Development and application of molecular tools for the detection of the human pathogenic protozoan *Giardia, Cryptosporidium* and *Toxoplasma*

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This dissertation is dedicated to

my parents and my sister

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Abbreviations

ATP	Adenosine Triphosphate
B3	Backward Outer Primer
BIP	Backward Inner Primer
c	Complementary
COWP	*Cryptosporidium* Oocyst Wall Protein
DDBJ	DNA Data Bank of Japan
EBI-EMBL	European Molecular Biology Laboratory -European Bioinformatics Institute
EF1α	Elongation Factor 1 Alpha
ESVs	Encystation Specific Vesicles
F3	Forward Outer Primer
FE-SEM	Field Emission Scanning Electron Microscopy
FIP	Forward Inner Primer
FITC	Fluorescein Isothiocyanate
GDH	Glutamate Dehydrogenase
gDNA	Genomic DNA
GP60	60 Kda Glycoprotein
HPLC	High-Performance Liquid Chromatography
HSP	Heat Shock Protein
ICZN	International Code of Zoological Nomenclature
IFT	Immunofluorescence Test
L	Loop Primers
LAMP	Loop Mediated Isothermal Amplification
LB	Loop Backward Primer

LDM	Limited Dilution Method
LF	Forward Loop Primer
Lsu rRNA	Long Subunit Rrna,
NASBA	Nucleic Acid Sequence Based Amplification
nPCR	Nested Polymerase Chain Reaction
nt	Nucleotides
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PCR−RFLP	Polymerase Chain Reaction−Restriction Fragment Length Polymorphism
PV	Parasitophorous Vacuole
PVM	Parasitophorous Vacuolar Membrane
SAM	S-Adenosyl-Methionine Synthetase
SNP	Single Nucleotide Polymorphism
SSU rRNA	Small Subunit Rrna
TAE	Tris-Acetate-Edta
TgOWP1	*Toxoplasma gondii* Oocyst Wall Protein
tpi	Triosephosphate Isomerase
TRAP C	Thrombospondin- Related Adhesive Protein of *Cryptosporidium*
wwPDB	Worldwide Protein Data Bank
β-giardin	Beta Giardin

1. Introduction

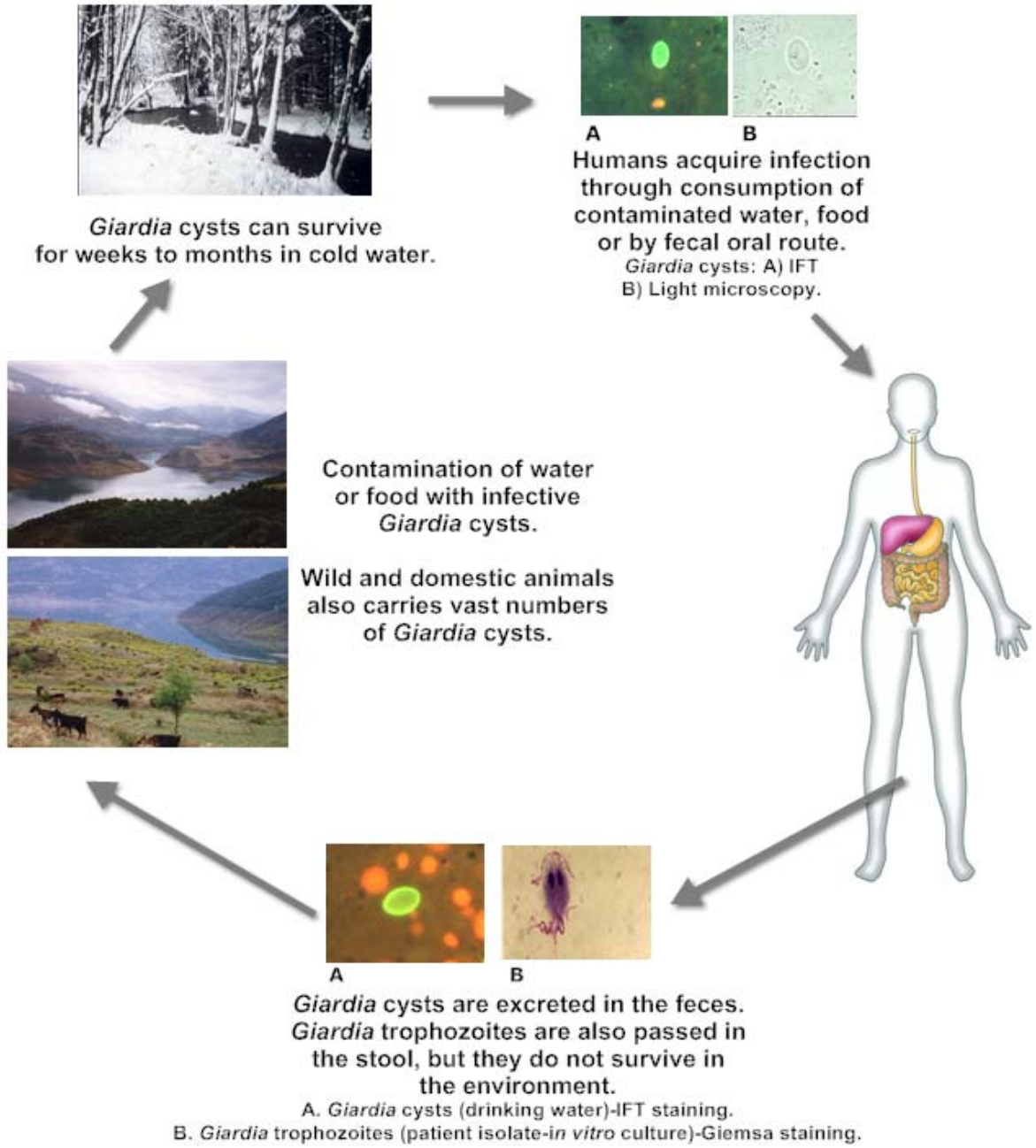
1.1 The life cycle of *Giardia* spp.

Giardia has a direct two-stage life cycle consisting of the trophozoites and cysts. The infectious stage of the parasite, the cyst, is encysted when released into the feces. It is the resistant form, immediately infectious and responsible for the transmission of the disease. The trophozoite stage of *Giardia lamblia* (*G. duodenalis*, *G. intestinalis*) has a characteristic flattened tear-drop shape and measures an average size of 15 x 9 µm x 3 µm. When stained, the trophozoites appear to be bilaterally symmetrical and dorsoventrally flattened having twin nuclei, two slender median rods (axostyles), which play an important role for differentiating between species, and 4 pairs of flagella - one arising from the anterior, two from the posterior and one from the caudal end of the body of the organism. The pair of anterior flagella, known as axoneme, is straight, closely approximated and parallel to each other, dividing the body of the organism into two halves longitudinally. Trophozoites lack mitochondria, endoplasmic reticulum, Golgi, peroxisomes and lysosomes (Elias et al., 2008; Abodeely et al., 2009).

The life cycle begins by the oral ingestion of a few cysts through contaminated water, food, or by the fecal-oral route (hands or fomites) (Rendtorff and Holt, 1954; Baldursson and Karanis, 2011) (Figure 1). The low pH of the stomach acid induces excystation, in which the activated flagellum breaks through the cyst wall and the proteolytic activity of the duodenum forces each cyst to produce two trophozoites. The trophozoites remain in the lumen of the proximal small bowel where they can be free or attached to the mucosa by a ventral sucking disk and multiply asexually by longitudinal binary fission.

The trigger for encystment is not clear, but the process occurs most likely as a result of exposure to and stimulation by bile salts and fatty acids, and a more alkaline environment. During encystation, developmentally regulated formation of large secretory compartments containing cyst-wall material occurs. Despite the lack of any morphological similarities, these encystation-specific vesicles (ESVs) show several biochemical characteristics of maturing Golgi cisternae (Marti and Hehl, 2003; Hehl and Marti, 2004).

Figure 1. *Giardia lamblia* life cycle stages and transmission routes.



Cysts are the stage found in normal and non-diarrheal feces, while both cysts and trophozoites can be found in feces, and are infectious immediately. The cysts are non-motile and egg-shaped, measuring an average of 12 µm x 9 µm and are encased by a smooth and colourless, thick and refractile wall (Ali and Hill, 2003). Immediately after encystation, like alldiplomonads, newly formed *Giardia* cysts contain two genetically identical diploid nuclei that are morphologically indistinguishable, replicate at approximately the same time, and are both transcriptionally active (Adam, 2000). However, each organelle duplicates so that in permanently stained mature cysts, four prominent nuclei and four median bodies are observed.

1.2 Phylogeny of *Giardia*

The phylogeny of *Giardia* is not completely clear since it has simple intracellular structures that might represent an early branching of the eukaryotic lineage that diverges before the acquisition of mitochondria. *Giardia* belongs to the Kingdom: Protista, Subkingdom: Protozoa, Phylum: Sarcomastigophora, Subphylum: Mastigophora, Class: Zommastigophora, Order: Diplomonadida, Family: Hexamitidae, Genus: *Giardia*, Species: lamblia (Adam, 2001; Morrison et al., 2007).

1.3 Nomenclature and taxonomy of *Giardia*

Giardia duodenalis, Giardia intestinalis and *Giardia lamblia* are species names interchangeably used in current literature referring to the same organism. *Giardia* has a global distribution and is a major contributor to the enormous burden of diarrheal diseases. Six different *G. intestinalis* assemblages have been defined (A-H) by DNA sequence analysis so far, of which assemblages A and B are mainly virulent for humans and are often referred to as ‘zoonotic’ assemblages (Caccio and Ryan, 2008; Lasek-Nesselquist et al., 2010). *Giardia* host-adapted assemblages have been found to be assemblages C and D primarily for dogs, assemblage E or livestock genotype for artiodactyl species and assemblage F for cats (Table 1).

Table 1. *Giardia* spp. names, assemblages, host-adapted assemblages and Polymerase Chain Reaction (PCR-based techniques to detect and differentiate *Giardia* spp. at species and assemblages level.

Species	Assemblages	Major host	PCR-based techniques	Reference
G. duodenalis (intestinalis, lamblia)	Assemblage A (Polish)	Humans, nonhuman primates, domestic and wild ruminants, alpacas, pigs, horses, domestic and wild canines, cats, ferrets, rodents, monkeys, marsupials, other mammals	GDH, β-giardin, tpi, SSU rRNA, kinesin-like protein 1, β-tubulin, cyst wall protein, giardin	Upcroft et al., 1996 Monis et al., 1996 Homan et al., 1998 Karanis & Ey, 1998 Iwabe and Miyata, 2001 Palm et al., 2003 Beck et al., 2011 Gomez-Munoz et al., 2012 Asher et al., 2012 Beck et al., 2012
	Assemblage B (Belgian)		GDH, tpi, β-giardin, EF1A, SSU rRNA	Monis et al.,1996 Homan et al., 1998 Karanis & Ey, 1998 Sulaiman et al., 2003 Breathnach et al., 2010, Lasek-Nesselquist et al., 2008 Bonhomme et al., 2011 Wielinga et al., 2011 Asher et al., 2012
	Assemblages C and D	Domestic and wild canines	GDH, β giardin, tpi	Monis et al., 1996 Sulaiman et al., 2003 Gaydos et al., 2008 Paz e Silva et al., 2012
	Assemblage E		GDH, β-giardin, tpi, SSU rRNA	Khan et al., 2011 Levecke et al., 2011 Ng et al., 2011
	Assemblage F	Cats	GDH, SSU rRNA, tpi, β-giardin	Lalle et al., 2005 Suzuki et al., 2011
	Assemblage G	Mice, rats	GDH, tpi,	Lasek-Nesselquist et al., 2010 Lebbad et al., 2010
	Assemblage H	Seals	GDH	Lasek-Nesselquist et al., 2010
G. agilis		Amphibians	none	Hegner, 1922
G. muris		Rodents	Lsu rRNA, variant-specific surface protein, tpi, EF1A, β-giardin	van Keulen et al., 1992 Monis et al., 1999 van Keulen et al., 2002 Guy et al., 2004 Ropolo et al., 2005
G. psittaci		Birds	SSU rRNA	van Keulen et al., 2002
G. ardeae		Birds	GDH SSU rRNA, tpi, EF1A, actin	van Keulen et al., 1993 Monis et al., 1999 Teodorovic et al., 2007
G. microti		Muskrats and voles	SSU rRNA, tpi	van Keulen et al., 1998 Sulaiman et al., 2003

The uncertain taxonomy affected negatively the understanding of giardiasis and was supported from the fact that several papers in the international literature do not consistently used the terms (sub-) assemblage and (sub-)genotype. It is suggested to use the terms genotype, assemblage and subassemblage for the characterisation of the different *Giardia* lineages (Plutzer et al., 2010).

The uniqueness of assemblages A and B was shown after phylogenetic analyses of nucleotide sequence obtained from the amplification of the small subunit (SSU) rRNA gene and housekeping genes such as glutamate dehydrogenase (GDH), beta giardin (β-giardin), elongation factor 1 alpha (EF1α), and triosephosphate isomerase (tpi) genes (Adams et al., 2004; Robertson et al., 2006; Gelanew et al., 2007; Caccio et al., 2008). Recent studies based on a multilocus approach defined that isolates could only be allocated to a specific group when polymorphisms of the three loci were combined, which makes the multilocus genotyping more difficult and complex (Levecke et al., 2009).

1.4 The life cycle of *Cryptosporidium* spp.

The life cycle of *Cryptosporidium* is complex with both sexual and asexual cycles, and there are six distinct developmental stages where it complete its entire cycle within a single host (Keusch et al., 1992). *Cryptosporidium* infection begins with ingestion of the sporulated oocyst, the resistant stage produced by *Cryptosporidium* that is found in the environment. The remarkably stable oocysts can survive for months or even years in the environment, while the infective dose is low, probably even a single oocyst can cause infection (Skotarczak, 2010) (Figure 2).

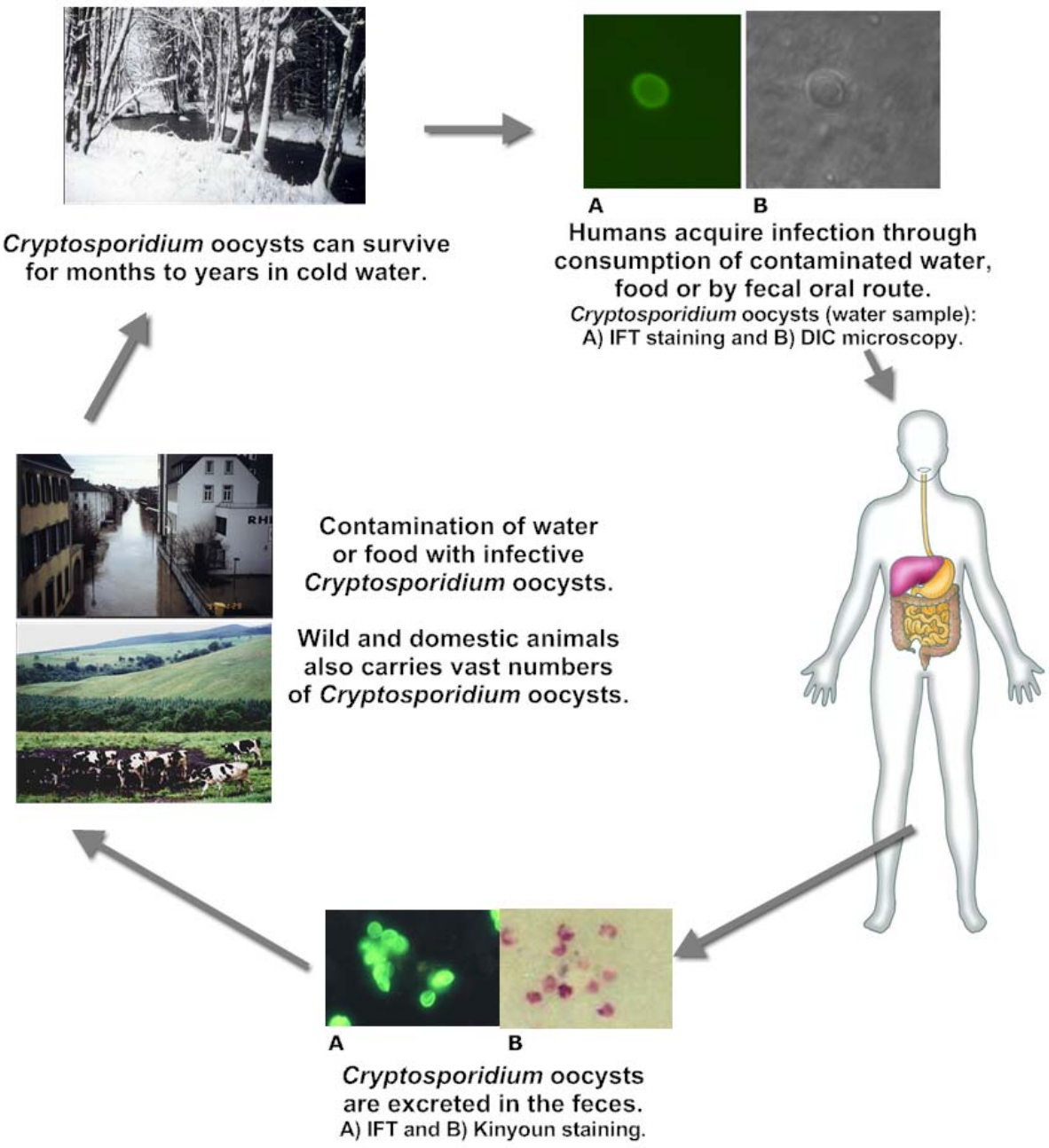
Each mature oocyst (5 μm) contains 4 infective stages termed sporozoites, which are rapidly expelled through an opening that is formed in the oocyst wall when the oocysts encounter appropriate biochemical and environmental factors (Fayer and Leek, 1984; Upton et al., 1994; Karanis et al., 2008; Karanis and Aldeyarbi, 2011). The residual body is either released or retained within the oocyst. In the intestine, sporozoites invade the epithelial linings and within these cells, the sporozoites undergo schizogony, or asexual reproduction.

When the sporozoites attach to the epithelial cells and invade the host cell, a parasitophorous vacuolar membrane (PVM) envelops them. At this stage the parasite is considered as being intracellular but extracytoplasmic, enclosed by a thin layer of host cell cytoplasm, as it remains in a highly polar location adjacent to the surface of the columnar host intestinal epithelial cell (O'Donoghue, 1995; Umemiya et al., 2005). Throughout the literature for the localisation of *Cryptosporidium* stages in the intestine, recently the findings of Valigurova et al. re-evaluated the term used up to now term "intracellular, but extra-cytoplasmatic" (Valigurova et al., 2007; Jirku et al., 2008). The newly introduced and suggested term is "epicellular" and is used to describe the parasite embracement by the host cell membrane (Valigurova et al., 2007).

Field Emission Scanning Electron Microscopy (FE-SEM) and comparative transmission electron microscopy observations, showed the evolutionary homology of *Gregarina steini* and *C. muris* and confirmed that the cryptosporidian developmental stages are not intracellular, do not penetrate under the host cell membrane and do not come into close contact with the host cell cytoplasm (Valigurova et al., 2007).

Cryptosporidium shares several characteristics similar to gregarines like the occupation of an extracytoplasmatic niche, concerning the feeding dependency and similar location after host cell invasion (Borowski et al., 2008). On the other hand the feature of *Cryptosporidium* to promote the encapsulation with the host cell apical membrane, when the parasite invides the host cell is a unique property in relation to gregarines and is closely dependent upon their connection with the host cell for nutrient acquisition (Borowski et al., 2010).

Figure 2. *Cryptosporidium* life cycle stages and transmission routes.



During the process of the developing of the parasite to trophozoite, the parasitophorous vacuole (PV) invaginates at the host cell interface and forms the so called “feeder organelle” responsible for nutrient uptake from the host cell (Spano and Crisanti, 2000) while materials appear to be released from the conoid at the anterior tip of the sporozoite (Xiao and Fayer, 2008). Within the Type I meront, 8 merozoites are formed as a result of the multiple fission (=merogony; =schizogony) releasing free merozoites from the PV. Type I merozoites can undergo two different cycles. Either they can further develop through type II merogony, or undergo another round of Type I merogony to form additional meronts by rising the potential of continuously existance of Type I meronts (Current and Reese,1986). Probably due to the recycling of the Type I merozoites through the Type I merogony the classical persistent *Cryptosporidium* infection is observed. Trophozoites undergoing asexual division produce from each meront 6-8 merozoites that can infect another host cell and to develop the Type II meronts. Type II meronts are capable either of initiating another round of type II merogony or type II merozoites develop into the sexual reproductive stages of the parasite known as gamonts. Controversial preliminary investigations suggest that extracellular gamont stages of the gregarines very closely resemble the extracellular forms of *Cryptosporidium* (Hijjawi et al., 2002; 2004; Rosales et al., 2005). These gamonts initiate sexual differentiation producing male microgamonts or female macrogamonts.

The sexual stage of development occurs upon the mating of the macrogamete, containing one large nucleus which does not divide, and the multinucleate microgamete incorporating at least 16 microgametes. The produced diploid zygote result to the formation of oocyst containing four haploid sporozoites. These oocysts are the environmentally resistant form and are passed in the feces after their release in the gastrointestinal tract. Oocysts can be differentiated into two types: the so called “thick-walled” oocysts composed of two outer membranes representing approximately 80% of the produced oocysts capable of infecting other hosts and the “thin-walled” composed of a single outer membrane not passed in the feces representing approximately 20% of the produced oocysts capable of infecting other hosts (Current and Reese,1986). This feature, along with recycled meronts, is probably responsible for the persistent infections that are not propagated by repeated

ingestion of oocysts. This often life-threatening non-resolving state of cryptosporidiosis is often observed in AIDS patients (Clark, 1999; Gookin et al., 2002).

1.5 Phylogeny of *Cryptosporidium*

The genus *Cryptosporidium* was discovered by Ernest E. Tyzzer as a peculiar parasite affecting the mouse stomach and is recognised as the first named species, *Crytosporidium muris*, as coccidian (Jirku et al., 2008). Later he described *C. parvum* which affected the mouse intestine (Tyzzer, 1912). In the following years, multiple new cryptosporidian species were described, infecting the majority of vertebrates, and the taxonomic status of the genus has been traditionally aligned to the coccidia. The taxonomic classification has been the subject of debate undergoing major revisions, because *Cryptosporidium* possesses features of both the coccidia and gregarines, and yet probably represents a distantly related lineage of apicomplexa (Barta and Thompson, 2006).

Within the genus, species designation has evolved with the application and interpretation of emerging technologies in the 100 years since the first named species, *C. muris,* was proposed (Tyzzer, 1910). Classical parasitological features of life cycle, host range, morphology and site of infection have more recently been complemented with genomic data. At the 6th Meeting on Molecular Epidemiology and Evolutionary Genetics in Infectious Disease, France, recommendations for the criteria for naming new species of *Cryptosporidium* were made (Xiao et al., 2004a). Fulfilment of four basic requirements was suggested: morphometric studies of oocysts, genetic characterisation, demonstration of natural and (if feasible) some experimental host specificity, and compliance with the International Code of Zoological Nomenclature (ICZN) (Xiao et al., 2004a, Plutzer and Karanis, 2009). On this basis, there are now 20 named species (22, if two fish species are accepted without genetic characterisation) (Fayer et al., 2010). The main human pathogens are considered to be *C. hominis*, *C. parvum* and *C. meleagridis*, with evidence for pathogenicity of *C. felis* and *C. canis* in some settings (Xiao et al., 2002; Xiao and Ryan, 2004; Cama et al., 2008). However, genetic analyses in the absence of other data have led to the temporary naming of “genotypes” unlinked to named species and of uncertain taxonomic position, now numbering more than 40 in the published literature (Fayer et al., 2010) of which 28 are valid names for distinct species

(Slapeta, 2012). One of these, the “rabbit genotype”, has been identified as a human pathogen of public health importance, having caused a waterborne outbreak (Chalmers et al., 2009).

