crypto*.	(-)	G &/or C	*Crypto*.	*Crypto*.	NA	59	M	none
128170	8.27.2009	“ . ”. "	(-)	(-)	G &/or C	(-)	(-)	NA	46	M	none
128495	9.2.2009	“ . ”. "	*Crypto*.	(-)	G &/or C	*Crypto*.	*Crypto*.	NA	2	F	none
128436	9.2.2009	“ . ”. "	*Crypto*.	(-)	G &/or C	*Crypto*.	*Crypto*.	NA	54	M	none
128495	9.2.2009	“ . ”. "	*Crypto*.	(-)	G &/or C	*Crypto*.	*Crypto*.	NA	2	F	none
128893	9.8.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	NA	43	F	none
128538	9.3.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	44	M	none

ID	D.O.C. M/D/Y	D.O.E. M/D/Y	*Crypto*. spp. (*u*PCR)	*G. lamblia* (*u*PCR)	Screening kit[1]	Discriminatory kits[2]	*m*PCR	Sequencing	Age	Sex	Co- infection
128703	9.4.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	0	M	none
128304	8.20.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	14	F	none
128224	8.28.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	53	M	none
128153	8.27.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	48	F	none
127995	8.25.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	NA	3	M	none
128562	9.3.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	NA	57	F	none
128930	9.9.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	NA	4	M	*Salmonella* species
129095	9.11.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	NA	34	M	none
129080	9.11.2009	“ . ”. "	(-)	(-)	(-)	NA	(-)	NA	66	M	none
129089	9.11.2009	“ . ”. "	(-)	*G. lamblia*	(-)	*Giardia*	(-)	assemblage B	40	F	none
128565	9.11.2009	“ . ”. "	(-)	(-)	G &/or C	(-)	(-)	NA	0	M	none
107056	9.23.2008	“ . ”. "	*Crypto*	*G. lamblia*	G &/or C	G & C	C & G	assemblage A	5	M	none
107903	10.7.2008	“ . ”. "	*Crypto*	*G. lamblia*	G &/or C	G & C	C & G	*C. hominis*	11	F	none
120263	4.19.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Giardia*	*G. lamblia*	NA	68	F	none
127554	8.17.2009	“ . ”. "	(-)	*G. lamblia*	G &/or C	*Crypto*	*G. lamblia*	NA	51	M	none
509820	9.15.2007	“ . ”. "	(-)	(-)	G &/or C	(-)	(-)	NA	0	M	none
108774	10.18.2008	“ . ”. "	*Crypto*	(-)	G &/or C	*Crypto*	*Crypto.*	*C. hominis*	1	F	none

Abbreviations:

ID; the identification number of the clinical sample

D.O.C.; the date of collection

D.O.E.; the date of sample extraction

*u*PCR; the uniplex PCR

*m*PCR; the multiplex PCR

G/ C; *Giardia*/*Cryptosporidium*

NA; not applied

F; female, M; male

[1] *Giardia*/*Cryptosporidium* Chek™ kit (TechLab)

[2] RIDA®Quick *Cryptosporidium* and RIDA®Quick *Giardia* (R- Biopharm)

Appendix B: Laboratory reagents

Material	Working solution preparation and/or storage	Company
Methanol 1 %	Stored at RT	Fisher
Hydrochloric acid 1%	Used directly	Sigma (UK)
Carbol fuchsin reagent	Carbol fuchsin reagent 3% (1000 ml): • Basic fuchsin (10 g) • Absolute Ethanol (100 ml) • Phenol (50 g) • dH2O up to 1000 ml	Sigma (UK)
Malachite green 0.4 %	Stored at RT	
1 % Lugol's Iodine	Freshly prepared (2 weeks) and stored at RT	
10 % formal-saline	Stored at RT	(E and O Laboratories)
Ethyl-ether	Stored at RT	Fischer Scientific
Saturated salt solution	Saturated salt solution with specific gravity of 1.2 • 200 g of sodiumchloride • 200 ml of dH2O • 10g /10 min with continous stirring until saturation • With the hydrometer, the specific gravity was adjusted to 1.2 by adding dH2O Stored at 4°C t	Sigma (UK)
Giardia/*Cryptosporidium* Check™	Stored at 4°C	(TechLab)
RIDA® Quick *Cryptosporidium* kit	Stored at 4°C	(Biopharm)
RIDA® Quick *Giardia* kit	Stored at 4°C	(Biopharm)
E.histolytica II test	Stored at 4°C	(TechLab)
QIAamp® Stool Mini Kit	Stored at RT	(Qiagen)