1.6 Nomenclature and taxonomy of *Cryptosporidium*

An endless debate associated with the nomenclature and the taxonomy of the different *Cryptosporidium* spp. has begun, where researchers all over the world quote their arguments and defending the newly characterised species. According to the fourth edition of the ICZN rules governing the taxonomy of protozoan parasites and other animals taking effect on 1 January 2000 (www.nhm.ac.uk/hosted-sites/iczn/code/) for the naming of new species of *Cryptosporidium* there are criteria to be met such as morphometric (i.e. microscopic observations of oocysts - size and morphology) description of the morphology and unique features of the oocysts, cross-infectivity experiments to demonstrate natural or to conduct forced host infections, the development of numerous molecular biological techniques experimental host specificity and genetic characterisation to differentiate the new nominal taxon from related or similar taxa (Egyed et al., 2003; Slapeta, 2006; Xiao et al., 2004a; 2012; Slapeta, 2012). There are unsolved problems about the precise number of species infecting humans, complexity, the attribution of different species or strains/genotypes to sporadic or outbreak events, the virulence or transmission of the species or strains/genotypes in humans and the abscence of diagnostic features that allow the differentiation of *Cryptosporidium* spp..

Traditional, microscopic, biochemical and serological techniques have shown significant limitations on the specific diagnosis of cryptosporidiosis and there has been substantial need for improved molecular detection techniques (Cacciò and Pozio, 2006). Epidemiological investigations are helpful to define the transmission routes of cryptosporidiosis, but traditional diagnostic tools do not have the ability to differentiate sources of parasites, and epidemiologic investigations are expensive to detect and differentiate *Cryptosporidium* spp. at species/genotype and subtype levels as shown in Table 2. Although the present table focuses predominantly on PCR-based methods, other approaches such nucleic acid sequence based amplification (NASBA) have been proposed (Compton, 1991; van Belkum and Neisters, 1995).

Table 2. *Cryptosporidium* spp. names, host-adapted species and PCR-based techniques to detect and differentiate *Cryptosporidium* spp. at species and genotype level.

Species	Major Host	PCR-based techniques	Reference
*C. agni**	Sheeps	-	Barker & Carbonell, 1974
*C. ameivae**	Lizards	-	Arcay-de-Peraza & Bastardo-de-San-Jose,1969
C. andersoni	Cattles, Calves Bactrin Camels	HSP90, microsatellite & minisatellite, SSU rRNA, COWP loci, 18s rRNA, actin	Lindsay et al., 2000 Xiao et al., 2000 Sulaiman et al., 2002 Fayer et al., 2006 Wang et al., 2008 Feng et al., 2011 Wang et al., 2011
*C. anserinum**	Geeses	-	Proctor & Kemp,1974
C. baileyi	Birds Ducks Geeses	18s rRNA, SSU rRNA and HSP70, COWP, actin	Current et al., 1986 Sulaiman et al., 2000 Xiao et al., 2000 Gasser et al., 2001 Sulaiman et al., 2002 Chvala et al., 2006 Amer et al., 2010
C. blagburni	Birds	HSP70, 18s rRNA	Morgan et al., 2001
C. bovis	Calves Cattles	SSU rRNA, 18s rRNA HSP-70, actin	Fayer et al., 2005, Fayer et al., 2006 Wang et al., 2011 Santín & Zarlenga, 2009
C. canis	Dogs Humans	Hsp70, SSU rRNA, COWP, cHSP70	Xiao et al., 1999 Xiao et al., 2000 Sulaiman et al., 2000 Lindergard et al., 2003
*C. cichlidis**	Fishes	-	Paperna & Vilenkin, 1996
C. crotali	Snakes	-	Triffit, 1925
C. cuniculus	Rabbits	SSU rRNA, 60kDa GP60, HSP70, actin, CWOP	Rehg et al., 1979 Chalmers et al., 2009 Nolan et al., 2010 Robinson et al., 2010
C. curyi	Cats	-	Ogassawara et al., 1986
C. fayeri	Koalas Kangaroos	GP60, 18s rRNA, actin	Ryan et al. 2008 Power et al., 2009 Power et al., 2011 Waldron et al., 2010
C. felis	Cats	18S rRNA SSU rRNA, COWP TRAP-C Cpgp40/15, HSP70, actin	Sulaiman et al., 2000 Xiao et al., 2000 Sulaiman et al., 2002 Lindergard et al., 2003 Santin et al., 2006 Muthusamy et al., 2006

Species	Major Host	PCR-based techniques	Reference
C. fragile	Frogs	SSU rRNA	Jirku et al., 2008
C. galli	Birds	18s rRNA	Pavlasek, 1999 da Silva et al., 2010
C. hominis	Humans Monkeys	SSU rRNA, COWP, TRAP-C, Cpgp40/15 (	Morgan-Ryan et al., 2002 Muthusamy et al., 2006
C. macropodum	Kangaroos		Power & Ryan 2008
C. meleagridis	Birds Turkeys Humans	SSU rRNA COWP, TRAP-C, Cpgp40/15, HSP70, actin	Sulaiman et al., 2000 Sulaiman et al., 2002 Xiao et al., 2000 Muthusamy et al., 2006
C. molnari	Fishes	SSU rRNA, actin	Alvarez-Pellitero & Sitjà-Bobadilla, 2002 Palenzuela et al, 2010 Barugahare et al., 2011
C. muris	Rodents Humans Mices Giraffe Monkeys Snakes Cattles Bovine Cats Bactrin Camels	18S rRNA, SSU rRNA, GP60, 18S rDNA and ITS1 regions, HSP-70, TRAP C, COWP, pgp40/15, microsatellite and minisatellite, HSP70, actin	Tyzzer, 1907 Morgan et al., 2000 Sulaiman et al., 2000 Xiao et al., 2000 Dubey etal., 2002 Gatei et al., 2002 Sulaiman et al., 2002 Palmer et al., 2003 Hikosaka & Nakai, 2005 Muthusamy et al., 2006 Santín et al., 2006 Lv et al., 2009 Pedraza-Díaz et al., 2009 Kodadkova et al., 2010 Feng et al., 2011
C. nasorum	Fishes	-	Levine, 1984
C. parvum	Humans Snakes Cattles Mammals	SSU rRNA, COWP TRAP-C, CpGP40/15, hsp 70, 18s rRNA	Tyzzer, 1912, Sulaiman et al., 2002 Lindergard et al., 2003 Xiao et al., 2004b Fayer et al., 2006
C. pestis	Tortoises Cattles	SSU rRNAr	Slapeta, 2006, Traversa et al., 2008
C. ryanae	Cattles Calves	SSU rRNA	Fayer et al., 2008 Santín & Zarlenga, 2009 Wang et al., 2011
C. reichenbachklinkei	Fish	-	Paperna et al., 1986
*C. rhesi**	Monkeys	-	Levine, 1980
C. saurophilum	Reptiles Lizards	actin	Koudela & Modry 1998 Sulaiman et al., 2002 Xiao et al., 2004b Plutzer & Karanis, 2007 Pavlasek & Ryan, 2008
C. scophthalmi			Alvarez-Pellitero et al. 2004; 2009

Species	Major Host	PCR-based techniques	Reference
C. serpentis	Snakes	COWP, HSP70, actin	Levine, 1980, Sulaiman et al., 2000 Xiao et al., 2000 Sulaiman et al., 2002 Xiao et al., 2004b
C. suis	Pigs Cattles	18s rRNA	Ryan et al. 2004 Fayer et al., 2006
C. tyzzeri n. sp.	Mices	SSU rRNA, GP60	Ren et al., 2012 Xiao et al., 2012
C. ubiquitum	Goats Sheeps Calves Humans	SSU rRNA, COWP HSP70, actin	Robinson et al., 2008 Fayer et al., 2010 Pollock et al., 2010 Robinson et al., 2010 Fiuza et al., 2011
*C. varanii**	Lizards	18S rRNA, actin	Pavlasek & Ryan, 2008
C. villithecum	Fishes	-	Paperna et al., 1986
C. wrairi	Guinea pigs	COWP, HPS70, actin	Vetterling et al. 1971 Xiao et al., 2000 Sulaiman et al., 2000 Sulaiman et al., 2002
C. xiaoi	Sheep	SSU rRNA, HSP-70, actin	Fayer & Santin, 2009

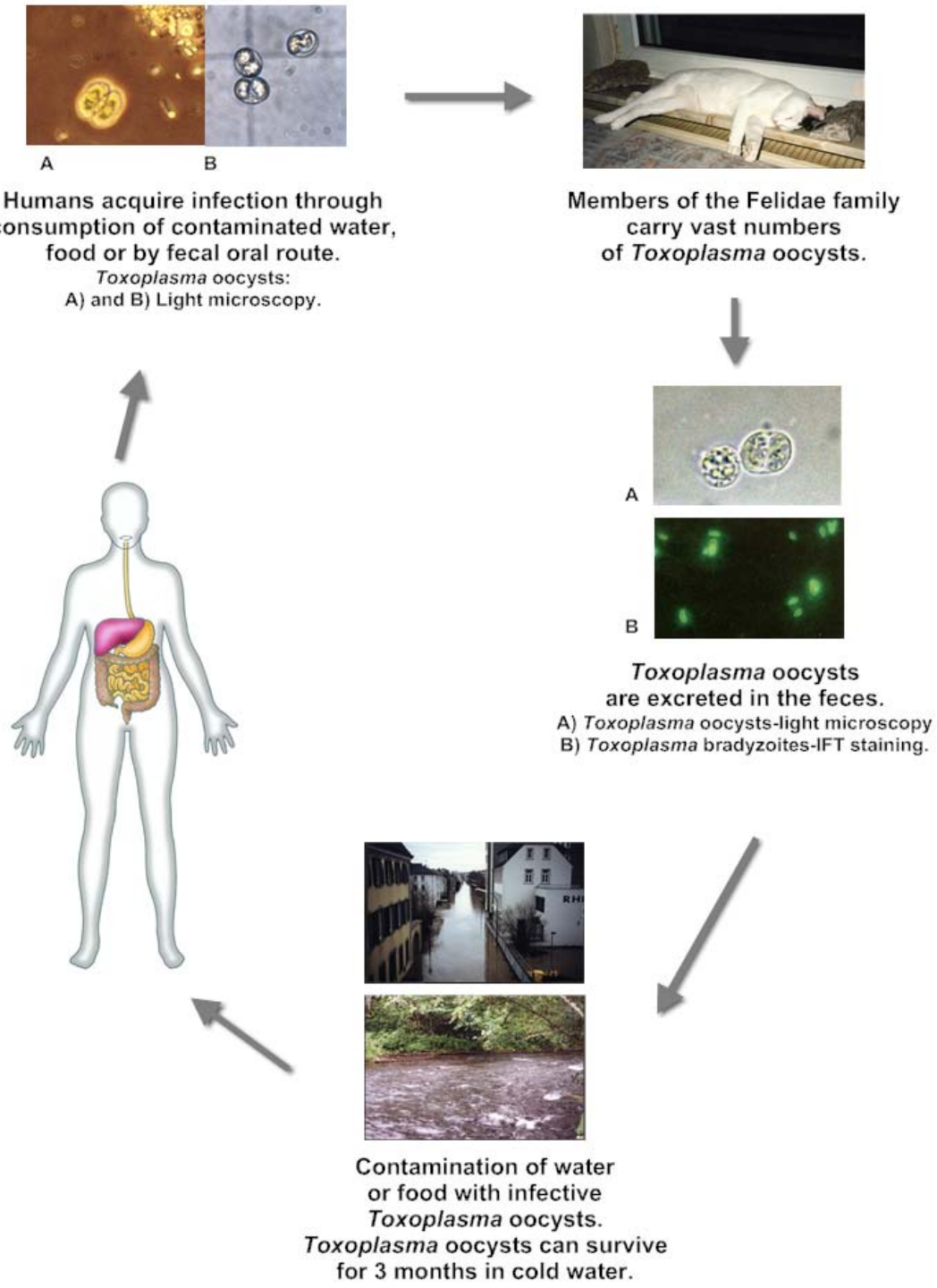
- : only morphological data; *removed from *Cryptosporidium*

1.7 The life cycle of *Toxoplasma*

The life cycle of *Toxoplasma* consists of two phases and exists in three infectious forms: oocysts, tachyzoites, and bradyzoites virulent for both both intermediate and definitive host (Tender, 2009). The oocyst is the most environmentally resistant stage of *Toxoplasma.* Domestic cats and their relatives, members of the family Felidae, are the definitive host. They produce large numbers of unsporulated *Toxoplasma* oocysts in the environment after ingesting any of the three infectious stages of *T. gondii* (Fritz et al., 2012) (Figure 3). Intermediate hosts in nature are birds, rodents and humans as well, and they become infected after ingesting soil, water or plant material contaminated with *Toxoplasma* oocysts. Shortly after ingestion, the oocysts develop into tachyzoites, which are the rapidly multiplying trophozoite form of *Toxoplasma,* by invading the epithelial cells and forming the PV. At this stage the parasites divide rapidly and convert to tissue cysts bradyzoites, which are the slowly replicating versions of the parasite and represent the encysted stage of the parasite in tissue. Tissue cysts are more prevalent in the neural and muscular tissues, but also emerge in brain, eyes, and skeletal and cardiac muscles (Dubey, 1997a and b). The life cycle

completes when Felidae become infected by ingestion of bradyzoite tissue cysts of infected intermediate hosts (Afonso et al., 2006). Sporulation occurs outside the cat within 1-5 days depending upon the ground temperatures. Environmental conditions are important determinants of oocyst survival in soil and water (Frenkel et al., 1975; Sukthana et al., 2003).

Figure 3. *Toxoplasma gondii* life cycle stages and transmission routes.



Additionally, humans can be infected by any of several routes including ingestion of tissue cysts or tachyzoites contained in meat, primary offal (visceral) or meat-derived products of many different mammals and birds, or the ingestion of sporulated oocysts that are contained in the environment (Tenter et al., 2000; Tenter, 2009). Blood transfusion, organ transplantation and transplacental transmission and intrauterine infection from mother to foetus are characteristical features in the life cycle of *Toxoplasma* (Abbasi et al., 2003).

1.8 Phylogeny of *Toxoplasma*

Toxoplasma gondii belongs to the Kingdom: Alveolata, Phylum: Apicomplexa, Class: Sporozoea, Subclass: Coccidia, Order: Eucoccidia, Suborder: Eumeriina, Family: Sarcocystidae Genus: *Toxoplasma* (Mehlhorn and Piekarski, 1998). *T. gondii* is the only species of this genus. *T. gondii* was first described in a North African desert rodent and is related to the organism that *T. gondii* was originally found in (Nicolle and Manceaux, 2009).

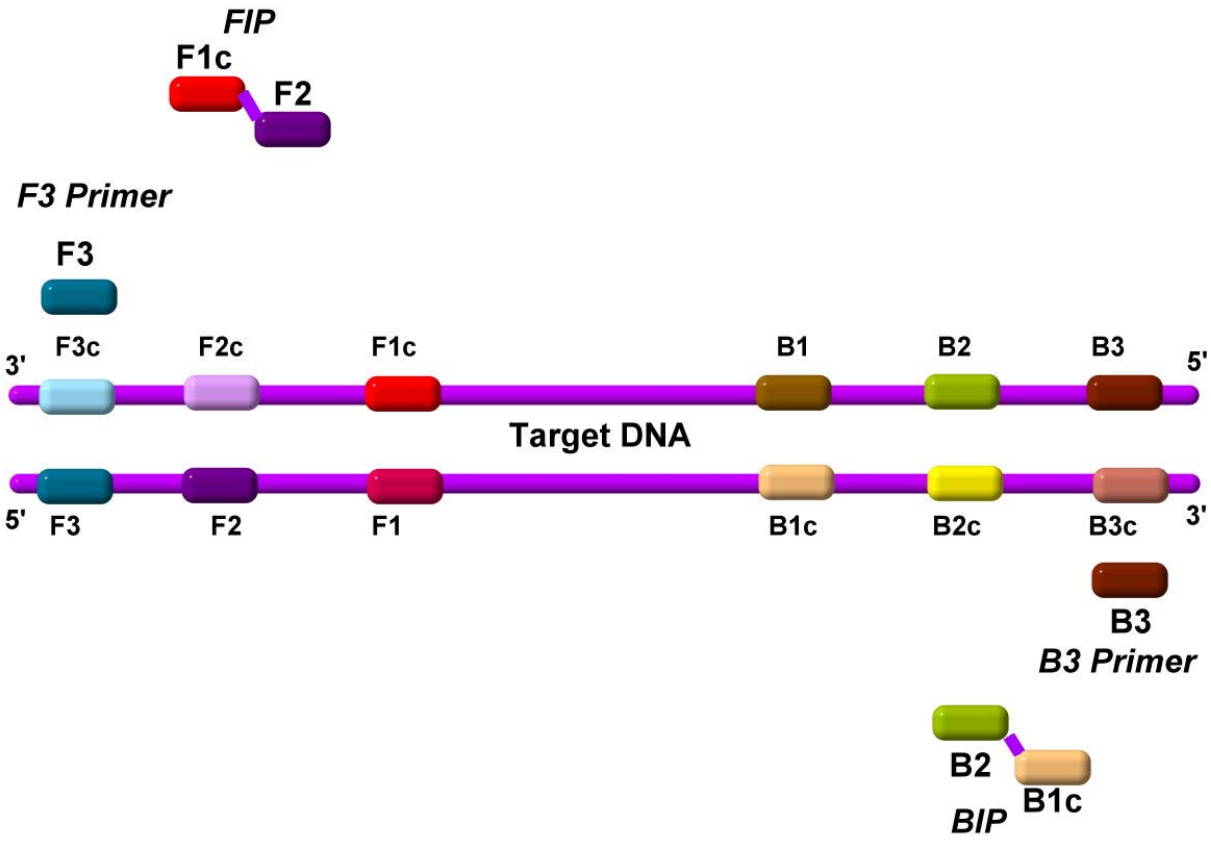
1.9 The LAMP principle

Loop-mediated isothermal amplification (LAMP) method is a one-step amplification reaction amplifying a target DNA sequence with high specificity, sensitivity, high efficiency and rapidity under isothermal conditions and is performed at a constant temperature (in the region of 60 °C – 65 °C) using a DNA polymerase (Bst) with strand displacement activity (Notomi et al., 2000). The mechanism of the LAMP reaction is consisting of 3 major steps, an initial step, a cycling amplification step, and an elongation step. Along with the *Bst* polymerase, LAMP employs two inner primers (FIP, BIP, typically length ~40 – 42 bp) and two outer primers (F3, B3 typically length ~17 -20 bp) which are able to recognize a total of 6 distinct regions within the target DNA (Figure 4). The 6 recognised distinct sites of the target sequence are mentioned in the figure as Forward (F) and Backward (B) and complementary (c). In detail at the 3’ end the sites F1c, F2c, F3c sites are recognised and on the 5’ end the B1, B2, B3 sites are recognised.

The role of the F3 and B3 primers is similar to the ordinary and single domain primers used for the usual PCR amplification by recognising each one of the six regions and resulting in the amplification of the entire target DNA sequence.

The Forward inner primer (FIP) are composite, double-domain primers consisting of the F2 region that is complementary to the F2c region on the target gene at the 3' end, and the same sequence as the F1c region on the target gene at the 5' end. The forward outer primer (F3) consists of the F3 region that is complementary to the F3c region on the target gene. Similar to the FIP primers, the backward inner primer (BIP) consists of the B2 region that is complementary to the B2c region on the target gene at the 3' end, and the same sequence as the B1c region on the target gene at the 5' end. The backward outer primer (B3) consists of the B3 region that is complementary to the B3c region on the target gene.

Figure 4. Schematic representation and localisation of the 6 (inner and outer) LAMP primers on the target DNA sequence.



As mentioned in the original publication of Notomi et al. (2000), a TTTT spacer was joined between F1c and F2 sequences of FIP as well as B1c and B2 sequences of BIP primers.

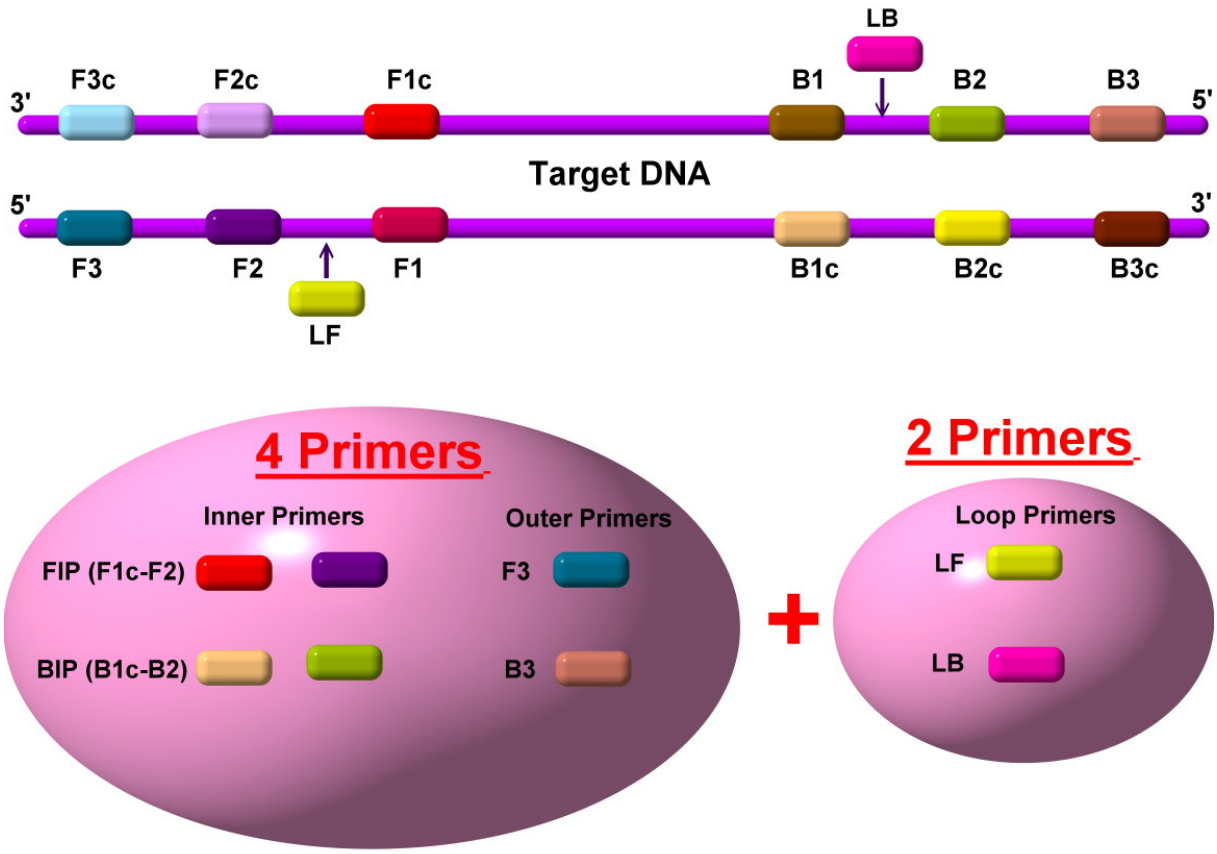
Consequently, the first developed and evaluated protocols for the detection of pathogens such as African *Trypanosoma* species (Kuboki et al., 2003) and *Toxoplasma gondii* (Sotiriadou and Karanis 2008) used this spacer. The use of the spacer was optional as its presence or absence does not affect the activity of FIP and BIP primers.

Additionally, a third pair of primers named Loop primers (L) can be designed in the region between F1 and F2 sites regarding the forward loop primer (LF) and between (B1c) and (B2c) sites regarding the loop backward primer (LB). The role of the Loop primers is that they accelerate the reaction by hybridizing to the stem loops and significantly reducing the reaction time (Nagamine et al., 2002) (Figure 5).

The production of the starting structure requires the participation of all four (or 6 primers when loop primers are included) primers. The initial step of the LAMP reaction begins by the use of all four primers, but later and during the cycling reaction only the inner primers are used for strand displacement DNA synthesis. Firstly, one inner FIP (BIP) primer binds at the starting structure, producing the replicative DNA synthesis by Bst DNA polymerase. F3 (B3) primer binds immediately after the FIP (BIP) primer, displacing the newly synthesized DNA strand and releasing the target DNA. Similar and simultaneously BIP and B3 primers bind to the target DNA as mentioned in the brackets above, resulting in the formation of the single-stranded dumbbell-like starting structure with loops at both ends. By the use of Bst polymerase in the reaction mix and its displacement activity the F3 site of the target DNA sequence is replaced with the F1c, which is complementary to F1 forming the initial stem loop-loop structure.

The cycling amplification step uses this DNA construct as starting material for the further amplification of the LAMP reaction. During the cycling amplification step only the inner primers (FIP and BIP) are used.

Figure 5. Schematic representation and localisation of the 8 (inner, outer and loop) LAMP primers on the target DNA sequence.



The initial stem–loop DNA synthesised strand by self-primed DNA synthesis, hybridizes again with the FIP (BIP) primer and through the displacement DNA synthesis. One complementary structure of the original stem-loop DNA is yielded. One gap repaired stem-loop DNA with a stem elongated to twice as long (double copies of the target sequence) and a loop at the opposite end yields one complementary structure of the original stem–loop DNA. The elongated products are produced by the elongation and recycling step reactions.

1.10 Bioinformatics and primer design

The construction and design of primer sets for applications such as nested PCR and LAMP becomes a challenging approach in comparison to the trivial single primer set for PCR. Efficacy, sensitivity and specificity of the PCR and LAMP reactions is largely dependent on the efficiency of primers and is crucial for obtaining high-quality

sequence data for target regions. The success rate of any primer design should not only include the amplification success rate of the designed primers through computational analysis, but should also take into account the target regions that could have been covered. Potentially identifiable factors which contribute to overall success of the PCR and LAMP reactions were tried to be elucidated taking into consideration a list of criteria need to be established for target sequence analysis and primer design.

1.11 Web-based analysis of target gene sequences

Numerous web-based resources for analysis of target sequences are freely available which contain in some cases hundreds of sequences that may be targeting one gene. Free analyses of proteins and nucleic acid sequences can be performed by simple log in to the established servers and web-based interfaces (Singh and Kumar, 2001). Often such sequence resources are of variable quality, not well maintained leading to missing links and sites and probably not functional in a later date.

When scheduling important primer design it is worthwhile to evaluate sequences present in mumerous databases and consider predictions for numerous factors probably affecting the primer design when using the common sense and laboratory experience to evaluate the suggested primers before committing to their synthesis (Binas, 2000; Abd-Elsalam, 2003).

To select primer sets for the detection of *Toxoplasma* and *Cryptosporidium* species, we analysed only entries present in the National Center for Biotechnology Information (NCBI). The NCBI database is considered be a reliable resource by providing full access to biomedical and genomic information of deposited sequences database (www.ncbi.nlm.nih.gov) (Altschul et al., 1997). Table 3 demonstrates the calculated statistical significance matches of the analysis of the S-adenosyl-methionine synthetase (SAM) gene of *C. parvum* (GenBank accession number AY161084) against all existing *Cryptosporidium* SAM-sequences comparing enties present also in the DNA Data Bank of JAPAN (DDBJ), European Molecular Biology Laboratory-European Bioinformatics Institute (EBI-EMBL) and Worldwide Protein Data Bank (wwPDB).

The analysis of the target gene of *Toxoplasma* is presented in Table 4. The table demonstrates the calculated statistical significances of matches and sequences producing significant alignments concerning the *Toxoplasma* B1 (GenBank

accession number AF179871) and *Tg*OWP (GenBank accession number AY465428) genes.

Table 3. The table demonstrates the calculated statistical significance of matches and sequences producing significant alignments. Accession numbers marked in bold are chosen for *Cryptosporidium* LAMP primer design.

Accession Number	Description	Query coverage	E value	Max ident
AY161084	*C. parvum* methionine adenosyltransferase gene, complete cds	100%	0.0	100%
XM_628434	*C. parvum* Iowa II s-adenosylmethionine synthetase (cgd7_2650) partial mRNA	100%	0.0	100%
XM_662396	*C. hominis* TU502 methionine adenosyltransferase (Chro.70301) partial mRNA	100%	0.0	98%
AB119646	*C. parvum* SAMs gene for methionine adenosyltransferase, complete cds, human genotype	100%	0.0	98%
AB119647	*C. parvum* SAMs gene for methionine adenosyltransferase, complete cds, ferret genotype	100%	0.0	95%
AB119648	*C. meleagridis* SAMs gene for methionine adenosyltransferase, complete cds	100%	0.0	95%
AB513799	*Cryptosporidium* sp. skunk genotype MAT gene for methionine adenosyltransferase, partial cds	78%	0.0	89%
AB513797	*C. suis* MAT gene for methionine adenosyltransferase, partial cds	79%	0.0	85%
AB513796	*C. canis* MAT gene for methionine adenosyltransferase, partial cds	80%	0.0	80%
AB513798	*C. felis* MAT gene for methionine adenosyltransferase, partial cds	71%	4e-180	75%
XM_002141401.1	*C. muris* RN66 S-adenosylmethionine synthetase family protein, mRNA	76%	5e-154	73%

Table 4. The table demonstrates the calculated statistical significance of matches and sequences producing significant alignments. Accession numbers marked in bold are chosen for *Toxopasma* LAMP primer design.

Accession Number	Description	Query coverage	E value	Max ident
AY465428	*T. gondii* putative oocyst wall protein COWP mRNA, complete cds	100%	0.0	100%
XM_002367673	*T. gondii* ME49 oocyst wall protein COWP, putative, mRNA	99%	0.0	99%
EU851867	*T. gondii* oocyst wall protein-1 (OWP-1) mRNA, complete cds	99%	0.0	99%
FR823388	*Neospora caninum* Liverpool complete genome, chromosome VIIa	24%	2e-10[7]	86%

1.12 Criteria and key factors in the LAMP primer design

The LAMP primer design software Primer Explorer V4 (http://primerexplorer.jp) was used to design the primer set for the detection of *Toxoplasma* and *Cryptosporidium* species. We advocated the adoption of standardized criteria for primer selection within our laboratory.

1.12.1 Melting temperature (Tm) of the primers

The nearest-Neighbor method is automatically integrated and used in the Primer Explorer V4 for the estimation of the Tm, which approximates the value closest to the actual value. Tm can be affected by the salt and oligo concentration and was designed to be for each region about 65 °C (64 – 66 °C) for F1c and B1c, about 60 °C (59 – 61 °C) for F2, B2, F3, and B3, and about 60 °C for the loop primers.

1.12.2 Stability of the end of the primers

A certain degree of stability is essential for less false priming since the end of the primers serves as the starting point of the DNA synthesis and thus must have a certain degree of stability. The 3' ends of F2/B2, F3/B3, and LF/LB and the 5' end of F1c/B1c are designed so that the free energy is –4 kcal/ mol or less. The 5' end of F1c after amplification corresponds to the 3' end of F1, so that stability is important. ΔG is the free energy of the primer and measures the spontaneity of the reaction. The annealing between the primer and the target gene is an equilibrium reaction, and the annealing reaction proceeds with a smaller ΔG.

1.12.3 GC% primer content

GC% is the percentage of G and C in the primer and is calculated by dividing the sum of G and C with the total number of bases present in the primer. The GC-pair is bound by three hydrogen bonds, DNA with high GC-content is more stable than DNA with low GC-content; however, the hydrogen bonds do not stabilize the DNA significantly, and stabilization is due mainly to stacking interactions (Yakovchuk et al., 2006).

PCR primers should maintain a reasonable GC-content and Tm should be well matched. Poorly matched primer pairs with less similar physical characteristics can be less efficient and specific because loss of specificity arises with a greater chance of mispriming under these conditions (Dieffenbach et al., 1993). Primers with a GC content between 45% and 60% tend to give relatively good primers, while primers ending in a G or C, or CG or GC (3') prevent "breathing" of ends and increase efficiency of priming.

1.12.4 Formation of secondary structures

The presence of primer secondary structures produced by inter- or intramolecular interactions can lead to poor or no yield of the product and greatly reduces the availability of primers to the reaction. It is significant that the inner primers are so designed that they do not form secondary structures, because they adversely affect primer template annealing and thus the amplification.

Formed hairpins are usually the result of the intramolecular interaction within the primer and often occurs when complementary (palindromic) sites leading to the fold back of the primer onto itself (Breslauer et al., 1986).

A primer self-dimer formation is characterised by the intermolecular interaction between the two (same sense) primers, where the primer is homologous and anneal to itself or to other primers (primer dimer formation) used in the LAMP reaction. Generally a large amount of primers are used in LAMP compared to the amount of target gene. When primers form intermolecular dimers much more readily than hybridizing to target DNA, they produce mispriming and reduce the product yield.
To prevent the formation of secondary structures, it is also important to ensure that the 3' ends are not complementary and the presence of G or C bases within the last five bases from the 3' end of primers (GC-clamp) helps promote specific binding at the 3' end due to the stronger binding of G and C bases. More than 3 G's or C's should be avoided in the last 5 bases at the 3' end of the primer.

1.12.5 Improved specificity of the primers

The improved specificity of the primers often relates to the fact that the primers are chosen and designed to a unique target region within the DNA template. Regions of homology must be avoided and primers designed for a sequence must not amplify other genes in the mixture.

1.13 The investigated gene for the development of the LAMP technique for the detection of *Cryptosporidium*

SAM is an enzyme present in all living organisms and was reported to play central role in biological methylation and was directly linked to polyamine metabolism. SAM enzyme catalyses the synthesis of S-adenosyl-methionine from L-methionine and adenosine triphosphate (ATP) (Catoni, 1953). Cellular organisms use SAM in many transmethylation reactions as it acts as a methyl group donor important in the cell metabolism (Lu, 2000; Tabor and Tabor, 1985). *C. parvum* was identified as a single copy, AT-rich (61.8%) and intronless gene consisting of 1221 bp and encoding a polypeptide of 406 amino acids with a molecular mass of 44.8 kDa (Slapeta et al., 2003). The differential expression of *C. parvum* in infected HCT-8 cell cultures over 72 h was assayed and the results indicated that SAM is a differentially expressed gene throughout the life cycle of *C. parvum*. The strongest expression as measured by RT-PCR occured at 6 h post infection and 24-48 h post infection. The biphasic character of the expression correlated with the late trophozoite development and

transformation to early meronts at 6 h post infection, followed by the first generation of merozoites at 12 h post infection and with the late meront development into gametocytes, to yield the sexual part of the life cycle. In both time points *C. parvum* undergoes morphological changes that require S-adenosylmethionine production to yield methylation and polyamine synthesis (Slapeta et al., 2003). The SAM amino acid sequence of *C*. *parvum* shares conserved and essential domains with homologs from other organs and is phylogenetically related to other cytosolic eukaryotic and prokaryotic organisms, but distinct from homologues in plants, fungi, mammals and eubacteria (Slapeta et al., 2003).

1.14 The investigated genes for the development of the LAMP technique for the detection of *Toxoplasma*

The sequence of the B1 gene and its copy number was determined by comparative hybridization using a titration of plasmid DNA (Burg et al., 1989). Because of its repetitive nature, the B1 gene is an attractive target for detecting *T. gondii* parasites through amplification of B1-specific DNA. The same authors performed quantitative Southern blot analysis and the autoradiograph showed that the B1 gene was between 25- and 50-fold repetitive. By quantitation of the radioactivity present in each band they indicated more precisely that there are about 35 copies of the B1 gene in the *T. gondii* genome. For the realisation of our approaches, we utilised the complete sequence deposited in the gene bank under the GenBank accession number AF179871.

Templeton et al. (2004) screened first the *Cryptosporidium* database, resulting in the identification of eight additional genes encoding similar arrays of cysteine-rich type I and/or type II domains. The authors showed that all eight *Cryptosporidium* COWP (*Cryptosporidium* Oocyst Wall Protein) genes were abundantly expressed at a time when developing oocysts are observed, approximately 48 to 72 h after inoculation of *in vitro* cultures. The hypothesis that multiple COWPs play a role in the oocyst wall structure was supported. Subsequently Templeton et al. screened by BLAST the *T. gondii* genome sequence database, which resulted in identification of a gene encoding at least one COWP homolog (*Tg*OWP1), and this multiexon sequence information was used to isolate a full-length cDNA.

1.15 Selection and comparison of sequences for primer design

The selection of sequences for primer design for the *Crytosporidium* SAM gene were firstly subject to discovery of phylogenetically related organisms expressing the SAM gene. Template sequences of *C. parvum* were compared by BLAST against the appropriate non-redundant databases and the software interpreted the results. Such phylogenetically related organisms were found to be *Homo sapiens*, *Entamoeba, Giardia, Toxoplasma* and *Plasmodium*. Sequences of *Isospora*, *Cyclospora, Hammondia, Cystoisospora, Sarcocystis* and *Neospora* were not deposited in the GenBank so far (Table 5).

Table 5. Organisms analysed against the concrete target region for primer design of the SAM gene (217 bp) of *Cryptosporidium parvum* (GenBank accession number AY161084).

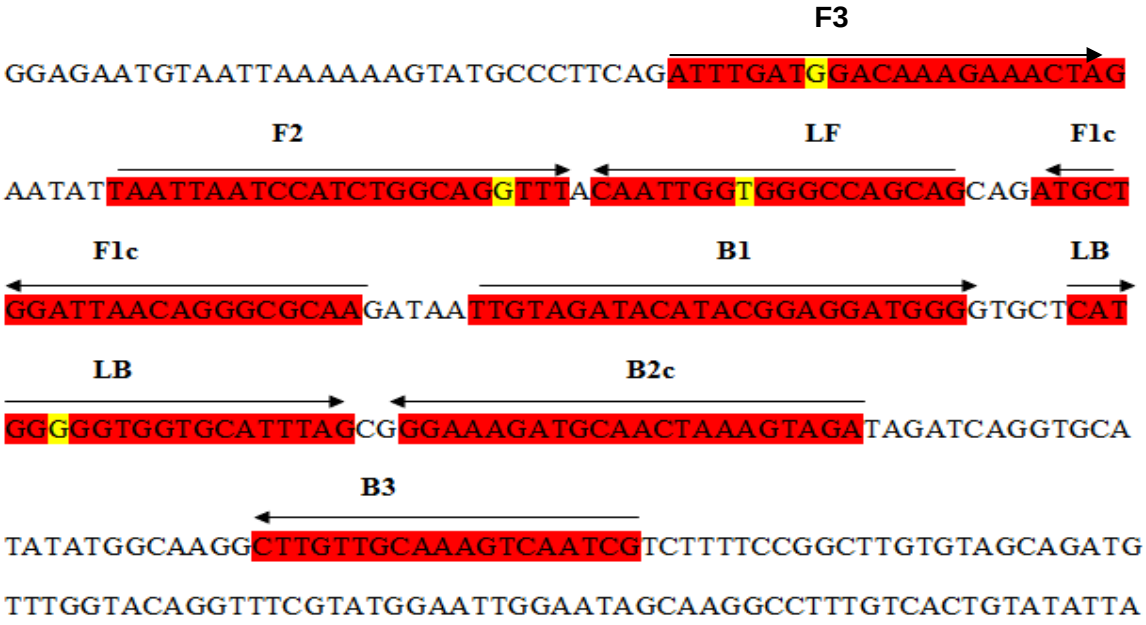
Organism	Gen Bank Accession Number	Aligment similarity score in EMBL-EBI database (%)	Aligment similarity score GenomeNet (%)
Homo sapiens	**NM_005911**	**86**	**63**
Toxoplasma gondii	**XM_002366647**	**83**	**59**
Plasmodium falciparum	**AF097923**	**82**	**68**
Entamoeba dispar	**XM_001738614**	**82**	**70**
Entamoeba hystolitica	**XM_001913755**	**81**	**70**
Entamoeba histolytica	**XM_647762**	**81**	**70**
Giardia lamblia	**XM_001704282**	**76**	**59**
Isospora	**-**		**-**
Cyclospora	**-**		**-**
Hammondia	**-**		**-**
Cystoisospora	**-**		**-**
Sarcocystis	**-**		**-**
Neospora	**-**		**-**

Two freely available databases and their bioinformatic services were used for the computational analysis and aligment of the interested gene sequences by the ClustalW (Thompson et al., 1994): the European Bioinformatics Institute operated from the EBI-EMBL *(*www.ebi.ac.uk/Tools/msa/clustalw2*)* and GenomeNet operated by the Kyoto University Bioinformatics Center *(*www.genome.jp/tools/clustalw/).

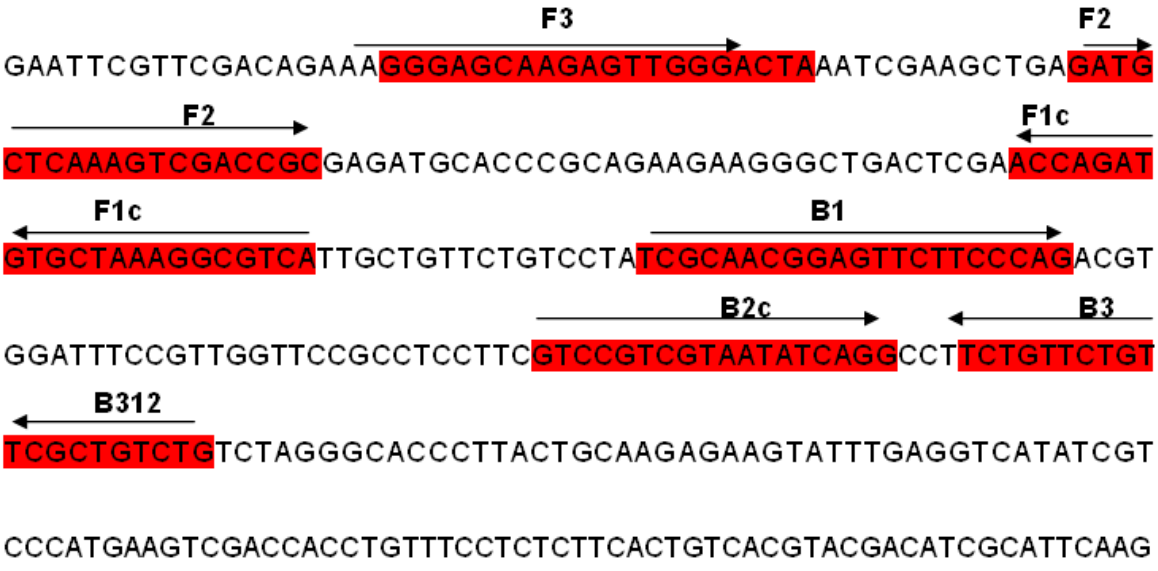
For primer design the area between the nucleotides 725 and 940 was chosen, representing an identity of 95-100% among *Cryptosporidium* SAM sequences but with 59%-86% identity with human and other organisms listed in Table 5. The sequence of *C. parvum* (GenBank accession number AY161084) and the LAMP primer design software Primer Explorer V4 (http://primerexplorer.jp/e/) were used to design the primer set.
A similar analysis strategy was applied for the development of the LAMP technique for the detection of *Toxoplasma*. The analysis of the selected sequences for the LAMP development of the *T. gondii* B1 and *Tg*OWP showed that both genes are selective genes and only a part (24%) of the B1 gene is preservative in *Neospora caninum*.

Figure 6. Schematic representation and localisation of the LAMP primers on the target DNA sequence for A) *C. parvum* (GenBank accession number AY161084) designed within the SAM gene, B) *Toxoplasma gondii* GenBank accession number AF179871) within the B1 gene and C) *Toxoplasma gondii* (GenBank accession number AY465428) within the *Tg*OWP gene. Recognition sequences of the primers are shown in red color and degenerate primers are mentioned with yellow color. Right arrows indicate that a sense sequence is used for primer design and left arrows indicate the usage of complementary sequence.

A. *Cryptosporidium parvum SAM gene* (GenBank accession number AY161084)



B. *Toxoplasma gondii* B1 gene (GenBank accession number AF179871)



1.16 LAMP vs PCR

The most widely used, often indispensable technique and a workhorse tool in the fields of biotechnology and biomedical sciences is the PCR. Since PCR is closely tied to the generation of the genetic database information most of the medical and biological research labs for a variety of applications are using this technique. PCR is a nucleic acid-based detection technique, which relies on the thermal cycling, by repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA, supported by the enzymatic activity provided by the polymerase for primer-directed target amplification.

PCR is one of the most sensitive techniques, but is labor-intensive and often requires extensive sample preparation to eliminate, sometimes unsuccesfully, amplification inhibitors (Nichols and Smith, 2004). Most PCR-based identification assays rely on the amplification of species-specific genes (Johnson et al., 2012) such as Thrombospondin-related attachment protein (TRAP) genes, loci including ribosomal RNA genes and spacers, the COWP, the 70 kDa heat shock protein (Sulaiman et al., 2000), and the 60 kDa glycoprotein (GP60) (O'Brien et al., 2008). The SSU rRNA is a genetic marker for the specific identification of *Cryptosporidium*, having relatively low intraspecific and relatively high interspecific sequence variation (Morgan et al., 1999, Fayer et al., 2000, Xiao et al., 2004b, Jex and Gasser, 2008). These targets offer enough sequence variation and degree of variability for species identification and require post PCR based target identification techniques for characterisation and

classification of *Cryptosporidium* species or genotypes. Such laborious and time-consuming post-PCR tools are considered gel electrophoresis, enzymatic cleavage, amplicon sequencing or the use of specific labeled identification reagents in real-time PCR systems, which are expensive and also sensitive to contamination and inhibition by compounds present in the template material (Corless et al., 2000). PCR is considered as a simple reaction but is not a cost-effective choice for laboratories, because all the post-PCR techniques require several automated instruments and skilled operators (Corless et al., 2000; Francois et al., 2007).

In conducting the LAMP method parameters such as the fundamental experiment conditions including essential simple precausions and measurements to prevent contamination such as keeping separate the preparation of reaction solutions, addition of template, and detection equipment, are considered. Sufficient care is needed when performing electrophoresis or restriction enzyme digests for the purpose of identifying the amplification products. The LAMP amplification conditions, the amount of the enzyme, high-performance liquid chromatography (HPLC) purification of the primers reaction can increase the amplification speed or sensitivity.

The presence of turbidity due to the magnesium pyrophosphate produced can be an excellent indicator of the presence of the target gene, allowing easily visual detection with the naked eye for the confirmation and visualization of the LAMP products. Moreover the fluorescent dye added to the reaction mix reacts with the magnesium pyrophosphate, a fact that further simplifies the detection by the naked eye or under UV-light. Electrophoresis, a basic procedure for confirmation of LAMP products, similar to PCR, is often recommended after the initial observation by the naked eye serving as an indicator of the high degree of reproducibility. As with all PCR protocols, certain precautions for the reagents need to be followed to prevent the deterioration of the reagents to ensure accurate amplification of the target DNA. All the above criteria are important and should be fullfilled otherwise they can become competitive enough to suppress product formation.