Material	Working solution preparation and/or storage	Company
Phosphate Buffer Saline (PBS)	<u>Phosphate Buffer Saline (PBS):</u> • NaCl (8 g)+ KC (l0.2g) + Na_2HPO_4 (1.44 g) • + KH_2PO_4 (0.24 g) • pH 7.4 • Made up to 500 ml in dH2O • Autoclaved at 121°C at 15 psi for 15 min • Stored at RT	Sigma, (UK)
Go Taq and Go Taq Hot Start DNA polymerase	Small aliquots were prepared and stored at -20°C	Promega
Polymerase buffers and $MgCl_2$	Stored at −20°C	Promega
Deoxynucleotides triphosphates (dNTPs)	Aliquots of 10 µl of 10 mM concentration of dNTPs mix were prepared and stored at −20°C	Bioline (UK).
Dimethyl sulfoxide (DMSO)	99.9 % molecular grade solution	Sigma (UK)
DNA Zap™ solutions and DNA off solution	Stored at RT	Bioline (UK)
Molecular biology grade agarose	Stored at RT	Bioline (UK)
TAE buffer (Tris/acetate/EDTA)	<u>1 liter of 50x stock solution of TAE</u> • 242 g Tris base • 57.1 ml glacial acetic acid • 37.2 g Na_2EDTA • Adding H_2O to 1 liter • Stored at RT	(Sigma, UK)
Ethidium bromide	-	(Sigma, UK)
λ-*Hind*III and GeneRuler DNA ladders	Stored at 4°C	(Fermentas, UK)

Material	Working solution preparation and/or storage	Company
DNA sample dye buffer	0.1% (w/v) DNA sample buffer preparation: • Bromophenol blue (20 mg) • Xylene cyanole(20 mg) • 400µl 0.5 M EDTA (pH 8.0) • 8 ml Glycerol (99%) • dH2O up to 12 ml • Stored at 4°C	(Sigma, UK)
YORBIO Gel/PCR DNA Purification Kit	Stored at RT	Yorkshire Bioscience
Ethanol	Stored at RT	(Sigma, UK)
*Eco*RI , *Dpn*I and *Hind* III restriction enzymes	Stored at −20°C	Roche or New England Biolabs
pGEM-T-Easy vector system	Stored at −20°C	Invitrogen
TOPO-TA Cloning Kit® for Sequencing	Stored at −20°C	Invitrogen
T4 DNA ligase and 2x Rapid ligation buffer	Stored at −20°C	Promega
RNase	Stored −20°C	(Sigma, UK)
Isopropanol	Stored at RT	Fisher (UK).
QIAprep - Spin Miniprep and Midiprep kits	Stored at RT	(Qiagen, UK)
SOC medium	SOC medium: • 4g Bacto-tryptone • 1 g Bacto-yeast extract • 0.1g NaCl • Made up to 200 ml with dH2O • Autoclaved at 121 ˚C and 15 psi for 15 min • To 10 ml of the medium, add : • 50µl of filter sterilized 2M MgCl2 • 200 µl of filter sterilized 1 M glucose • Stored at RT	(Sigma, UK)
Luria Bertani agar (LA)	500 ml of Luria Bertani agar (LA):	(Sigma, UK)

Material	Working solution preparation and/or storage	Company
	• 5 g Bacto-tryptone (Difco) • 2.5 g Bacto-yeast extract (Difco) • 5 g NaCl2 • 7.5 g Bacto-agar (Difco) • dH2O up to 500 ml Autoclaved at 121˚C and 15 psi for 15 min	
Luria Bertani broth (LB)	500 ml of Luria Bertani broth (LB): • 5 g Bacto-tryptone (Difco) • 2.5 g Bacto-yeast extract (Difco) • 5 g NaCl2 • dH2O up to 500 ml Autoclaved at 121˚C and 15 psi for 15 min	(Sigma, UK)
Ampicillin	100 mg/ml Ampicillin stock solution: • 1g Ampicillin • dd H_2O up to 10 ml • Filter sterilization • Stored at -20°C	(Sigma, UK)
Kanamycin (40mg/ml) and X-gal (40 mg/ml)*	Stored −20°C	(Sigma, UK)
Alkaline solution	Alkaline solution (10ml): • 0.2 M NaOH (0.4 ml 5 M) • 1% Sodium dodecyl sulphate SDS (1ml 10% SDS) • dd H_2O (8.6 ml)	(Sigma, UK)
Lysozyme	Lysozyme solution (10 ml): • 50 mM Glucose • 25 mM Tris HCL (pH 8.0) • 10 mM EDTA • dH2O up to 10 ml 4mg of Lysozyme per 1 ml Lysozyme solution (prepared fresh)	(Sigma, UK)