1.17 Aim of the present thesis

There is no doubt that the advances of the molecular techniques are of significance but there is an ongoing demand for development of molecular tools toward achieving a better fundamental method that overcomes the limitation barriers and the drawbacks of other methods for a precise detection of the disease. The different and complex transmission routes of *Toxoplasma*, *Giardia* and *Cryptosporidium* forced the development of alternative approaches for the accurate detection of the species of public health significance able to define contamination sources. Simple, cheap, and rapid assays for the diagnosis of the aforementioned protozoan parasites are attractive mostly in poor countries where facilities are minimal. The detection of *Cryptosporidium*, *Giardia* and *Toxoplasma* through the use of molecular techniques in human, environmental and food samples is offten problematic. Standardisation and validation of the protocols remain even nowdays an important issue as they are often affected by different factors. DNA extraction methods, PCR interferents, low numbers of parasites present in the sample, choice of loci, genes and primers, differences in the nucleotide sequences and their reproducibility in the protozoan parasites, multiple species, genotypes, assemblages or strains present in the samples are the most inportant and well documented factors affecting the molecular methods.

Taken together all these parameters, we aimed for the development of an accelerated, cost effective, specific and sensitive LAMP technique with valuable diagnostic potential for the detection of protozoan parasites. This has been performed in the present thesis for *Cryptosporidium* and *Toxoplasma*.

The second part of this work describes the development and evaluation of the LAMP technique able to detect single nucleotide polymorphisms of the human pathogenic species of *Cryptosporidium* on the S-adenosyl-methionine synthetase (SAM) *Cryptosporidium* gene. The SNP based SAM-LAMP technique was developed to detect *C. parvum, C. hominis* and *C. meleagridis* and subsequently was firstly tested in water sample pellets spiked with known numbers of *Cryptosporidium* oocysts. Consequently the SNP-based SAM-LAMP was applied in environmental water, food and animal fecal samples, which is able to detect the three species as mentioned above. Phylogenetic analysis for *Cryptosporidium* species was conducted by the application of two different nPCR assays amplifying the SSU rRNA gene.

The third section reports the development of the LAMP method on B1 and *Tg*OWP genes for the detection of *Toxoplasma* in difficult templates derived from environmental water samples. The LAMP detection was firstly evaluated and compared with nested PCR in water sample pellets spiked with known numbers of *Toxoplasma* oocysts. All natural water samples included in the study were investigated and compared by IFT, nPCR and LAMP for the presence of *Toxoplasma* DNA. The LAMP assays were based on the B1 and *Tg*OWP genes.

The fourth and last section of this work is related to the molecular identification and phylogenetic analysis of *Giardia* and *Cryptosporidium* from dogs and cats from Germany as important contaminants of household animals. The *Cryptosporidium* genotypes were determined by sequencing a fragment of the SSU rRNA gene, while the *Giardia* assemblages were determined through analysis of the GDH locus.

1.18 References

Abbasi M, Kowalewska-Grochowska K, Bahar MA, Kilani RT, Winkler-Lowen B, Guilbert LJ (2003) Infection of placental trophoblasts by *Toxoplasma gondii. J Infect Dis* 188: 608-616.

Abd-Elsalam KA (2003) Non-gel based techniques for plant pathogen genotyping. *Acta Microbiol Pol* 52: 329-341.

Abodeely M, DuBois KN, Hehl A, Stefanic S, Sajid M, DeSouza W, Attias M, Engel JC, Hsieh I, Fetter RD, McKerrow JH (2009) A contiguous compartment functions as endoplasmic reticulum and endosome/lysosome in *Giardia lamblia*. *Eukaryot Cell* 8: 1665-1676.

Adam RD (2000) The *Giardia lamblia* genome. *Int J Parasitol* 30: 475-484.

Adam RD (2001) Biology of *Giardia lamblia*. *Clin Microbiol Rev* 14: 447-475.

Adams PJ, Monis PT, Elliot AD, Thompson RC (2004) Cyst morphology and sequence analysis of the small subunit rDNA and ef1 alpha identifies a novel *Giardia* genotype in a quenda (*Isoodon obesulus*) from Western Australia. *Infect Genet Evol* 4: 365–370.

Afonso E, Thulliez P, Gilot-Fromont E (2006) Transmission of *Toxoplasma gondii* in an urban population of domestic cats (*Felis catus*). *Int J Parasitol* 36: 1373-1382.

Ali SA, Hill DR (2003) *Giardia* intestinalis. *Curr Opin Infect Dis* 16: 453-460.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25: 3389-3402.

Alvarez-Pellitero P, Sitjà-Bobadilla A (2002) *Cryptosporidium molnari* n. sp. (Apicomplexa: Cryptosporidiidae) infecting two marine fish species, *Sparus aurata L*. and *Dicentrarchus labrax L*. *Int J Parasitol* 32: 1007-1021.

Alvarez-Pellitero P, Quiroga MI, Sitjà-Bobadilla A, Redondo MJ, Palenzuela O, Padrós F, Vázquez S, Nieto JM (2004) *Cryptosporidium scophthalmi* n. sp. (Apicomplexa: Cryptosporidiidae) from cultured turbot *Scophthalmus maximus*. Light and electron microscope description and histopathological study. *Dis Aquat Organ* 62: 133-145.

Alvarez-Pellitero P, Perez A, Quiroga MI, Redondo MJ, Vázquez S, Riaza A, Palenzuela O, Sitjà-Bobadilla A, Nieto JM (2009) Host and environmental risk

factors associated with *Cryptosporidium scophthalmi* (Apicomplexa) infection in cultured turbot, *Psetta maxima* (L.) (*Pisces, Teleostei*). *Vet Parasitol* 165: 207-215.

Amer S, Wang C, He H (2010) First detection of *Cryptosporidium baileyi* in Ruddy Shelduck (*Tadorna ferruginea*) in China. *J Vet Med Sci* 72: 935-938.

Arcay-de-Peraza L, Bastardo-de-San-Jose T (1969) *Cryptosporidium ameivae* sp. nov. (Coccidia, Cryptosporidiidae) del intestino delgado de Ameiva ameiva de Venezuela. *Acta Cient Venez* 20: 125.

Asher AJ, Waldron LS, Power ML (2012) Rapid identification of *Giardia duodenalis* assemblages in NSW using terminal-restriction fragment length polymorphism. *Parasitol* doi:10.1017/S0031182012000388

Baldursson S, Karanis P (2011) Waterborne transmission of protozoan parasites: review of worldwide outbreaks - an update 2004-2010. *Water Res* 45: 6603-6614.

Barker IK, Carbonell PL (1974) *Cryptosporidium agni* sp.n. from lambs, and *Cryptosporidium bovis* sp.n. from a calf, with observations on the oocyst. *Z Parasitenkd* 44: 289-298.

Barta JR, Thompson RC (2006) What is *Cryptosporidium*? Reappraising its biology and phylogenetic affinities. *Trends Parasitol* 22:463-468.

Barugahare R, Dennis MM, Becker JA, Slapeta J (2011) Detection of *Cryptosporidium molnari* oocysts from fish by fluorescent-antibody staining assays for *Cryptosporidium* spp. affecting humans. *Appl Environ Microbiol* 77: 1878-1880.

Beck R, Sprong H, Lucinger S, Pozio E, Caccio SM (2011) A large survey of Croatian wild mammals for *Giardia duodenalis* reveals a low prevalence and limited zoonotic potential. *Vector Borne Zoonotic Dis* 11: 1049-1055.

Beck R, Sprong H, Pozio E, Caccio SM (2012) Genotyping *Giardia duodenalis* isolates from dogs: Lessons from a multilocus sequence typing study. *Vector Borne Zoonotic Dis* 12: 206-213.

Biñas M (2000) Designing PCR primers on the Web. *Biotechniques* 29: 988-990.

Bonhomme J, Le Goff L, Lemee V, Gargala G, Ballet JJ,Favennec L (2011) Limitations of tpi and bg genes sub-genotyping for characterization of human *Giardia duodenalis* isolates. *Parasitol Int* 60: 327-330.

Borowski H, Clode PL, Thompson RC (2008) Active invasion and/or encapsulation? A reappraisal of host-cell parasitism by *Cryptosporidium*. *Trends Parasitol* 24: 509-516.

Borowski H, Thompson RC, Armstrong T, Clode PL (2010) Morphological characterization of *Cryptosporidium parvum* life-cycle stages in an in vitro model system. *Parasitol* 137:13-26.

Breathnach AS, McHugh TD, Butcher PD (2010) Prevalence and clinical correlations of genetic subtypes of *Giardia lamblia* in an urban setting. *Epidemiol Infect* 138: 1459-1467.

Breslauer KJ, Frank R, Blöcker H, Marky LA (1986) Predicting DNA duplex stability from the base sequence. *Proc Natl Acad Sci U S A* 83: 3746-3750.

Burg JL, Grover CM, Pouletty P, Boothroyd JC (1989) Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. *J Clin Microbiol* 27: 1787-1792.

Caccio SM, Ryan U (2008) Molecular epidemiology of giardiasis. *Mol Biochem Parasitol* 160: 75-80.

Caccio SM, Beck R, Lalle M, Marinculic A, Pozio E (2008) Multilocus genotyping of *Giardia duodenalis* reveals striking differences between assemblages A and B. *Int J Parasitol* 38: 1523–1531.

Cama VA, Bern C, Roberts J, Cabrera L, Sterling CR, Ortega Y, Gilman RH, Xiao L (2008) *Cryptosporidium* species and subtypes and clinical manifestations in children, Peru. *Emerg Infect Dis* 14: 1567-1574.

Catoni GL (1953) S-Adenosylmethionine; a new intermediate formed enzymatically from L-methionine and adenosinetriphosphate. *J Biol Chem* 204: 403-416.

Chalmers RM, Robinson G, Elwin K, Hadfield SJ, Xiao L, Ryan U, Modha D, Mallaghan C (2009) *Cryptosporidium* sp. rabbit genotype, a newly identified human pathogen. *Emerg Infect Dis* 15: 829-830.

Chvala S, Fragner K, Hackl R, Hess M, Weissenbo H (2006) *Cryptosporidium* infection in domestic geese (*Anser anser f. domestica*) detected by in-situ hybridization. *J Comp Pathol* 134: 211–218.

Clark DP (1999) New insights into human cryptosporidiosis. *Clin Microbiol Rev* 12: 554-563.

Compton J (1991) Nucleic acid sequence-based amplification. *Nature* 350: 91–92.

Corless CE, Guiver M, Borrow R, Edwards-Jones V, Kaczmarski EB, Fox AJ (2000) Contamination and sensitivity issues with a real-time universal 16S rRNA PCR. *J Clin Microbiol* 38: 1747-1552.

33: 98-108.

Current WL, Reese NC (1986) A comparison of endogenous development of three isolates of *Cryptosporidium* in suckling mice. *J Protozool* 33: 98-108.

Current WL, Upton SJ, Haynes TB (1986) The life cycle of *Cryptosporidium baileyi* n. sp. (Apicomplexa, Cryptosporidiidae) infecting chickens. *J Protozool* 33: 289-296.

da Silva DC, Homem CG, Nakamura AA, Teixeira WF, Perri SH, Meireles MV (2010) Physical, epidemiological, and molecular evaluation of infection by *Cryptosporidium galli* in Passeriformes. *Parasitol Res* 107: 271-277.

Dieffenbach CW, Lowe TM, Dveksler GS (1993) General concepts for PCR primer design. *PCR Methods Appl* 3: S30-7.

Dubey JP (1997a) Distribution of tissue cysts in organs of rats fed *Toxoplasma gondii* oocysts. *J Parasitol* 83: 755–757.

Dubey JP (1997b) Tissue cyst tropism in *Toxoplasma gondii*: a comparison of tissue cyst formation in organs of cats, and rodents fed oocysts. *Parasitol* 115: 15–20.

Dubey JP, Markovits JE, Killary KA (2002) *Cryptosporidium muris*-like infection in stomach of cynomologus monkeys (*Macaca fascicularis*). *Vet Pathol* 39: 363–371.

Egyed Z, Sréter T, Széll Z, Varga I (2003) Characterization of *Cryptosporidium* spp. - recent developments and future needs. *Vet Parasitol* 111: 103–114.

Elias EV, Quiroga R, Gottig N, Nakanishi H, Nash TE, Neiman A, Lujan HD (2008) Characterization of SNAREs determines the absence of a typical Golgi apparatus in the ancient eukaryote *Giardia lamblia. J Biol Chem* 283: 35996-36010.

Fayer R, Leek RG (1984) The effects of reducing conditions, medium, pH, temperature, and time on in vitro excystation of *Cryptosporidium*. *J Protozool* 31: 567-569.

Fayer R, Santín M, Trout JM, Greiner E (2006) Prevalence of species and genotypes of *Cryptosporidium* found in 1-2-year-old dairy cattle in the eastern United States. *Vet Parasitol* 135: 105-112.

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

Fayer R, Santín M (2009) *Cryptosporidium xiaoi* n. sp. (Apicomplexa: Cryptosporidiidae) in sheep (*Ovis aries*).*Vet Parasitol* 64: 192-200.

Fayer R, Santín M, Macarisin D (2010) *Cryptosporidium ubiquitum* n. sp. in animals and humans. *Vet Parasitol* 172: 23-32.

Fiuza VR, Cosendey RI, Frazão-Teixeira E, Santín M, Fayer R, de Oliveira FC (2011) Molecular characterization of *Cryptosporidium* in Brazilian sheep. *Vet Parasitol* 175: 360-362.

Francois P, Garzoni C, Bento M, Schrenzel J (2007) Comparison of amplification methods for transcriptomic analyses of low abundance prokaryotic RNA sources. *J Microbiol Methods* 68: 385-391.

Frenkel JK, Ruiz A, Chinchilla M (1975) Soil survival of *Toxoplasma* oocysts in Kansas and Costa Rica. *Am J Trop Med Hyg* 24: 439–443

Fritz HM, Buchholz KR, Chen X, Durbin-Johnson B, Rocke DM, Conrad PA, Boothroyd JC (2012) Transcriptomic analysis of *Toxoplasma* development reveals many novel functions and structures specific to sporozoites and oocysts. *PLoS One* 7: 29998.

Gasser RB, Zhu X, Caccio S, Chalmers R, Widmer G, Morgan UM, Thompson RC, Pozio E, Browning GF (2001) Genotyping *Cryptosporidium parvum* by single-strand conformation polymorphism analysis of ribosomal and heat shock gene regions. *Electrophoresis* 22: 433-7.

Gatei W, Suputtamongkol Y, Waywa D, Ashford RW, Bailey JW, Greensill J, Beeching NJ, Hart CA (2002) Zoonotic species of *Cryptosporidium* are as prevalent as the anthroponotic in HIV-infected patients in Thailand. *Ann Trop Med Parasitol* 96: 797-802.

Gaydos JK, Miller WA, Johnson C, Zornetzer H, Melli A, Packham A, Jeffries S.J, Lance MM, Conrad PA (2008) Novel and canine genotypes of *Giardia duodenalis* in harbor seals (*Phoca vitulina richardsi*). J Parasitol 94: 1264–1648.

Gelanew T, Lalle M, Hailu A, Pozio E, Cacciò SM (2007) Molecular characterization of human isolates of *Giardia duodenalis* from Ethiopia. *Acta Trop* 102: 92–99.

Gomez-Munoz MT, Camara-Badenes C, Martinez-Herrero MC, Dea-Ayuela MA, Perez-Gracia MT, Fernandez-Barredo S, Santin M, Fayer R (2012) Multilocus

genotyping of *Giardia duodenalis* in lambs from Spain reveals a high heterogeneity. *Res Vet Sci.* 10.1016/j.rvsc.2011.12.012.

Gookin JL, Nordone SK, Argenzio RA (2002) Host responses to *Cryptosporidium* infection. *J Vet Intern Med* 1: 12-21

Guy RA, Xiao C, 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.

Hegner RW 1922. The systematic relationship of *Giardia lamblia*, Stiles 1915 from man and *G. agilis* Kunstler, 1882 from the tadpole. *Am J Hyg* 2: 435-441.

Hehl AB, Marti M (2004) Secretory protein trafficking in *Giardia intestinalis*. *Mol Microbiol* 53: 19-28.

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

Hijjawi NS, Meloni BP, Ryan UM, Olson ME, Thompson RC (2002) Successful in vitro cultivation of *Cryptosporidium andersoni*: evidence for the existence of novel extracellular stages in the life cycle and implications for the classification of *Cryptosporidium*. *Int J Parasitol* 32: 1719-1726.

Hijjawi NS, Meloni BP, Ng'anzo M, Ryan UM, Olson ME, Cox PT, Monis PT, Thompson RC (2004) Complete development of *Cryptosporidium parvum* in host cell-free culture. *Int J Parasitol* 34: 769-777.

Hikosaka K, Nakai Y (2005) A novel genotype of *Cryptosporidium muri*s from large Japanese field mice, *Apodemus speciosus*. *Parasitol Res* 97: 373-379.

Iwabe N Miyata T (2001) Overlapping genes in parasitic protist *Giardia lamblia*.Gene 280: 163-167.

Jex AR, Gasser RB (2008) Analysis of the genetic diversity within *Cryptosporidium hominis* and *Cryptosporidium parvum* from imported and autochthonous cases of human cryptosporidiosis by mutation scanning. *Electrophoresis* 29: 4119–4129.

Jirku M, Valigurova A, Koudela B, Krizek J, Modry D, Slapeta J (2008) New species of *Cryptosporidium* Tyzzer, 1907 (Apicomplexa) from amphibian host: morphology, biology and phylogeny. *Folia Parasitol (Praha)* 55: 81-94.

Johnson AM, Giovanni GD, Rochelle PA (2012) Comparison of assays for sensitive and reproducible detection of cell culture-infectious *Cryptosporidium parvum*

and *Cryptosporidium hominis* in drinking water. *Appl Environ Microbiol* 78: 156-162.

Karanis P, Ey PL (1998) Characterization of axenic isolates of *Giardia intestinalis* established from humans and animals in Germany. *Parasitol Res* 84: 442-449.

Karanis P, Aldeyarbi HM (2011) Evolution of *Cryptosporidium* in vitro culture. *Int J Parasitol* 41: 1231-1242.

Karanis P, Kimura A, Nagasawa H, Igarashi I, Suzuki N (2008) Observations on *Cryptosporidium* life cycle stages during excystation. *J Parasitol* 94: 298-300.

Keusch GT, Thea DM, Kamenga M, Kakanda K, Mbala M, Brown C, Davachi F (1992) Persistent diarrhea associated with AIDS. *Acta Paediatr Suppl* 381: 45-48.

Khan SM, Debnath C, Pramanik AK, Xiao L, Nozaki T, Ganguly S (2011) Molecular evidence for zoonotic transmission of *Giardia duodenalis* among dairy farm workers in West Bengal, India. *Vet Parasitol* 178: 342-345.

Kodádková A, Kváč M, Ditrich O, Sak B, Xiao L (2010) *Cryptosporidium muris* in a reticulated giraffe (*Giraffa camelopardalis reticulata*). *J Parasitol* 96: 211–212.

Kuboki N, Inoue N, Sakurai T, Di Cello F, Grab DJ, Suzuki H, Sugimoto C, Igarashi I (2003) Loop mediated isothermal amplification for detection of African trypanosomes. *J Clin Microbiol* 41: 5517–5524.

Lalle M, Pozio E, Capelli G, Bruschi F, Crotti D, Caccio SM (2005) Genetic heterogeneity at the beta-giardin locus among human and animal isolates of *Giardia duodenalis* and identification of potentially zoonotic subgenotypes. *Int J Parasitol* 35: 207-213.

Lasek-Nesselquist E, Bogomolni AL, Gast RJ, Welch DM, Ellis JC, Sogin ML, Moore MJ (2008) Molecular characterization of *Giardia intestinalis* haplotypes in marine animals: variation and zoonotic potential. *Dis Aquat Org* 81: 39-51.

Lasek-Nesselquist E, Welch DM, Sogin ML (2010) The identification of a new *Giardia duodenalis* assemblage in marine vertebrates and a preliminary analysis of *G. duodenalis* population biology in marine systems. *Int J Parasitol* 40: 1063-1074.

Lebbad M, Mattsson JG, Christensson B, Ljungstrom B, Backhans A, Andersson JO Svard SG (2010) From mouse to moose: Multilocus genotyping of *Giardia* isolates from various animal species. *Vet Parasitol* 168: 231-239.

Levecke B, Geldhof P, Claerebout E, Dorny P, Vercammen F, Cacciò SM, Vercruysse J, Geurden T (2009) Molecular characterisation of *Giardia*

duodenalis in captive non-human primates reveals mixed assemblage A and B infections and novel polymorphisms. *Int J Parasitol* 39: 1595-1601.

Levecke B, Meulemans L, Dalemans T, Casaert S, Claerebout E, Geurden T (2011) Mixed *Giardia duodenalis* assemblage A, B, C and E infections in pet chinchillas (*Chinchilla lanigera*) in Flanders (Belgium). *Vet Parasitol* 177: 166-170.

Levine ND (1980) Some correlations of coccidian (Apicomplexa: Protozoa) nomenclature. *J Parasitol* 66: 830–834.

Levine ND (1984) Taxonomy and review of the coccidian genus *Cryptosporidium* (protozoa, apicomplexa). *J Protozool* 31: 94-98.

Lindergard G, Nydam DV, Wade SE, Schaaf SL, Mohammed HO (2003) A novel multiplex polymerase chain reaction approach for detection of four human infective *Cryptosporidium* isolates: *Cryptosporidium parvum*, types H and C, *Cryptosporidium canis*, and *Cryptosporidium felis* in fecal and soil samples. *J Vet Diagn Invest* 15: 262-267.

Lindsay DS, Upton SJ, Owens DS, Morgan UM, Mead JR, Blagburn BL (2000) *Cryptosporidium andersoni* n. sp. (Apicomplexa: Cryptosporidiidae) from cattle, *Bos taurus. J Eukaryot Microbiol* 47: 91–95.

Lu SC (2000) S-Adenosylmethionine. *Int J Biochem Cell Biol* 32: 391-395.

Lv C, Zhang L, Wang R, Jian F, Zhang S, Ning C, Wang H, Feng C, Wang X, Ren X, Qi M, Xiao L (2009) Prevalence and molecular characterization of *Cryptosporidium* spp. in wild, laboratory, and pet rodents in China. *Appl Environ Microbiol* 75: 7692-7699.

Marti M, Hehl AB (2003) Encystation-specific vesicles in *Giardia*: a primordial Golgi or just another secretory compartment? *Trends Parasitol* 19: 440-446.

Mehlhorn H, Piekarski G (1998) Grundriß der Parasitenkunde: Parasiten des Menschen und der Nutztiere. *Spektrum Akad Vlg Hdg* Auflage: 5 überarb. A.

Monis PT, Mayrhofer G, Andrews RH, Homan WL, Limper L, Ey PL (1996) Molecular genetic analysis of *Giardia intestinalis* isolates at the glutamate dehydrogenase locus. *Parasitol* 112: 1-12.

Monis PT, Andrews RH, Mayrhofer G, Ey PL (1999) Molecular systematics of the parasitic protozoan *Giardia intestinalis. Mol Biol Evol* 16: 1135-1144.

Morgan UM, Monis PT, Fayer R, Deplazes P, Thompson RC (1999) Phylogenic relationships among isolates of *Cryptosporidium*: evidence for several new species. *J Parasitol* 85: 1126–1133.

Morgan UM, Xiao L, Monis P, Sulaiman I, Pavlasek I, Blagburn B, Olson M, Upton SJ, Khramtsov NV, Lal A, Elliot A, Thompson RC (2000) Molecular and phylogenetic analysis of *Cryptosporidium muris* from various hosts. *Parasitol* 120: 457–464.

Morgan UM, Monis PT, Xiao L, Limor J, Sulaiman I, Raidal S, O'Donoghue P, Gasser R, Murray A, Fayer R, Blagburn BL, Lal AA, Thompson RC (2001) Molecular and phylogenic characterisation of *Cryptosporidium* from birds. *Int J Parasitol* 31: 289–296.

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

Morrison HG, McArthur AG, Gillin FD, Aley SB, Adam RD, Olsen GJ, Best AA, Cande WZ, Chen F, Cipriano MJ, Davids BJ, Dawson SC, Elmendorf HG, Hehl AB, Holder ME, Huse SM, Kim UU, Lasek-Nesselquist E, Manning G, Nigam A, Nixon JE, Palm D, Passamaneck NE, Prabhu A, Reich CI, Reiner DS, Samuelson J, Svard SG, Sogin ML (2007) Genomic minimalism in the early diverging intestinal parasite *Giardia lamblia*. *Science* 317: 1921-1926.

Muthusamy D, Rao SS, Ramani S, Monica B, Banerjee I, Abraham OC, Mathai DC, Primrose B, Muliyil J, Wanke CA, Ward HD, Kang G (2006) Multilocus genotyping of *Cryptosporidium* sp. isolates from human immunodeficiency virus-infected individuals in South India. *J Clin Microbiol* 44: 632-634.

Nagamine K, Hase T, Notomi T (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Mol Cell Probes* 16: 223-229.

Ng J, Yang R, McCarthy S, Gordon C, Hijjawi N, Ryan U (2011) Molecular characterization of *Cryptosporidium* and *Giardia* in pre-weaned calves in Western Australia and New South Wales. *Vet Parasitol* 176: 145-150.

Nichols RA, Smith HV (2004) Optimization of DNA extraction and molecular detection of *Cryptosporidium* oocysts in natural mineral water sources. *J Food Prot* 67: 524-532.

Nicolle C, Manceaux LH (2009) On a leishman body infection (or related organisms) of the gondi. 1908. *Int J Parasitol* 39: 863-864.

Nolan MJ, Jex AR, Haydon SR, Stevens MA, Gasser RB (2010) Molecular detection of *Cryptosporidium cuniculus* in rabbits in Australia. *Infect Genet Evol* 10:1179-1187.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T, (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* 28: E63.

O'Brien E, McInnes L, Ryan U (2008) *Cryptosporidium* GP60 genotypes from humans and domesticated animals in Australia, North America and Europe. *Exp Parasitol* 118: 118-121.

O'Donoghue PJ (1995) *Cryptosporidium* and cryptosporidiosis in man and animals. *Int J Parasitol* 25: 139-195.

Ogassawara S, Benassi S, Larsson CE, Hagiwara MK (1986) *Cryptosporidium curyi sp.n.*, in the feces of cats in the city of Sao Paulo, Brazil. *Revista de Microbiologia* 17: 346-349.

Palenzuela O, Alvarez-Pellitero P, Sitjà-Bobadilla A (2010) Molecular characterization of *Cryptosporidium molnari* reveals a distinct piscine clade. *Appl Environ Microbiol* 76: 7646-7649.

Palm JE, Weiland ME, Griffiths WJ, Ljungstrom I, Svard SG (2003) Identification of immunoreactive proteins during acute human giardiasis. *J Infect Dis* 187: 1849-1859.

Palmer CJ, Xiao L, Terashima A, Guerra H, Gotuzzo E, Saldías G, Bonilla JA, Zhou L, Lindquist A, Upton SJ (2003) *Cryptosporidium muris*, a rodent pathogen, recovered from a human in Perú. *Emerg Infect Dis* 9: 1174-1176.

Paperna I, Landsberg JH, Ostrovska K (1986) Cryptosporidia of lower vertebrates. *J. Protozool Soc Protozool Abstracts* 145.

Pavlasek I (1999) Cryptosporidia: biology, diagnostics, host spectrum, specificity and relation to the environment. *Remedia-Klin Mikrobiol* 3: 290–301.

Pavlasek I, Ryan U (2008) *Cryptosporidium varanii* takes precedence over *C. saurophilum*. *Exp Parasitol* 118: 434–437.

Paz e Silva FM, Monobe MM, Lopes RS, Araujo JPJr (2012) Molecular characterization of *Giardia duodenalis* in dogs from Brazil. *Parasitol Res* 110: 325-334.

Pedraza-Dıaz S, Ortega-Mora LM, Carrion BA, Navarro V, Gomez Bautista M (2009) Molecular characterisation of *Cryptosporidium* isolates from pet reptiles. *Vet. Parasitol* 160: 204-210.

Plutzer J, Karanis P (2007) Molecular identification of a *Cryptosporidium saurophilum* from corn snake (*Elaphe guttata guttata*). *Parasitol Res* 101: 1141-1145.

Plutzer J, Karanis P (2009) Genetic polymorphism in *Cryptosporidium* species: an update. *Vet Parasitol* 165: 187-99.

Plutzer J, Ongerth J, Karanis P (2010) *Giardia* taxonomy, phylogeny and epidemiology: Facts and open questions. *Int J Hyg Environ Health* 213: 321-333.

Pollock KG, Ternent HE, Mellor DJ, Chalmers RM, Smith HV, Ramsay CN, Innocent GT (2010) Spatial and temporal epidemiology of sporadic human cryptosporidiosis in Scotland. *Zoonoses Public Health* 57: 487-492.

Power ML, Cheung-Kwok-Sang C, Slade M, Williamson S (2009) *Cryptosporidium fayeri*: diversity within the GP60 locus of isolates from different marsupial hosts. *Exp Parasitol* 121: 219-223.

Power ML, Holley M, Ryan UM, Worden P, Gillings MR (2011) Identification and differentiation of *Cryptosporidium* species by capillary electrophoresis single-strand conformation polymorphism. *FEMS Microbiol Lett* 314: 34-41.

Proctor SJ, Kemp RL (1974) *Cryptosporidium anserinum* sp. n. (Sporozoa) in a domestic goose *Anser anser L*., from Iowa. *J Protozool* 21: 664-666.

Rehg JE, Lawton GW, Pakes SP (1979) *Cryptosporidium cuniculus* in the rabbit (*Oryctolagus cuniculus*). *Lab Anim Sci* 29: 656-560.

Ren X, Zhao J, Zhang L, Ning C, Jian F, Wang R, Lv C, Wang Q, Arrowood MJ, Xiao L (2012) *Cryptosporidium tyzzeri* n. sp. (Apicomplexa: Cryptosporidiidae) in domestic mice (*Mus musculus). Exp Parasitol* 130: 274-281.

Rendtorff RC, Holt CJ (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.

Robertson LJ, Hermansen L, Gjerde BK, Strand E,. Alvsvag J.O, Langeland N (2006) Application of genotyping during an extensive outbreak of waterborne giardiasis in Bergen, Norway, during autumn and winter 2004. *Appl Environ Microbiol* 72: 2212–2217.

Robinson G, Elwin K, Chalmers RM (2008) Unusual *Cryptosporidium* genotypes in human cases of diarrhea. *Emerg Infect Dis* 14: 1800-1802.

Robinson G, Wright S, Elwin K, Hadfield SJ, Katzer F, Bartley PM, Hunter PR, Nath M, Innes EA, Chalmers RM (2010) Re-description of *Cryptosporidium cuniculus* Inman and Takeuchi, 1979 (Apicomplexa: Cryptosporidiidae): morphology, biology and phylogeny. *Int J Parasitol* 40: 1539-1548.

Ropolo AS, Saura A, Carranza PG, Lujan HD (2005) Identification of variant-specific surface proteins in *Giardia muris* trophozoites. *Infect Immun* 73: 5208-5211.

Rosales MJ, Cordon GP, Moreno MS, Sanchez CM (2005) Extracellular like-gregarine stages of *Cryptosporidium parvum*. *Acta Trop* 95: 74-78.

Ryan UM, Monis P, Enemark HL, Sulaiman I, Samarasinghe B, Read C, Buddle R, Robertson I, Zhou L, Thompson RC, Xiao L (2004a) *Cryptosporidium suis* n. sp. (Apicomplexa: Cryptosporidiidae) in pigs (*Sus scrofa*). *J Parasitol* 90: 769–773.

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

Santin M, Trout JM, Vecino JA, Dubey JP, Fayer R (2006) *Cryptosporidium*, *Giardia* and *Enterocytozoon bieneusi* in cats from Bogota (Colombia) and genotyping of isolates. *Vet Parasitol* 141: 334-339.

Santin M, Zarlenga DS (2009) A multiplex polymerase chain reaction assay to simultaneously distinguish *Cryptosporidium* species of veterinary and public health concern in cattle. *Vet Parasitol* 166: 32-37.

Singh GS, Kumar B (2001) Transport tensors in perfectly aligned low-density fluids: self-diffusion and thermal conductivity. *Phys Rev E Stat Nonlin Soft Matter Phys* 63: 061707.

Skotarczak B (2010) Progress in the molecular methods for the detection and genetic characterization of *Cryptosporidium* in water samples. *Ann Agric Environ Med* 17: 1-8.

Slapeta J, Stejskal F, Keithly JS (2003) Characterization of S-adenosylmethionine synthetase in *Cryptosporidium parvum* (Apicomplexa). *FEMS Microbiol Lett* 225: 271-277.

Slapeta J (2006) *Cryptosporidium* species found in cattle: a proposal for a new species. Trends Parasitol 22: 469–474.

Slapeta J (2012) The name *Cryptosporidium tyzzeri* Ren, Zhao, Zhang, Ning, Jian, Wang, Lv, Wang, Arrowood and Xiao, 2012 is permanently invalid. *Exp Parasitol* 130: 306-307.

Sotiriadou I, Karanis P (2008) Evaluation of loop-mediated isothermal amplification for detection of *Toxoplasma gondii* in water samples and comparative findings by polymerase chain reaction and immunofluorescence test (IFT). *Diagn Microbiol Infect Dis* 62: 357-365.

Spano F, Crisanti A (2000) *Cryptosporidium parvum*: the many secrets of a small genome. *Int J Parasitol* 30: 553-565.

Sukthana Y, Kaewkungwal, J, Jantanavivat C, Lekkla A, Chiabchalard R, Aumarm W (2003) *Toxoplasma gondii* antibody in Thai cats and their owners. *Southeast Asian J Trop Med Public Health* 34: 733–738.

Sulaiman IM, Morgan UM, Thompson RC, Lal AA, Xiao L (2000) Phylogenetic relationships of *Cryptosporidium* parasites based on the 70-kilodalton heat shock protein (HSP70) gene. *Appl Environ Microbiol* 66: 2385-2391.

Sulaiman IM, Lal AA, Xiao L (2002) Molecular phylogeny and evolutionary relationships of *Cryptosporidium* parasites at the actin locus. *J Parasitol* 88: 388–394.

Sulaiman IM, Fayer R, Bern C, Gilman RH, Trout JM, Schantz PM, Das P, Lal AA, Xiao L (2003) Triosephosphate isomerase gene characterization and potentialzoonotic transmission of *Giardia duodenalis*. *Emerging Infect Dis* 9: 1444-1452.

Suzuki J, Murata R, Kobayashi S, Sadamasu K, Kai A, Takeuchi T (2011) Risk of human infection with *Giardia duodenalis* from cats in Japan and genotyping of the isolates to assess the route of infection in cats. *Parasitol* 138: 493-500.

Tabor CW, Tabor H (1985) Polyamines in microorganisms. *Microbiol Rev* 49: 81-99.

Templeton TJ, Lancto CA, Vigdorovich V, Liu C, London NR, Hadsall KZ, Abrahamsen MS (2004) The *Cryptosporidium* oocyst wall protein is a member of a multigene family and has a homolog in *Toxoplasma*. *Infect Immun* 72: 980-987.

Tenter AM, Heckeroth AR, Weiss LM (2000) *Toxoplasma gondii*: from animals to humans. *Int J Parasitol* 30: 1217–1258.

Tenter AM (2009) *Toxoplasma gondii* in animals used for human consumption. *Mem Inst Oswaldo Cruz* 104: 364–369.

Teodorovic S, Braverman JM, Elmendorf HG (2007) Unusually low levels of genetic variation among *Giardia lamblia* isolates. *Eukaryotic Cell* 6: 1421-1430.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22: 4673-4680.

Traversa D, Iorio R, Otranto D, Modry D, Slapeta J (2008) *Cryptosporidium* from tortoises: Genetic characterisation, phylogeny and zoonotic implications. *Mol Cell Probes* 22: 122-128.

Triffit MJ (1925) Observations on two new species ofcoccidia parasitic in snakes. *Protozoology* 1: 19-26.

Tyzzer E (1907) A sporozoan found in the peptic glands of the common mouse. *Proc Soc Exp Biol Med* 5: 12–13.

Tyzzer EE (1910) An extracellular Coccidium, *Cryptosporidium muris* (gen. et sp. nov.), of the gastric glands of the common mouse. *J Med Res* 23: 487-510.

Tyzzer E (1912) *Cryptosporidium parvum* (sp. nov.), a coccidium found in the small intestine of the common mouse. *Arch Protis* 26: 394–412.

Umemiya R, Fukuda M, Fujisaki K, Matsui T (2005) Electron microscopic observation of the invasion process of *Cryptosporidium parvum* in severe combined immunodeficiency mice. *J Parasitol* 91: 1034-1039.

Upcroft J, Mitchell R, Chen N, Upcroft P (1996) Albendazole resistance in *Giardia* is correlated with cytoskeletal changes but not with a mutation at amino acid 200 in beta-tubulin. *Microb Drug Resist* 2: 303-308.

Upton SJ, Tilley M, Brillhart DB (1994) Comparative development of *Cryptosporidium* parvum (Apicomplexa) in 11 continuous host cell lines. *FEMS Microbiol Lett* 118: 233-236.

Valigurova A, Hofmannova L, Koudela B, Vavra J (2007) An ultrastructural comparison of the attachment sites between *Gregarina steini* and *Cryptosporidium* muris. J Eukaryot Microbiol 54: 495-510.

van Belkum A, Neisters HG (1995) Nucleic acid amplification and related techniques in microbiological diagnostics and epidemiology. *Cell Mol Biol* 41: 615–623.

van Keulen H, Gutell RR, Campbell SR, Erlandsen SL, Jarroll EL (1992) The nucleotide sequence of the entire ribosomal DNA operon and the structure of the large subunit rRNA of *Giardia muris*. *J Mol Evol* 35: 318-328.

van Keulen H, Gutell RR, Gates MA, Campbell SR, Erlandsen SL, Jarroll EL, Kulda J, Meyer EA (1993) Unique phylogenetic position of Diplomonadida based on the complete small subunit ribosomal RNA sequence of *Giardia ardeae, G. muris, G. duodenalis* and *Hexamita* sp. *FASEB J* 7: 223-231.

van Keulen H, Feely DE, Macechko PT, Jarroll EL, Erlandsen SL (1998) The sequence of *Giardia* small subunit rRNA shows that voles and muskrats are parasitized by a unique species *Giardia microti*. *J Parasitol* 84: 294-300.

van Keulen H, Macechko PT, Wade S, Schaaf S, Wallis PM, Erlandsen SL (2002) Presence of human *Giardia* in domestic, farm and wild animals, and environmental samples suggests a zoonotic potential for giardiasis. *Vet Parasitol* 108: 97-107.

Waldron LS, Cheung-Kwok-Sang C, Power ML (2010) Wildlife-associated *Cryptosporidium fayeri* in human, Australia. *Emerg Infect Dis* 16: 2006-2007.

Wang R, Zhang L, Feng Y, Ning C, Jian F, Xiao L, Zhao J, Wang Y (2008) Molecular characterization of a new genotype of *Cryptosporidium* from American minks (*Mustela vison*) in China. *Vet Parasitol* 154: 162-166.

Wang R, Wang H, Sun Y, Zhang L, Jian F, Qi M, Ning C, Xiao L (2011) Characteristics of *Cryptosporidium* transmission in preweaned dairy cattle in Henan, China. *J Clin Microbiol* 49: 1077-1082.

Wielinga C, Ryan U, Thompson RCA, Monis P (2011) Multi-locus analysis of *Giardia duodenalis* intra-Assemblage B substitution patterns in cloned culture isolates suggests sub-Assemblage B analyses will require multi-locus genotyping with conserved and variable genes. *Int J Parasitol* 41: 495-503.

Xiao L, Escalante L, Yang C, Sulaiman I, Escalante AA, Montali RJ, Fayer R, Lal AA (1999) Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene locus. *Appl Environ Microbiol* 65: 1578–1583.

Xiao L, Limor J, Morgan UM, Sulaiman IM, Thompson RC, Lal AA (2000) Sequence differences in the diagnostic target region of the oocyst wall protein gene of *Cryptosporidium* parasites. *Appl Environ Microbiol* 66: 5499–5502.

Xiao L, Sulaiman IM, Ryan UM, Zhou L, Atwill ER, Tischler ML, Zhang X, Fayer R, Lal AA (2002) Host adaptation and host-parasite co-evolution in *Cryptosporidium*: implications for taxonomy and public health. *Int J Parasitol* 32: 1773-1785.

Xiao LH, Ryan UM (2004) Cryptosporidiosis: an update in molecular epidemiology. *Current Opinion in Infectious Diseases* 17: 483-490.

Xiao L, Fayer R, Ryan U, Upton SJ (2004a) *Cryptosporidium* taxonomy: recent advances and implications for public health. *Crit Rev Microbiol* 17: 72–97.

Xiao L, Ryan UM, Graczyk TK, Limor J, Li L, Kombert M, Junge R, Sulaiman IM, Zhou L, Arrowood MJ, Koudela B, Modry D, Lal AA (2004b) Genetic diversity of *Cryptosporidium* spp. in captive reptiles. *Appl Environ Microbiol* 70: 891-899.

Xiao L, Fayer R (2008) Molecular characterisation of species and genotypes of *Cryptosporidium* and *Giardia* and assessment of zoonotic transmission. *Int J Parasitol* 38: 1239-1255.

Xiao L, Ryan UM, Fayer R, Bowman DD, Zhang L (2012) *Cryptosporidium tyzzeri* and *Cryptosporidium pestis*: which name is valid? Exp Parasitol 130: 308-309.

Yakovchuk P, Protozanova E, Frank-Kamenetskii MD (2006) Base-stacking and base-pairing contributions into thermal stability of the DNA double helix. *Nucleic Acids Res* 34: 564-574.

2. Single nucleotide polymorphism (SNP)-based loop-mediated isothermal amplification (LAMP) assay for the detection of human pathogenic *Cryptosporidium* species – clinical and environmental verification and first detection of *C. fragile* in water

Abstract

Here, the development and evaluation of an SNP-based LAMP assay for the rapid detection of *Cryptosporidium* human pathogenic species in water samples is described. The method has been evaluated based on the amplification of the S-adenosyl-methionine synthetase (SAM) *Cryptosporidium* gene, and it demonstrated a sensitivity detection limit of 800 fg/µl. SNP-based LAMP detection was compared with two nested polymerase chain reaction (nPCR) assays that detect the small subunit (SSU) rRNA gene and a GP60-LAMP assay. Testing was carried out on 53 water sample pellets spiked with known numbers of *Cryptosporidium* oocysts. After DNA extraction, the detection sensitivity for the spiked pellets was 100% for the SNP-based SAM-LAMP assay and the GP60-LAMP assay, while for the two applied nPCRs based on the SSU rRNA gene, the sensitivity was only 3% and 7%, respectively. In the same water samples prior to spiking, 50% were found to be positive for *Cryptosporidium* DNA by the SNP-based LAMP assay, and 24% were positive according to the GP60-LAMP assay; the nPCR assays generated products in only 5% and 7% of the samples, respectively.

The SNP-based SAM-LAMP assays showed positive results in 12 of 15 water samples (80%) from Thailand, 4 of 8 mussel samples (50%), 11 of 44 HIV/AIDS patient fecal samples (25%) and 12 of 22 animal fecal samples (55%). The GP60-LAMP assay specific to *C. parvum* amplified *Cryptosporidium* DNA in 7 of 15 water samples (46%), in 1 of 8 mussel samples (12%), in 6 of 44 HIV/AIDS patient fecal samples (13%) and in 8 of 22 animal fecal samples (36%). nPCRs shown to amplify the SSU rRNA gene amplified *Cryptosporidium* in 2 or 3 of 15 water samples (13% and 20%, respectively), 4 of 8 mussel samples (50% by both assays), 1 or 7 of 44 HIV/AIDS patient fecal samples (2% and 16%, respectively) and 8 or 5 of 22 animal

fecal samples (36% and 22%, respectively). Among the species identified by sequencing analysis, the most commonly detected species was *C. parvum*, whereas *C. hominis* was detected in material collected from one mussel, *C. andersoni* was detected in 1 animal fecal sample, and *C. fragile* was detected in 1 water sample. This is the first description of *C. fragile* in water samples. The results clearly indicate that the above tested method is an effective tool for the detection of *Cryptosporidium* species in fecal clinical samples and environmental water samples.

2.1. Introduction

Cryptosporidium species are frequently found in the stool of humans, domestic and wild ruminants, birds and fishes. They have gained attention as a possible cause of gastrointestinal complaints and are therefore important for differential diagnostic purposes. The aquatic transmission of *Giardia* and *Cryptosporidium* causes significant public concern, which is justified by the waterborne transmission of both parasites in numerous well-documented outbreaks in the USA, Europe, Asia, Australia and Canada (Schuster et al., 2005; Karanis et al., 2007a; Smith et al., 2007; Yoshida et al., 2007; Blasi et al., 2008; Dale et al., 2010; Baldursson and Karanis, 2011; Rimšelienė et al., 2011; Serdarevic et al., 2011). *Cryptosporidium* has been classified as a possible zoonotic disease that is carried in vast numbers by wild and domestic animals (Xiao et al., 2002; Monis and Thompson, 2003; Plutzer and Karanis 2009a; Ng et al., 2011).

The low infectious dose and the resistance of *Cryptosporidium* oocysts to chlorine disinfection in water treatment plants, public water supplies or recreational waters can lead to diarrheal diseases in normal and immunocompromised hosts (Korich et al., 1990; DuPont et al., 1995; Pereira et al., 2008; Shields et al., 2008; Lim et al., 2011). It is acknowledged that individuals with an impaired immune system, such as patients with AIDS, can face a chronic and life-threatening course of *Cryptosporidium* disease (Bruce et al., 2000; Chalmers et al., 2002; Nahrevanian and Assmar, 2008).

Molecular methods have shown considerable genetic variation among human isolates. Humans are primarily infected by *C. parvum* and *C. hominis* (Morgan-Ryan et al., 2002; Plutzer and Karanis, 2009a). Other species, including *C. meleagridis*, *C. canis*, *C. cuniculus*, *C. felis* and *C. suis*, have also been detected, of which *C. meleagridis* is encountered most frequently (Cama et al., 2007; Llorente et al., 2007; Xiao et al., 2001, 2004; Hadfield and Chalmers, 2012).

The diagnosis of cryptosporidiosis in clinical and environmental samples is usually made by detecting the parasites by microscopy and confirmed by nested PCR. Both of these methodologies commonly require special and expensive equipment and well-trained and experienced staff, which makes the diagnostic criteria difficult and subjective to apply, either due to their tediousness or inherent sensitivity problems. The microscopic identification of *Cryptosporidium* in both environmental and fecal material by conventional staining methods remains difficult and subjective due to the debris, primeses and other possible microorganisms present in the collected samples. These limitations of the standard method of diagnosis necessitated the development of a definitive, fast, highly reliable method. Loop-mediated isothermal amplification of DNA has already been successfully applied to the detection of other microorganisms, and it is likely to be rapid, specific, sensitive and simple compared to the other methods. That LAMP overcomes the disadvantages of other methods is confirmed by the growing number of reference publications covering different applications and areas (Mori and Notomi, 2009; Karanis and Ongerth, 2009).

LAMP has already found applications in the diagnosis of several parasitic diseases, including malaria (Pöschl et al., 2010) toxoplasmosis (Sotiriadou and Karanis, 2008), trypanosomiasis (Thekisoe et al., 2010) schistosomiasis (Xu et al., 2010) and others (Karanis and Ongerth, 2009). LAMP assays for the detection of *Cryptosporidium* species have been previously applied (Karanis et al., 2007b; Inomata et al., 2009; Plutzer et al., 2010) based on the amplification of the single copy 60-kDa glycoprotein (GP60) gene (Strong et al. 2000) of *C. parvum* (Karanis et al. 2007b) and on the SAM gene (Bakheit et al., 2008).

Here, we investigated the conditions of the LAMP reaction and evaluated the sensitivity and sensitivity of the SNP-based LAMP technique in reference isolates. The method was also evaluated in environmental water samples of different origins that were spiked with *Cryptosporidium* oocysts. Moreover, the evaluated assay was also verified in environmental water samples, animal and human fecal samples, and food material. All samples used in the study were also tested in parallel with the GP60-LAMP assay and two nPCRs. Subsequently, we identified the different *Cryptosporidium* genotypes by sequencing.

2.2 Materials and methods

After DNA extraction, the developed LAMP assay was tested first against simulated environmental samples, and subsequently, the assay was applied to the detection of cryptosporidial DNA extracted from fecal samples collected from various hosts and natural environmental samples of different origins. Two nPCRs targeting the SSU rRNA gene were performed, and the results were analyzed by sequencing and compared with those from the GP-60 assay and the SNP-based SAM-LAMP assay.

2.2.1 Origins of samples

Environmental water samples from Bulgaria (n=37) and Russia (n=16) (Karanis et al., 2006) were randomly selected from the sample bank of our laboratory and were used for the evaluation of the LAMP method and the nested PCR reactions in the present study. Additional environmental water samples (n=15) from the Thailand tsunami area (Srisuphanunt et al., 2010), mussel tissues (n=8) (Srisuphanunt et al., 2009) and fecal samples from HIV/AIDS patients (n=44) or animals (n=22) in Thailand (Srisuphanunt et al., 2011) were included in the study.

2.2.2 Reference isolates used

We used as our reference DNA template isolates of *C. parvum* Hungarian isolate (Plutzer and Karanis, 2007a), *C. parvum* (IOWA isolate) and *C. parvum* genotype II (collectively referred to hereinafter as *C. parvum*) to test the specificity and sensitivity of the method. The reference DNA was also used as a positive control for all PCR and LAMP experiments. As a negative control, we used distilled water. As internal controls to demonstrate the specificity of the SAM-LAMP assay, we used the DNA of various parasites, including *Trypanosoma brucei* (GUTat 1.3), *Babesia gibsoni, Toxoplasma gondii* (RH and PLK strains from the National Research Center for Protozoan Diseases, Obihiro University for Agriculture), *Giardia intestinalis* Assemblage-A (Karanis & Ey, 1998), *Leishmania major* (VL 39, Pasteur Institute, Athens, Greece) and *Neospora caninum* (NRCPD, Obihiro University for Agriculture and Veterinary Medicine, Japan).

2.2.3 Sample analysis

All environmental samples included in the study from different sources and quality were concentrated using $Al_2(SO_4)_3$-flocculation, as described by Karanis and Kimura (2002) and as later applied by Kourenti et al. (2003), and concentrated using the Sheather sugar gradient centrifugation method according to Karanis et al. (2006).

2.2.4 Detection and identification of *Cryptosporidium* oocysts by immunofluorescence test (IFT) and differential interference contrast (DIC) microscopy

All water, fecal and mussel tissue samples included in the study were stained with fluorescently labeled (FITC) monoclonal antibodies (Waterborne, Inc, New Orleans, LA) according to the recommendations of the manufacturer for the detection of *Cryptosporidium* oocysts. The method was slightly modified by performing the staining in an Eppendorf tube instead of on slides to minimize the loss of oocysts during the washing step. Microscopic identification of *Cryptosporidium* oocysts was performed independently by two well-trained and experienced individuals who screened all of the samples using an epifluorescent and DIC microscope (Olympus, Germany), fulfilling the acceptance criteria (Plutzer et al., 2007, 2008). Internal structures of intact oocysts were confirmed by DIC (Karanis et al., 2006; Srisuphanunt et al., 2010).

2.2.5 Extraction of genomic DNA (gDNA)

DNA was extracted from the resultant pellets after concentration and purification of the samples and utilized for molecular analysis, sequencing of PCR-generated products and evaluation of the LAMP method. DNA from simulated and natural water samples was treated according to the modified extraction method of Plutzer et al. (2008) using the QIAamp DNA Mini Kit (Qiagen, Germany). The same method as described by Plutzer et al. (2010) for genomic DNA extraction from water pellets originating from Thailand was performed.

DNA from fecal samples was directly extracted using the modified method described by Plutzer and Karanis (2009b) followed by the use of the QIAamp Stool Kit (Qiagen GmbH, Hilden, Germany). Briefly, the oocysts in the pellets were ruptured using ten freeze-thaw cycles in the presence of lysis buffer in a Dry Thermo device (DTU-2B,

Taitec, Japan) and were further processed according to the manufacturer's instructions. DNA was eluted in 100 µl of AE buffer and stored at –20 °C for later use.

2.2.6 Development of the SNP-based LAMP assay for the detection of the SAM *Cryptosporidium* gene

The LAMP primer design software Primer Explorer V4 (http://primerexplorer.jp/e/) was used to design the primer set. To ensure perfect annealing of all the primers to all the above-mentioned sequences of the *Cryptosporidium* SAM gene, the primer length and location were edited, and degenerate nucleotides were introduced.

For the identification of our SAM-LAMP assay-based SNPs, we first performed a complete analysis of the known sequences deposited in the GenBank under the accession numbers for *C. parvum* (GenBank accession number AY161084, XM_628434, AB119646 and AB119647), *C. hominis* (GenBank accession number XM_662396) and *C. meleagridis* (GenBank accession number AB119648). Sequences were aligned along with human (GenBank accession number X68836) and *E. histolytica* (GenBank accession number XM_647762) sequences. The detailed analysis of the diverse sequences from GenBank demonstrated that the *Cryptosporidium* SAM gene was a well-conserved housekeeping gene with limited sequence variation between *C. parvum*, *C. hominis* and *C. meleagridis.* The extensive alignment of *Cryptosporidium* SAM sequences with other sequences of other human and pathogenic species conducted using MEGA 5 (Tamura et al., 2011) and ClustalW (Thompson et al., 1994) showed clear sequence variations and a low similarity rate. The SAM-LAMP primers were first constructed, and afterwards, degenerate nucleotide bases, to detect SNP sites, were introduced to include the amplification of *C. meleagridis*. It was essential to find a gene that enabled the design of primers that could selectively amplify these species. The selected gene's name, the length and the sequence of each primer are shown in Table 1.

Table 1. Primer sets used for *Cryptosporidium* DNA amplification in LAMP assays based on the SAM and GP60 genes.

Assay	Primer Type	Sequence (5'→ 3')	Length (nt[1])	Amplicon size	Target
SAM* LAMP	F3	ATTTGAT**R**GACAAAGAAACTAG	22	145	*Cryptosporidium parvum, C. hominis* and *C. meleagridis* S-adenosyl-methionine synthetase (SAM) gene
	B3	CGATTGACTTTGCAACAAG	19		
	FIP (F1c-F2)	TTGCGCCCTGTTAATCCAGCATTAATTAATCCATCTGGCAG**R**TTT	45		
	BIP (B1-B2c)	TTGTAGATACATACGGAGGATGGGTCTACTTTAGTTGCATCTTTCC	46		
	LF	CTGCTGGCCC**M**CCAATTG	18		
	LB	CATGG**R**GGTGGTGCATTTAG	20		
GP60 LAMP	F3	TCGCACCAGCAAATAAGGC	19	157	*Cryptosporidium parvum* GP60 gene
	B3	GCCGCATTCTTCTTTTGGAG	20		
	FIP (F1c-F2)	ACCCTGGCTACCAGAAGCTTCAGAACTGGAGACGCAGAA	39		
	BIP (B1-B2c)	GGCCAAACTAGTGCTGCTTCCCGTTTCGGTAGTTGCGCCTT	41		
	LF	GTACCACTAGAATCTTGACTGCC	23		
	LB	AACCCACTACTCCAGCTCAAAGT	23		

*degenerate primers introduced to detect SNP sites are underlined in the sequences shown in the table; [1]nt: nucleotides,

2.2.7 Application of the GP60-LAMP assay

The GP60-LAMP reaction was based on the previous report of Karanis et al. (2007b) to amplify the *C. parvum* 60-kDa glycoprotein gene. The assay used the previously described reaction mix and the loop-designed primers (LF and LB) at a final concentration of 20 pmol each. The samples were incubated at 63 °C for 40 min and then heated to 80 °C for 3 min to terminate the reaction.

2.2.8 SAM-LAMP reaction conditions

The LAMP reaction was carried out in 25 µL of reaction mixture. The primer mix (0.9 µL) contained 40 pmol of FIP and BIP, 20 pmol of LF and LB and 5 pmol of F3 and B3 each, 12.5 µL of reaction buffer (1.6 M of betaine, 40 mM of Tris–HCl [pH 8.8], 20 mM of KCl, 20 mM $(NH_4)_2SO_4$, 2.8 mM of each deoxyribonucleotide triphosphate (dNTP), 0.2% Tween-20, and 16 mM of $MgSO_4$), 1 µL (8U) of Bst DNA polymerase (NEB, Cat. No. M0275L), 1 µL of fluorescent detection dye, 2 µL of DNA, and 7.6 µL of distilled water. The mixture was incubated at 63 °C for 1 h and then heated to 80 °C for 5 min to complete the reaction.

2.2.9 nPCRs based on the 18S rRNA gene for the detection of *Cryptosporidium*

The DNA from all samples was tested using two nested PCR procedures targeting the SSU rRNA gene following the original reports (Xiao et al., 1999, 2001; Nichols et al., 2003). Both nPCRs were successfully standardized and established in our lab. The first, nPCR-1, was performed using primers as applied by Plutzer and Karanis (2007b). It results in a 826–864 bp PCR product. The second, nPCR-2, was performed as originally described by Nichols et al. (2003) and later applied by Karanis et al. (2007). It amplifies a 435 bp long polymorphous region of the gene, which is suitable for *Cryptosporidium* species or genotype identification.

2.2.10 Visualization of the nPCR and LAMP-generated products

Aliquots of both nPCR and LAMP products (10 µl) were subjected to electrophoresis on a 1.5% agarose gel (1 × Tris-Acetate-EDTA [TAE]) and visualized under ultraviolet (UV) light after 10 min of ethidium bromide (1 mg/ml, Sigma-Aldrich,

Germany) staining. Along with the samples, a 100 bp DNA ladder (Promega, Mannheim, Germany; G3161) was used as a standard for comparing molecular weights. To avoid contamination between samples, we used different laboratories to extract DNA and assemble the LAMP reactions and only used pipette tips with filters for aerosol protection. Moreover, the reaction tubes in which the LAMP reaction was performed were observed from the lateral side under daylight and by irradiating the tube under UV light to detect products colorimetrically. The turbidity due to the magnesium pyrophosphate produced was proportional to the amount of amplified product. The presence of the turbidity was an excellent indicator of the presence of the target gene, allowing us to make an easy visual detection with the naked eye (Mori et al. 2001; Tomita et al., 2008) (see Figures 1 and 2).

2.2.11 Sequencing of the positive *Cryptosporidium* productes detected by nPCR

After PCR amplification followed by agarose gel electrophoresis, the bands were purified using the QIAquick Gel Extraction Kit (Quiagen, Hilden, Germany). The sequencing reaction was carried out using the Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, CA, USA) as described by the manufacturer. The cycle sequencing reaction was followed by precipitation and washing steps with 100% and 70% ethanol, respectively. Afterwards, the samples were dried for 3 min at 85 °C, and formamide was added. Separation of the fragments was achieved using an automatic sequencer (Genetic Analyzer 3130 XL, Applied Biosystems, CA, USA). The standard sequencing protocol suggested by Applied Biosystems was used. The row data were collected with the 3130xl Data Collection v3.0 program (Applied Biosystems, CA, USA). The conversion of the raw data to chromatograms was performed using the Sequencing Analysis 5.2.0 program (Applied Biosystems, CA, USA).

2.2.12 Phylogenetic analysis of the *Cryptosporidium* species in positive samples

The accuracy of the data was confirmed by two-directional sequencing of the obtained sequences and by alignment of the nucleotide sequences of the SSU rRNA for *Cryptosporidium* oocysts against the reference sequences retrieved from

GenBank using the program ClustalW. Sequence similarity was also determined using the Basic Local Alignment Search Tool (BLAST), and the phylogenetic analyses were conducted using MEGA version 5. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987), and the respective evolutionary analyses were conducted using MEGA 5 (Tamura et al., 2011). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches in Figure 1 (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in units of the number of base substitutions per site.

2.2.13 Specificity and sensitivity of the SAM-LAMP assay

To assess the specificity of the SAM-LAMP assay, DNA templates obtained from *C. parvum*, *T. brucei*, *B. gibsoni, T. gondii, G. intestinalis*, *L. major* and *N. caninum* were used. The incubation was performed at 63 ºC for 60 min for the SAM-LAMP assay and at 63 ºC for 40 min for the GP60-LAMP assay.

Sensitivity was evaluated at up to six serial dilutions steps of DNA derived from *C. parvum* oocysts after being quantified using a spectrophotometer (Biophotometer, Eppendorf, Germany). The dilutions provided a concentration range for *C. parvum* DNA from 800 ng/µl down to 800 fg/µl, which corresponds to *C. parvum* oocyst numbers from 10^5 down to 0.1 oocysts. LAMP incubations were performed at 63 ºC for 60 min for the SAM-LAMP assay and for 40 min for the GP60-LAMP assay. The data represent three independent experiments for each LAMP assay.

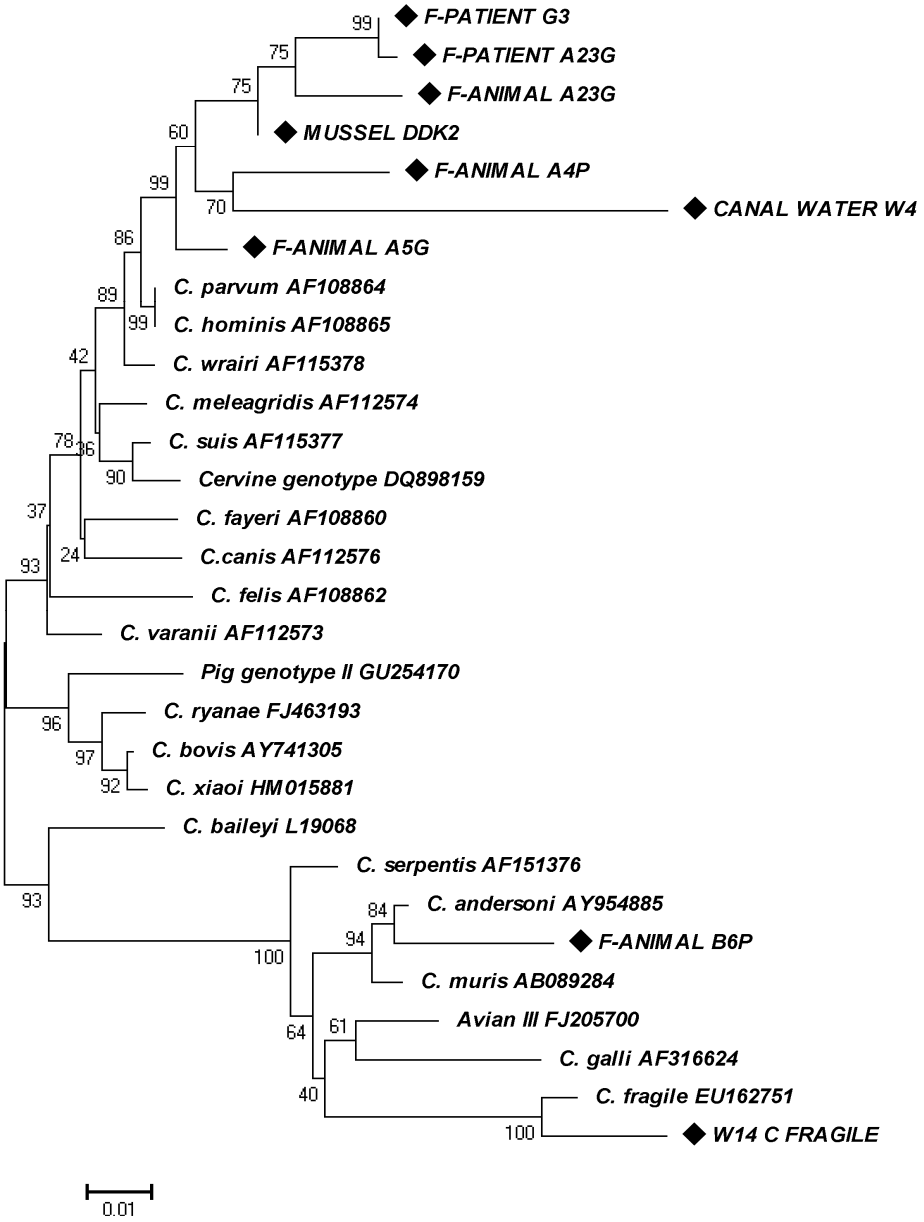
2.2.14 LAMP verification in simulated water samples

To verify the ability of the developed SAM-LAMP assay to detect *Cryptosporidium*, we tested randomly selected environmental water samples from Bulgaria (n=37) and Russia (n=16) (Karanis et al., 2006). The samples were taken from different areas with variable quality (see Table 2), and three replicates of each sample were tested.

C. parvum oocysts were counted using a hemocytometer, and 10 oocysts were added to 10% aliquots of the concentrated sample pellets. After DNA extraction, the SAM-LAMP assay was performed on all 53 simulated sediments. The results from the simulated samples were compared to the results of the original samples. The

experiment consisted of three independent trials for each LAMP assay. The PCR products were analyzed with 1.6% agarose gel electrophoresis and visualization with ethidium bromide staining.

Figure 1. Phylogenetic relationship of partial SSU rRNA sequences of 21 known *Cryptosporidium* species/genotype sequences retrieved from the GenBank database conducted by the program MEGA. Evolutionary distances were inferred by the neighbor-joining method, computed using the Kimura 2-parameter with the pairwise deletion option, and the bootstrap consensus tree was inferred from 1,000 replicates. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.



2.2.15 PCR verification in simulated water samples

The verification of the nPCR assays targeting the SSU rRNA gene was performed by testing 10% aliquots of the final sample pellets for the presence of inhibitors. We used randomly selected environmental water samples from Bulgaria (n=37) and Russia (n=16) that each contained 10 *C. parvum* oocysts from which the DNA was extracted. The SSU rRNA-based PCR was applied to all samples in triplicate. The results of the simulated and natural replicates, included to define the precision of the developed method, were visualized as mentioned before and were compared accordingly to the results of the original samples.

Figure 3 A. Visual detection under daylight of the SNP based SAM-LAMP assay. 1) negative control containing distilled water as template without fluorescent dye; 2) 8 pg/µl *Cryptosporidium* DNA in prescence of fluorescent dye; 3) 800 fg/µl *Cryptosporidium* DNA in prescence of fluorescent dye; 4) negative control containing distilled water as template in abscence of fluorescent dye. Arrows shows the formation magnesium pyrophosphate;

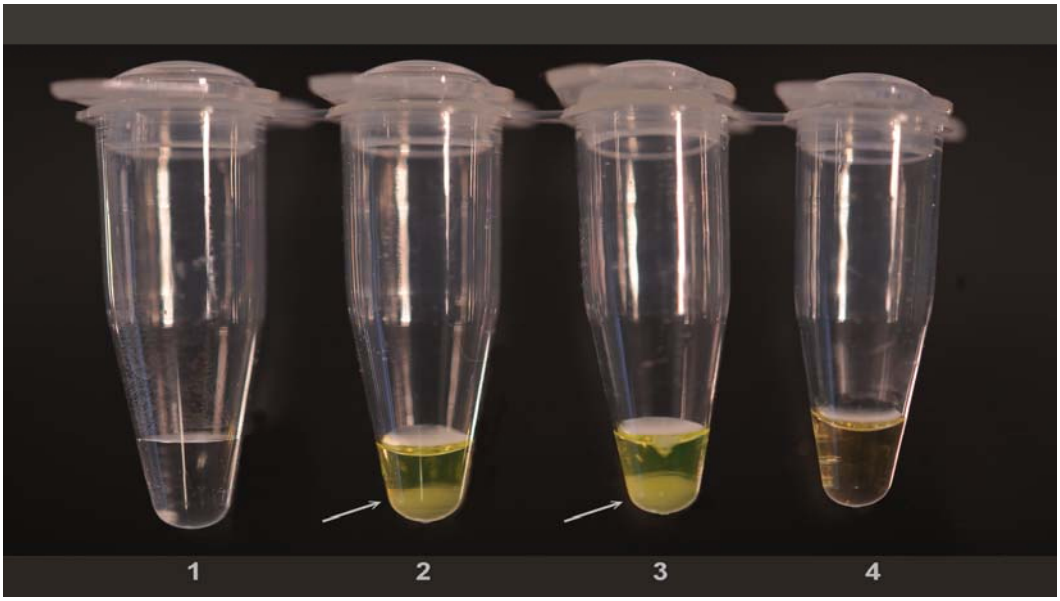
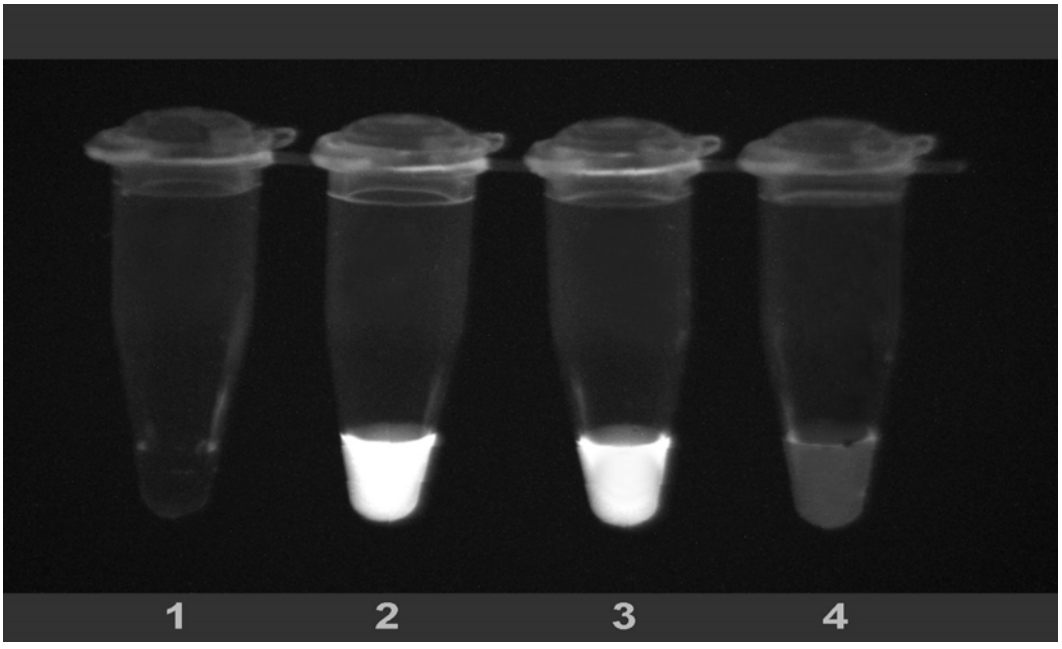


Figure 3 B. Irridiation of the same samples under UV-light.



2.3 Results

The detection sensitivity in spiked water pellets was 100% by the SNP-based SAM-LAMP assay and the GP60-LAMP assay, while for the two nPCRs based on the SSU rRNA gene, the sensitivities were only 5% and 13%. Using the same 53 natural water samples prior to spiking, the SNP-based LAMP assay detected *Cryptosporidium* DNA in 27 samples (50%), and the GP60-LAMP assay detected *Cryptosporidium* in only 13 samples (24%). *Cryptosporidium* nPCR products were generated in 5% and 7% of the samples, respectively. The overall detection rate of the applied method in samples examined from various hosts (human, cattle, mussels) and origins was 43% positive for *Cryptosporidium* spp. by the SNP-based SAM-LAMP assay, followed by 22% positive by the GP60-LAMP. The nPCRs detected *Cryptosporidium* DNA in only 16% and 21% of the examined samples.

The most commonly detected species determined by sequencing analysis was *C. parvum*, but *C. hominis, C. andersoni* and *C. fragile* were also detected in the examined material.

2.3.1 Specificity of the SNP-based SAM-LAMP assay for the detection of *Cryptosporidium* species DNA

The specificity of the assays was determined by testing DNA derived from different known *Cryptosporidium* isolates (Hungarian, IOWA and C. *parvum* genotype II) and from phylogenetically related protozoan parasites. The results demonstrated clearly that only *Cryptosporidium* DNA could be amplified and detected by this assay. No amplification was detected in samples containing DNA other than that of *Cryptosporidium.* The other parasites tested were *T. brucei, B. gibsoni, T. gondii*, *G. intestinalis*, *L. major*, and *N. caninum*.

2.3.2 Sensitivity of the SAM-LAMP SNP-based assay for the detection of *Cryptosporidium* species DNA

The analytical sensitivity of the serially diluted *C. parvum* detected by the SAM-LAMP SNP-based assay is shown in Figure 4. The concentrations tested for the SAM-LAMP SNP-based assay were positive in all cases, and the detection limit was found to be less than 800 fg/µl, which corresponds to 0.1 *Cryptosporidium* oocysts. Higher concentrations of *Cryptosporidium* DNA were also tested (data not shown) and were found to be positive.

2.3.3 Verification of the SNP-based SAM-LAMP assay in simulated and natural water samples in comparison with IFT and nPCR methods

To test the LAMP method for the detection of naturally occurring *Cryptosporidium* oocysts and to determine its applicability to samples with unknown content of oocysts, simulated water samples were tested with LAMP and PCR. Table 2 shows the results obtained from a total of 53 simulated environmental water samples and the comparison of the results with IFT and nPCR. Of the 53 simulated environmental water samples tested with the SNP-based LAMP method and the GP60 assay, 100% were positive, clearly demonstrating the advantage of the developed LAMP methods over nPCR. Both nPCR methods demonstrated significantly lower recovery efficiency in the simulated water samples (nPCR-1, 5% and nPCR-2, 13%) because inhibitors in such samples are common. The application of the same methods to the original samples showed that 50% were positive by the SNP-based LAMP method and that 11% were positive by the GP60-LAMP assay. Each of the nPCRs found only 7% positive samples.

There was a small discrepancy in the results of the nPCRs used on the simulated water samples. A simulated river water sample was found to be positive by nPCR-1, but it was negative by nPCR-2. A similar situation was seen in a simulated sewage water sample, which initially contained a large number of *Cryptosporidium* oocysts, but only 2 of the 3 examined aliquots yielded a positive result. There were also two sewage water samples in which one was positive by nPCR-1 but not by nPCR-2, and the opposite pattern was observed in a third sewage sample.

2.2.4 Application and comparison of the IFT, nPCR and LAMP assays in samples from different hosts and water samples from Thailand

2.2.4.1 Water samples

Table 3 shows the results obtained when water, mussel and fecal samples from different hosts and origins were tested with IFT and all of the molecular assays. In summary, of the 89 samples tested, 41 samples (46%) were positive by IFT and 15 samples (16%) and 19 samples (21%) were positive in the nPCR-1 and nPCR-2 assays, respectively. In total, 39 samples (43%) and 22 samples (24%) of the 89

analyzed samples were positive in the SNP-based SAM-LAMP and GP60 assays, respectively.

Of the 15 natural water samples, FITC staining indicated that all samples were *Cryptosporidium* positive, while 2 samples (13%) and 3 samples (20%) were *Cryptosporidium* positive using the nPCR-1 and nPCR-2 assays, respectively (Table 3). The detection rate by the GP60 assay was 46% positive when assaying 1 bore, 1 canal, 2 pond, 1 river and 2 well water samples. For samples examined with the SNP-based SAM-LAMP assay, 12 (2 bore, 1 canal, 3 pond, 2 reservoir, 1 river and 3 well water samples) yielded positive results, which corresponds to a detection rate of 80%.

Table 2. Application and verification of the IFT, nPCRs assays and LAMP methods in simulated and natural environmental water samples from Bulgaria (n=37) and Russia (n=16).

Sample origin	Sample number examined	IFT	SSU rRNA nPCR-1	SSU rRNA nPCR-2	SNP based SAM LAMP	GP60 LAMP
Simulated water samples from Bulgaria and Russia						
River water	23	8	2[a*]	2	23	23
Sewage water	7	6	-	1[b]	7	7
Tap water	14	9	-	3	14	14
Well water	4	1	1	2	4	4
Bottled water	4	1	-	-	4	4
Spring water	1	0	-	-	1	1
Total simulated samples	**53**	**25 (47%)**	**3 (5%)**	**7 (13%)**	**53 (100%)**	**53 (100%)**
Natural water samples from Sofia-Bulgaria						
River	23	8	1[a]	2	15	9
Sewage water	7	6	1	1[c]	5	2
Tap water	14	9	1	1	4	1
Bottled water	4	1	-	-	-	-
Well water	4	1	-	-	3	1
Spring	1	0	-	-	-	-
Total natural samples	**53**	**25 (47%)**	**3 (5%)**	**4 (7%)**	**27 (50%)**	**13 (24%)**

-: not detected; *: river sample named W14 originated from Russia was successfully sequenced.
a: another river water sample was found positive.
b: of one sample only 2 of the triplet were found positive.
c: a different sample was positive by the nPCR-2 assay.

Table 3. Comparative detection findings of *Cryptosporidium* by GP60-LAMP and SSU rRNA nPCR assays in water samples (n=15), mussel tissue samples (n=8) and fecal samples from various humans and animals (patients, n=44; animals n=22) from the Thailand Tsunami area.

Sample name (sample code microscopy)	Sample Name - origin	IFT	SSU rRNA nPCR-1	SSU rRNA nPCR-2	SNP based SAM LAMP	GP60 LAMP
	Bore water	2	-	1*	2	1
	Canal water	1	1*	1	1	1
	Pond water	6	1	1	3	2
	Reservoir water	2	-	0	2	0
	River water	1	-	0	1	1
	Well water	3	-	0	3	2
Subtotal	**15**	**15 (100%)**	**2 (13%)**	**3 (20%)**	**12 (80%)**	**7 (46%)**
N. n.	Mussel	+	+	-	+	+
BKK2 (8/02/07)	Mussel	+	+*	+	+	-
SP1 (25/11/06)	Mussel	+	+	+	+	-
SP2 (2/12/06)	Mussel	+	-	+	-	-
SP3 (14/01/07)	Mussel	+	-	-	-	-
SP4(4.1) (15/02/07)	Mussel	+	+	+	+	-
SP4(4.2) (15/02/07)	Mussel	+	-	-	-	-
SP5 (27/02/07)	Mussel	+	-	-	-	-
Total number of samples examined	**8**	**8 (100)**	**4 (50%)**	**4 (50%)**	**4 (50%)**	**1 (12%)**

Sample name (sample code microscopy)	Sample Name - origin	IFT	SSU rRNA nPCR-1	SSU rRNA nPCR-2	SNP based SAM LAMP	GP60 LAMP
G2	F-Patient	-	-	+	+	+
G3	F-Patient (16602)	-	+*	+	-	-
G4	F-Patient (-391)	-	-	-	+	+
G5	F-Patient (16637)	-	-	+	+	+
G6	F-Patient (L50 O HN 01482)	-	-	+	+	-
G7	F-Patient (16677 HN 85972/37)	-	-	+	+	-
68	F-Patient	-	-	-	-	-
G9	F-Patient (16745 HN 25576/48)	-	-	-	-	-
G10	F-Patient (16890 HN 74594/43)	-	-	-	-	+
63	F-Patient	-	-	+*	-	-
65	F-Patient	-	-	-	-	-
66	F-Patient	-	-	+	-	-
70	F-Patient	-	-	-	+	+
71	F-Patient	-	-	-	+	+
103	F-Patient	-	-	-	+	-
K13	F-Patient	-	-	-	+	-
K14	F-Patient	-	-	-	+	-
K15	F-Patient	-	-	-	+	-
A18P	F-Patient	+	-	-	-	-
Subotal	**44**	**1 (2%)**	**1 (2%)**	**7 (16%)**	**11 (25%)**	**6 (13%)**

Sample name (sample code microscopy)	Sample Name - origin	IFT	SSU rRNA nPCR-1	SSU rRNA nPCR-2	SNP based SAM LAMP	GP60 LAMP
A3G	F-Animal	+	+	-	-	-
A5G	F-Animal	+	+*	-	+	-
A23G	F-Animal	+	+*	+	+	+
A25G	F-Animal	+	+*	-	+	-
A2P	F-Animal	-	-	-	+	-
A4P	F-Animal	-	+*	+	+	+
A22P	F-Animal	+	+	+	+	-
B3G	F-Animal	+	-	-	-	+
B5G	F-Animal	+	-	-	+	+
B15G	F-Animal	+	-	-	-	+
B17G	F-Animal	+	-	-	+	+
B4P	F-Animal	+	-	-	-	+
B6P	F-Animal	+	+*	+*	-	-
B10P	F-Animal	+	-	-	+	+
B12P	F-Animal	+	+	+	-	-
B22P	F-Animal	+	-	-	-	-
A1G	F-Animal	+	-	-	-	-
B1G	F-Animal	+	-	-	+	-
C17P	F-Animal	-	-	-	+	-
A18P	F-Animal	+	-	-	+	-
Subtotal	**22**	**17 (77%)**	**8 (36%)**	**5 (22%)**	**12 (55%)**	**8 (36%)**
Total number of examined samples (%)	**89**	**41 (46%)**	**15 (16%)**	**19 (21%)**	**39 (43%)**	**22 (24%)**

F-Patient= Fecal material from HIV/AIDS patients; F-Animal=fecal material from animals; N. n.: not named; *Successfully sequenced samples.

2.2.4.2 Mussel samples

Regarding the mussel samples, both nPCRs detected the same number of positive samples, but the same result was confirmed in only 3 of the 4 positive samples (i.e., one mussel sample was found to be positive by nPCR-1 but not by nPCR-2 and another mussel sample was found to be negative by nPCR-1 but positive by nPCR-2). In summary, there were 3 mussel samples found to be positive by both nPCRs, and each assay detected an additional but different sample as positive. The SNP-based SAM-LAMP assay showed DNA amplification in two additional samples.

2.2.4.3 Human fecal samples

Of the 44 HIV/AIDS patient fecal samples, the lowest detection rate (2%) was found with the IFT and nPCR-1 assays, followed by the nPCR-2 and GP60 LAMP assays (16% and 13%, respectively), while the SNP-based LAMP assay demonstrated a positive LAMP signal in 11 samples (25%). The final group of samples analyzed included 22 animal fecal samples, which had the lowest detection rate with the nPCR assays and the GP60 LAMP assay. IFT detected 17 positive animal samples (77%), followed by the SNP-based SAM-LAMP assay, which indicated 12 samples as positive, corresponding to a detection rate of 55%.

2.2.4.4 Sequence and phylogenetic analysis

The phylogenetic relationships of the positive *Cryptosporidium* samples are shown in Figure 2. We used sequences of 21 different *Cryptosporidium* species downloaded from GenBank as reference sequences. Sequence analysis indicated that the most frequently observed species was *C. parvum*, which was identified by nPCR-1 in 7 of 8 samples. DNA sequencing of 4 animal fecal samples (A25G, A23G, A4P, A5G) indicated that that they were most closely related to *C. parvum* species, with similarity ranging from 96-100%. The HIV/AIDS patient fecal sample (G3) was also identified as *C. parvum* (96% similarity). However, the animal fecal sample (B6P) showed a similarity of 98% to *C. andersoni.* In the mussel tissue sample (DKK2), the genotype identified was *C. hominis* (99% similarity). *C. hominis* (92% similarity) was identified in the canal water sample (W4) from Thailand. One additional river sample (W14) from Russia was also successfully sequenced with a similarity of 97% to the *C. fragile* genotype. Other *Cryptosporidium* nPCR products demonstrated weak bands that could not be sequenced.

2.3 Discussion

The CDC included *Cryptosporidium* in the second highest priority biological agent category, which includes agents that are moderately easy to disseminate, result in moderate morbidity and low mortality rates in humans, and require specific enhancements of the CDC's diagnostic capacity and enhanced disease surveillance. The capacity of *Cryptosporidium* to infect human populations and cause death in immunocompromised hosts drives the demand for the development of a suitable and sensitive detection method for all human pathogenic species.

The microscopic identification of *Cryptosporidium* in both environmental and fecal material by conventional staining methods remains difficult and subjective to apply due to the debris, primeses and other possible microorganisms present in the collected crude samples. A number of molecular tests to detect parasitic protozoa (and specifically *Cryptosporidium*) have been designed and used, but no assay can equal the rapidity, specificity, and simplicity of the LAMP technique, which makes it appealing, as it is the most promising and robust assay when applied to difficult templates (Karanis et al., 2007b; Bakheit et al., 2008; Sotiriadou and Karanis, 2008; Karanis and Ongerth, 2009; Thekisoe et al., 2010).

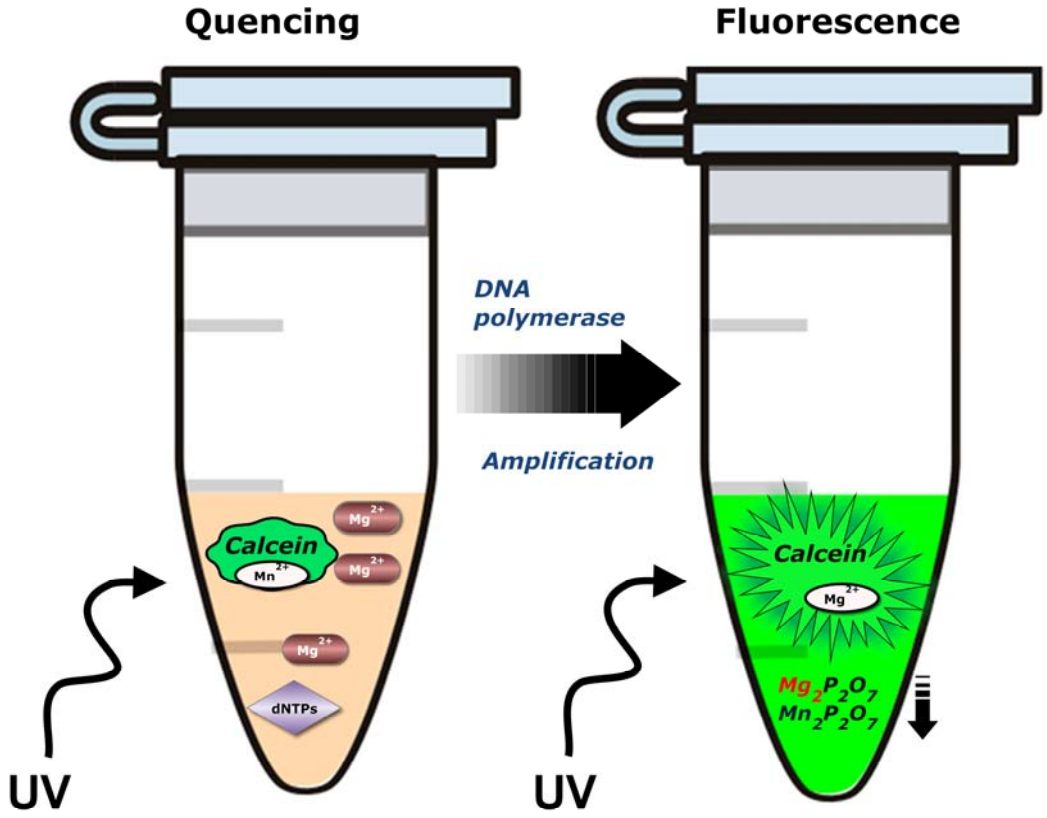
Recently, methods have been reported that incorporate the LAMP method for the detection of *Cryptosporidium* based on the amplification of the GP60 gene (Karanis et al., 2007b), HSP-70 gene (Bakheit et al., 2008) and part of the SAM gene. At this point, the significant differences between the previously developed *Cryptosporidium* LAMP assays focusing on the amplified genes should be noted. The GP60-LAMP assay was found to amplify only *C. parvum* DNA; the HPS-70 LAMP assay was found to amplify *C. andersoni*, *C. muris* and *C. serpentis*; and the SNP-based SAM-LAMP assay was able to specifically detect the human pathogenic species *C. parvum*, *C. hominis* and *C. meleagridis,* according to the analysis of the available *Cryptosporidium* sequences. Among the several types of genetic variation, SNPs are the most important and basic form of variation in the genome between members of a species. Numerous approaches to SNP genotyping have been developed, such as polymerase chain reaction−restriction fragment length polymorphism (PCR−RFLP) analysis and the TaqMan PCR method. In the present study, we tested only different *C. parvum* isolates, due to the absence of other *Cryptosporidium* spp. in our sample repository, while Bakheit et al. (2008) tested all 3 human pathogenic species using

the SNP-based LAMP assay. In the study by Bankheit et al. (2008), the SNP-based LAMP assay was applied with animal samples from South Africa, and the sensitivity and specificity were only briefly discussed. The same authors reported that 33.64% of cattle, 30.5% of sheep and 21.79% of horse samples examined were positive, and they also cloned and sequenced the LAMP products generated from the animal fecal samples.

In our study, the optimum SAM-LAMP SNP-based condition was found to be 63 ºC for 60 min, which was used for all assays performed in this study. We predict that the time to positivity of the SNP-based SAM-LAMP assay in samples with higher concentrations would be shorter. The reaction time was held constant for all the reactions tested in this study because the measured DNA concentrations of the animal fecal, environmental water and food samples varied significantly and other co-extracted DNA could influence the measured DNA concentrations.

Figures 3A and B show the difference in turbidity in the reaction tubes because of the formation of magnesium pyrophosphate, which can be observed with the naked eye or after addition of fluorescent dyes to positive and negative samples (Illustration 1). The negative sample showed no turbidity under exposure to UV light. The positive reaction could be detected visually unaided and under UV light exposure. The turbidity observed in the tubes is proportional to the amount of the amplified product produced because LAMP is able to synthesize an extremely large amount of DNA even from difficult DNA templates. Magnesium pyrophosphate, the characteristic by-product of the LAMP method, forms a white turbidity in the reaction tube, enabling detection by the naked eye, which makes it an excellent indicator of the presence of the target gene (Figure 3A and B and Illustration 1). Additionally, the fluorescent detection dyes added to the LAMP reaction mixture, which also react with the magnesium pyrophosphate, further simplified the detection by the naked eye or under UV light. Observation of the turbidity in positive reaction samples with or without fluorescent reagents/dyes was possible in all cases from reactions conducted with the laboratory thermal cycler.

Illustration 1. The formation of magnesium pyrophosphate, which can be observed with the naked eye or after addition of fluorescent dyes to positive and negative samples, enabling detection by the naked eye, which makes it an excellent indicator of the presence of the target gene.



The phylogenetic tree indicates that the sequences obtained in our study cluster mostly to *C. parvum.* Although the number of positive samples found with the nPCR assay was relatively low, these results clearly demonstrate the presence of different *Cryptosporidium* species in fecal samples from animals and humans and water samples of different origins. PCR amplification of the SSU rRNA gene was successful for 8 of 15 samples derived from Thailand. The following short literature review will help to illustrate the reasons for the development of the SNP-based SAM-LAMP technique and the significance of the sequencing results reported in this study, beginning with the most prevalent of the human pathogenic species of *Cryptosporidium*. The SAM amino acid sequence of *C. parvum* shares conserved and essential domains with homologs from other organisms and is phylogenetically related to other cytosolic eukaryotic and prokaryotic organisms, but it is distinct from

homologs in plants, fungi, ma0mmals and eubacteria (Slapeta, 2003). It is noteworthy that such phylogenetically related organisms include *Homo sapiens*, *Entamoeba, Giardia, Toxoplasma* and *Plasmodium*. SAM sequences of *Isospora*, *Cyclospora, Hammondia, Cystoisospora, Sarcocystis* and *Neospora* have not been deposited in the GenBank at this time, so for primer design, we chose the area representing an identity of 59%-86% with humans and other organisms.

C. andersoni infections have been reported mainly in cattle (Fiuza et al., 2011; Kváč et al., 2011; Wang et al., 2011); however, in the study by Leoni et al. (2006), only 3 (0.1%) *C. andersoni* infections in humans were detected by the amplification of the 18S rDNA gene, but these results were not confirmed upon testing for the *Cryptosporidium* oocyst wall protein. In the present report, we observed only one animal fecal sample infected by *C. andersoni*, and it was successfully sequenced by both nPCR assays.

C. fragile was first described in the stomachs of black-spined toads from the Malay Peninsula, which harbored *C. fragile* infection at the time of arrival of the frogs to Penisula (Jirku et al., 2008). In the present study, we identified only one sample that was positive for *C. fragile*, and this finding matches with the sampling site of the examined water sample from our previous publication (Karanis et al., 2006) in which the same sample was found to be positive for the presence of *Cryptosporidium* oocysts. The results indicate a correlation between the identified *Cryptosporidium* spp. found in the sample and the sample origin. The sample was collected from the Temernik River, which empties into the Don River and passes through the zoo; most of the zoo's sewage empties into it. It also passes through the botanic garden, the railway station and some other areas of the city of Rostov. The specific sample was taken from the river after it had passed through zoo at a point between the railway station and the bus station.

Only a few reports are available on the presence of *C. hominis* in shellfish, and the first reported presence of *C. hominis*/*C. parvum* genotype 1 was in sediment derived from filter-feeding mollusks from different European countries (Gómez-Couso et al., 2004). Molini et al. (2007) evaluated the temporal occurrence of *Cryptosporidium* oocysts in clams bred along the northeastern Italian Adriatic coast and showed that 1 of 36 pools was contaminated with *C. hominis.* A study was carried out in Thailand that investigated the occurrence of *Cryptosporidium* oocysts in green mussels in areas used for seafood harvesting and consumption because of its public health

significance. Of the samples examined, 12.5% were found to be positive by the IFT assay (Srisuphanunt et al., 2009). In the present study, we confirmed the presence of *C. hominis* in one mussel sample, and this result was verified by the SNP-based LAMP method. Despite the small number of reported cases of *C. hominis* in mussels, it should not be exempted from continuous vigilance by the authorities because mussels accumulate diverse agents, including viruses, bacteria and different protozoa, and organic and inorganic substances such as heavy metals that are contained in the water where they grow or filter.

The ability of the developed SNP-based method used in this study to detect human pathogenic *Cryptosporidium* species was evaluated by testing known reference isolates of three known *Cryptosporidium* species, and the method was consequently applied to define the detection limit of the developed SAM-LAMP assay.

In conclusion, the present study has demonstrated a specific, sensitive and rapid LAMP assay for the detection of human pathogenic species of *Cryptosporidium* that has been validated by its application to clinical and environmental materials. The additional advantage of the method is that the results can be easily observed immediately after the reaction with the use of fluorescent detection dyes in the reaction tubes. In this study, we show that the stand-alone application of the LAMP assay has the potential to become a rapid diagnostic tool for the control and prevention of disease and to determine the presence of the important anthroponotic species of *Cryptosporidium*. The application of the LAMP technique, coupled with the PCR-sequencing approach, would result in the rapid detection and differentiation of species of public health significance and the identification of the sources of contamination.

2.4 References

Bakheit MA, Torra D, Palomino LA, Thekisoe OM, Mbati PA, Ongerth J, Karanis P (2008) Sensitive and specific detection of *Cryptosporidium* species in PCR-negative samples by loop-mediated isothermal DNA amplification and confirmation of generated LAMP products by sequencing. *Vet Parasitol* 158: 11-22.

Baldursson S, Karanis P (2011) Waterborne transmission of protozoan parasites: review of worldwide outbreaks - an update 2004-2010. *Water Res* 45: 6603-6614.

Blasi MF, Carere M, Pompa MG, Rizzuto E, Funari E (2008) Water-related diseases outbreaks reported in Italy. *J Water Health* 6: 423-432.

Bruce BB, Blass MA, Blumberg HM, Lennox JL, del Rio C, Horsburgh CR Jr (2000) Risk of *Cryptosporidium parvum* transmission between hospital roommates. *Clin Infect Dis* 31: 947-950.

Cama VA, Ross JM, Crawford S, Kawai V, Chavez-Valdez R, Vargas D, Vivar A, Ticona E, Navincopa M, Williamson J, Ortega Y, Gilman RH, Bern C, Xiao L (2007) Differences in clinical manifestations among *Cryptosporidium* species and subtypes in HIV-infected persons. *J Infect Dis* 196: 684-691.

Chalmers RM, Elwin K, Thomas AL, Joynson DH (2002) Infection with unusual types of *Cryptosporidium* is not restricted to immunocompromised patients. *J Infect Dis* 185: 270-271.

Dale K, Kirk M, Sinclair M, Hall R, Leder K (2010) Reported waterborne outbreaks of gastrointestinal disease in Australia are predominantly associated with recreational exposure. *Aust N Z J Public Health* 34: 527-530.

DuPont HL, Chappell CL, Sterling CR, Okhuysen PC, Rose JB, Jakubowski W (1995) The infectivity of *Cryptosporidium parvum* in healthy volunteers. *N Engl J Med* 332: 855-859.

Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39: 783-791.

Fiuza VR, Almeida AJ, Frazão-Teixeira E, Santín M, Fayer R, Oliveira FC (2011) Occurrence of *Cryptosporidium andersoni* in Brazilian cattle. *J Parasitol* 97: 952-953. Hadfield SJ, Chalmers RM (2012) Detection and characterization of

Cryptosporidium cuniculus by real-time PCR. Parasitol ResDOI: 10.1007/s00436-012-2874-1.

Gómez-Couso H, Freire-Santos F, Amar CF, Grant KA, Williamson K, Ares-Mazás ME, McLauchlin J (2004) Detection of *Cryptosporidium* and *Giardia* in molluscan shellfish by multiplexed nested-PCR. *Int J Food Microbiol*. 91: 279-88.

Inomata A, Kishida N, Momoda T, Akiba M, Izumiyama S, Yagita K, Endo T (2009) Development and evaluation of a reverse transcription-loop-mediated isothermal amplification assay for rapid and high-sensitive detection of *Cryptosporidium* in water samples. *Water Sci Technol* 60: 2167-2172.

Jirku M, Valigurova A, Koudela B, Krizek J, Modry D, Slapeta J (2008) New species of *Cryptosporidium* Tyzzer, 1907 (Apicomplexa) from amphibian host: morphology, biology and phylogeny. *Folia Parasitol (Praha)* 55: 81-94.

Karanis P, Ey PL (1998) Characterization of axenic isolates of *Giardia intestinalis* established from humans and animals in Germany. *Parasitol Res* 84: 442-449.

Karanis P, Sotiriadou I, Kartashev V, Kourenti C, Tsvetkova N, Stojanova K (2006) Occurrence of *Giardia* and *Cryptosporidium* in water supplies of Russia and Bulgaria. *Environ Res* 102: 260-271.

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

Karanis P, Thekisoe O, Kiouptsi K, Ongerth J, Igarashi I, Inoue N (2007b) Development and preliminary evaluation of a loop-mediated isothermal amplification procedure for sensitive detection of *Cryptosporidium* oocysts in fecal and water samples. *Appl Environ Microbiol* 73: 5660-5662.

Karanis P, Ongerth J (2009) LAMP--a powerful and flexible tool for monitoring microbial pathogens. *Trends Parasitol* 25: 498-499.

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J Molecul Evol* 16: 111-120.

Korich D, Mead J, Madore M, Sinclair N, Sterling C (1990) Effects of ozone, chlorine dioxide, chlorine and monochloramine on *Cryptosporidium parvum* viability. *Appl Environ Microbiol* 56: 1423–1428.

Kourenti C, Heckeroth A, Tenter A, Karanis P (2003) Development and application of different methods for the detection of *Toxoplasma gondii* in water. *Appl Environ Microbiol* 69: 102-106.

Kváč M, Hromadová N, Květoňová D, Rost M, Sak B (2011) Molecular characterization of *Cryptosporidium* spp. in pre-weaned dairy calves in the Czech Republic: absence of *C. ryanae* and management-associated distribution of *C. andersoni*, C. bovis and C. parvum subtypes. *Vet Parasitol* 177: 378-382.

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

Lim YA, Iqbal A, Surin J, Sim BL, Jex AR, Nolan MJ, Smith HV, Gasser RB (2011) First genetic classification of *Cryptosporidium* and *Giardia* from HIV/AIDS patients in Malaysia. *Infect Genet Evol* 11: 968-974.

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

Molini U, Traversa D, Ceschia G, Iorio R, Boffo L, Zentilin A, Capelli G, Giangaspero A (2007) Temporal occurrence of *Cryptosporidium* in the Manila clam *Ruditapes philippinarum* in northern Adriatic Italian lagoons. *J Food Prot* 70: 494-499.

Monis PT, Thompson RC (2003) *Cryptosporidium* and *Giardia*-zoonoses: fact or fiction?, *Infect Genet Evol* 3: 233–244.

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

Mori Y, Nagamine K, Tomita N, Notomi T (2001) Detection of loop mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochem Biophys Res Commun* 289: 150-154.

Mori Y, Notomi T (2009) Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. J *Infect Chemother* 15: 62-69.

Nagamine K, Hase T, Notomi T (2002) Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Mol Cell Probes* 16: 223-229.

Nahrevanian H, Assmar M (2008) Cryptosporidiosis in immunocompromised patients in the Islamic Republic of Iran. *J Microbiol Immunol Infect* 41: 74-77.

Ng J, Yang R, McCarthy S, Gordon C, Hijjawi N, Ryan U (2011) Molecular characterization of *Cryptosporidium* and *Giardia* in pre-weaned calves in Western Australia and New South Wales. *Vet Parasitol* 176: 145-150.

Nichols RA, Campbell BM, Smith HV (2003) Identification of *Cryptosporidium* spp. oocysts in United Kingdom noncarbonated natural mineral waters and drinking waters by using a modified nested PCR-restriction fragment length polymorphism assay. *Appl. Environ. Microbiol* 69: 4183-4189.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* 28: E63.

Pereira JT, Costa AO, de Oliveira Silva MB, Schuchard W, Osaki SC, de Castro EA, Paulino RC, Soccol VT (2008) Comparing the efficacy of chlorine, chlorine dioxide, and ozone in the inactivation of *Cryptosporidium parvum* in water from Parana State, Southern Brazil. *Appl Biochem Biotechnol* 151: 464-73.

Plutzer J, Karanis P, (2007a) Genotype and subtype analyses of *Cryptosporidium* isolates from cattle in Hungary. *Vet Parasitol* 146; 357-362.

Plutzer J, Karanis P (2007b) Molecular identification of a *Cryptosporidium saurophilum* from corn snake (*Elaphe guttata guttata*). *Parasitol Res* 101: 1141-1145.

Plutzer J, Takó MH, Márialigeti K, Törökné A, Karanis P (2007) First investigations into the prevalence of *Cryptosporidium* and *Giardia* spp. in Hungarian drinking water. *J Water Health* 5: 573-584.

Plutzer J, Karanis P (2009a) Genetic polymorphism in *Cryptosporidium* species: an update. *Vet Parasitol* 165: 187-99.

Plutzer J, Karanis P (2009b) Rapid identification of *Giardia duodenalis* by loop-mediated isothermal amplification (LAMP) from faecal and environmental samples and comparative findings by PCR and real-time PCR methods. *Parasitol Res* 104: 1527-33.

Plutzer J, Törökné A, Karanis P (2010) Combination of ARAD microfibre filtration and LAMP methodology for simple, rapid and cost-effective detection of human pathogenic *Giardia duodenalis* and *Cryptosporidium* spp. in drinking water. *Lett Appl Microbiol* 50: 82-88.

Pöschl B, Waneesorn J, Thekisoe O, Chutipongvivate S, Karanis P (2010) Comparative diagnosis of malaria infections by microscopy, nested PCR, and LAMP in northern Thailand. *Am J Trop Med Hyg* 83: 56-60.

Rimšelienė G, Vold L, Robertson L, Nelke C, Soli K, Johansen OH, Thrana FS, Nygård K (2011) An outbreak of gastroenteritis among schoolchildren staying in a wildlife reserve: thorough investigation reveals Norway's largest cryptosporidiosis outbreak. *Scand J Public Health* 39: 287-295.

Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecul Biol Evolution 4: 406-425.

Schuster CJ, Ellis AG, Robertson WJ, Charron DF, Aramini JJ, Marshall BJ, Medeiros DT (2005) Infectious disease outbreaks related to drinking water in Canada, 1974-2001. *Can J Public Health* 96: 254-258.

Serdarevic F, Jones RC, Weaver KN, Black SR, Ritger KA, Guichard F, Dombroski P, Emanuel BP, Miller L, Gerber SI (2011) Multi-pathogen waterborne disease outbreak associated with a dinner cruise on Lake Michigan. *Epidemiol Infect* 19: 1-5.

Shields JM, Hill VR, Arrowood MJ, Beach MJ (2008) Inactivation of *Cryptosporidium parvum* under chlorinated recreational water conditions. *J Water Health* 6: 513-520.

Slapeta, J., Stejskal, F., Keithly, J.S (2003) Characterization of S-adenosylmethionine synthetase in *Cryptosporidium parvum* (Apicomplexa). *FEMS Microbiol. Lett* 225: 271-277.

Smith HV, Cacciò SM, Cook N, Nichols RA, Tait A (2007) *Cryptosporidium* and *Giardia* as foodborne zoonoses. *Vet. Parasitol* 149: 29-40.

Sotiriadou I, Karanis P (2008) Evaluation of loop-mediated isothermal amplification for detection of *Toxoplasma gondii* in water samples and comparative findings by polymerase chain reaction and immunofluorescence test (IFT). *Diagn*

Srisuphanunt M, Karanis P, Charoenca N, Boonkhao N, Ongerth JE (2010) *Cryptosporidium* and *Giardia* detection in environmental waters of southwest coastal areas of Thailand. *Parasitol Res* 106: 1299-1306.

Srisuphanunt M, Saksirisampant W, Karanis P (2011) Prevalence and genotyping of *Cryptosporidium* isolated from HIV/AIDS patients in urban areas of Thailand. *Ann Trop Med Parasitol* 105: 463-468.

Srisuphanunt M, Wiwanitkit V, Saksirisampant W, Karanis P (2009) Detection of *Cryptosporidium* oocysts in green mussels (Perna viridis) from shell-fish markets of Thailand. *Parasite* 16: 235-239.

Strong WB, Gut J, Nelson RG (2000) Cloning and sequence analysis of a highly polymorphic *Cryptosporidium parvum* gene encoding a 60-kilodalton glycoprotein and characterization of its 15- and 45-kilodalton zoite surface antigen products. *Infect Immun* 68: 4117-4134.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28: 2731-2739.

Thekisoe OM, Rodriguez CV, Rivas F, Coronel-Servian AM, Fukumoto S, Sugimoto C, Kawazu S, Inoue N (2010) Detection of *Trypanosoma cruzi* and T. rangeli infections from Rhodnius pallescens bugs by loop-mediated isothermal amplification (LAMP). Am J Trop Med Hyg 82: 855-60.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22: 4673-4680.

Tomita N, Mori Y, Kanda H, Notomi T (2008) Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nat Protoc. 2008;3(5):877-82.

Wang R, Ma G, Zhao J, Lu Q, Wang H, Zhang L, Jian F, Ning C, Xiao L (2011) *Cryptosporidium andersoni* is the predominant species in post-weaned and adult dairy cattle in China. Parasitol Int 60: 1-4.

Xiao L, Escalante L, Yang C, Sulaiman I, Escalante AA, Montali RJ, Fayer R, Lal AA (1999) Phylogenetic analysis of *Cryptosporidium* parasites based on the small-

Xiao L, Limor J, Morgan UM, Sulaiman I, Thompson RCA, Lal AA (2000) Sequence differences in the diagnostic target region of the oocyst wall protein gene of *Cryptosporidium* parasites. *Appl Environ Microbiol* 66: 5499–5502.

Xiao L, Singh A, Limor J, Graczyk TK, Gradus S, Lal AA (2001) Molecular characterization of *Cryptosporidium* oocysts in samples of raw surface water and wastewater. *Appl Environ Microbiol* 67: 1097-1101.

Xu J, Rong R, Zhang HQ, Shi CJ, Zhu XQ, Xia CM (2010) Sensitive and rapid detection of *Schistosoma japonicum* DNA by loop-mediated isothermal amplification (LAMP). Int J Parasitol 40: 327-331.

Yoshida H, Matsuo M, Miyoshi T, Uchino K, Nakaguchi H, Fukumoto T, Teranaka Y, Tanaka T (2007) An outbreak of cryptosporidiosis suspected to be related to contaminated food, October 2006, Sakai City, Japan. *Jpn J Infect Dis* 60: 405-407.

3. Evaluation of LAMP for detection of *Toxoplasma gondii* in water samples and comparative findings by PCR and IFT

Abstract

The development and evaluation of an one-step, single-tube accelerated loop-mediated isothermal amplification (LAMP) assay for the rapid detection of *Toxoplasma* in water samples is described. The method has been evaluated based on the amplification of B1 and *Tg*OWP *Toxoplasma* genes, and demonstrated a sensitivity detection limit of 0.1 tachyzoites' DNA for both genes. LAMP detection was evaluated and compared with nested PCR in 26 water samples pellets spiked with known numbers of *Toxoplasma* oocysts. After DNA extraction the detection sensitivity in spiked pellets was 100% by LAMP and 53.8% by PCR. Subsequently, fifty-two natural water samples of different origin were directly investigated by three assays LAMP, PCR and IFT. 25 out of 52 (48%) have been found positive for *Toxoplasma*-DNA by LAMP, while nested PCR products were generated in 7 out of 52 (13.5%) water samples. All 52 water samples were negative for *Toxoplasma* by IFT. This data clearly indicate LAMP as a rapid, specific and sensitive tool for the detection of *Toxoplasma* contamination in water samples.

3.1 Introduction

Infections by the protozoan parasite *Toxoplasma gondii* are prevalent worldwide in both animals and humans. The final host of *T. gondii* is the cat, but it can also be carried by the vast majority of warm blooded animals and humans. Toxoplasmosis can have serious, sometimes even fatal effects on a foetus, whose mother has first contact with the disease during pregnancy, or on an immunocompromised human or cat (Abbasi et al., 2003; Afonso et al., 2006; Kasper and Buzoni-Gatel, 1998; Pradhan et al., 2007). Seroprevalence studies are most widely used for the detection of *Toxoplasma* infections from humans and animals. The seroprevalence of *T. gondii* infection among the European population ranges between 12% (Norway, Sweden) and $>$ 50% (France, Belgium, Switzerland). Regional variation has been attributed to climate (Jenum et al., 1998), cultural differences in the amount and type of raw meat consumed (Dupouy-Camet et al., 1993), and the increased consumption of meat from animals farmed indoors and frozen meat (Gilbert et al., 1993). According to a European multi-center case-control study, eating undercooked, raw or cured meat contributed to between 30% and 63% of infections, while soil contact contributed to up to 17% of infections. In contrast, contact with cats was not a risk factor for infection (Cook et al., 2000).

The waterborne transmission of *T. gondii* to humans through oocyst-contaminated water and its epidemiological impact is now considered to be more significant than previously believed (Benenson et al., 1982; Aramini et al., 1998; 1999; Bahia-Oliveira et al., 2003). The toxoplasmosis outbreaks in humans of a western Canadian city (Bowie et al., 1997; Burnett et al., 1998), in Panama (Benenson et al., 1982) and in Atlanta (Dubey et al., 1981) at different time points were linked epidemiologically to oocyst contamination of a municipal water supply. Even *Toxoplasma* oocysts could not be identified in the municipal reservoir runoff from soil contaminated with faeces of infected domestic cats or cougars were considered the likely source (Aramini et al., 1998; 1999; Isaac-Renton et al., 1998). In Brazil, approximately 60% of 6–8-year-old children were found to have antibodies to *T. gondii* linked to the ingestion of oocysts in a heavily contaminated environment with *T. gondii* oocysts. Epidemiological evidence indicated that drinking water contaminated with *Toxoplasma* oocysts was the primary source of infection (Bahia-Oliveira et al., 2003). Felids are the only known hosts that can excrete environmentally resistant oocysts, which are able to excrete millions of

oocysts, and the prevalence of *T. gondii* in feral cats in USA is very high (Dubey et al., 2002a; 2002b). Deaths of marine mammals, including sea otters, have been associated with toxoplasmosis, which has been considered to be the cause of death in marine vertebrates (Miller et al., 2002a; 2002b).

Few research articles have described protocols for the detection of *Toxoplasma* in water (Dumetre et al., 2003; Kourenti et al., 2003; Kourenti and Karanis, 2006; Villena et al., 2004). These investigations forced research on water analysis for *Toxoplasma* detection, however, the main obstacle still is the absence of an effective method for the water detection. This is not only due to *Toxoplasma* epidemiology and distribution of oocysts in nature, but also due to the general limitations of the methodology for the detection of waterborne protozoan (Zarlenga and Trout, 2004; Weintraub, 2006).

A Loop-Mediated Isothermal Amplification (LAMP) is a newly introduced technique which amplifies target nucleic acids with high sensitivity, specificity, efficiency, and rapidity under isothermal conditions. It has been already evaluated for the detection of *Cryptosporidium parvum* oocysts (Karanis et al., 2007) and in other areas in parasitology including the diagnosis of trypanosomosis (Kuboki et al., 2003; Thekisoe et al., 2005; Njiru et al., 2008a; 2008b) and piroplasmosis (Ikadai et al., 2004; Alhassan et al., 2007). LAMP was firstly developed by Notomi et al. (2000) and the method employs a DNA polymerase called *Bst* polymerase with displacement activity and a set of four specially designed primers which recognize a total of six or eight distinct sequences of the target DNA (Negamine et al., 2002). One of the characteristics of the LAMP method is its ability to synthesize an extremely large amount of DNA even in difficult DNA templates. The sensitivity of the technique is not significantly influenced by the presence of non target DNA.

In the present article we report the evaluation of a LAMP specific protocol based on 2 *Toxoplasma* specific genes for the detection of *Toxoplasma* DNA under isothermal conditions in bench scale experiments using spiked water samples and direct application of the developed assay in environmental samples. The detection strategy includes comparison with nested PCR and IFT assays.

3.2 Materials and methods

3.2.1 Sources of DNA material for PCR and LAMP evaluation experiments; DNA templates selection for sensitivity and specificity evaluation assays

We used a DNA template obtained from *Toxoplasma* tachyzoites (strains RH and PLK), National Research Center Protozoan Diseases, Obihiro University for Agriculture and Veterinary Medicine, Japan) as well as DNA from oocysts of a *Toxoplasma* non-virulent strain (AHC1). DNA derived from *Giardia lamblia, Cryptosporidium parvum, Neospora caninum, Trypanosoma brucei* and *Babesia gibsoni* has been used to prove the specificity of *Toxoplasma* LAMP assay.

3.2.2 Target genes and primers design for the detection of *Toxoplasma* DNA

For the realisation of our LAMP assay, we utilised the complete sequence deposited in the GenBank under the accession number AF179871. BLAST screening of the *T. gondii* genome sequence database resulted in identification of a gene encoding at least one COWP homolog (*Tg*OWP1), and this multiexon sequence information was used to isolate a full-length cDNA. Essential sequence analysis by BLAST and exhaustive screening of the database showed that both the B1 gene (AF179871; 2.214 bp) and the *Tg*OWP gene (AY465428; 1.500 bp) are selective genes.

LAMP assay requires a set of four specific designed primers (B3, F3, BIP, and FIP) which recognize a total of six distinct sequences (B1, B2c, B3, F1c, F2, and F3) in the target DNA. Primers were designed against the *Toxoplasma* B1 and *Tg*OWP genes by using Primer Explorer V2 Software. The selected gene's name, the length and the sequence of each primer are shown in Table 2 and Figure 1.

Table 1. Primer sets used for *Toxoplasma* DNA amplification in LAMP assays based on the B1 and *Tg*OWP genes.

LAMP Assay	Primer Type	Sequence (5'→ 3')	Primer length (nt[1])	Amplicon size[2]	Target
Toxo B1	BIP (B1-B2c)	TCGCAACGGAGTTCTTCCCAGTTTTGGCCTGATATTACGACGGAC-	45	212	*Toxoplasma gondii* B1 gene GenBank Accession Number AF179871
	FIP (F1c-F2)	TGACGCCTTTAGCACATCTGGTTTTTGATGCTCAAAGTCGACCGC	45		
	B3	CAGACAGCGAACAGAACAGA	20		
	F3	GGGAGCAAGAGTTGGGACTA	20		
*Tg*OWP	BIP (B1-B2c)	TGTCCTCAAGGATTCGCCTTCGTTTTAGACTCTAGGCGGCACTG	44	236	*Toxoplasma gondii* putative oocyst wall protein COWP GenBank Accession Number AY465428
	FIP (F1c-F2)	CGTAGCCTCGTGGACAGCTGTTTGCAAGGGATGTGTGACCG	42		
	B3	TCCGTTGTCTTGTCGTTTGT	20		
	F3	CCCCCTAAGAGCAAGCAC	18		

[1]nt: nucleotides, [2]The length between F3 and B3.

3.2.3 Cultivation of *Toxoplasma* tachyzoites

Toxoplasma RH strain was maintained in monkey kidney *VERO* cells (T25) in tissue culture flasks according to Evans et al. (1999). Tachyzoites were removed from their feeder cell culture with a scraper and harvested by passing a needle through three times. The suspension (containing *Toxoplasma* tachyzoites and host cell debris) was filtrated through Millex SV filters (pore size 5 µm, Millipore) and only pure tachyzoites suspended in pre-warmed, sterile phosphate buffered saline (PBS) and washed twice by centrifugation at 1.500 × *g* for 10 min. The supernatant was discarded and a final 1 ml pellet was stored at -20°C for later use of DNA extraction (see below).

3.2.4 DNA extraction from *Toxoplasma* tachyzoites (culture) and oocysts (stock)

Toxoplasma DNA from tachyzoites and from oocysts was extracted with conventional phenol/chloroform extraction as described previously by Kourenti and Karanis (2004; 2006). In addition DNA has been extracted from in vitro cultivated RH *Toxoplasma* tachyzoites used in a serial limited dilution method (LDM) from $1x10^4$ to $1x10^{-1}$ tachyzoites.

3.2.5 Reaction mix for LAMP detection of the DNA

The LAMP reaction was conducted according to the original reports described by Notomi et al. (2000) and as applied in previous work by Karanis et al. (2007).

LAMP was carried out in a total 25 µL reaction mixture. 0.9 µL primer mix containing 40 pmol FIP and BIP each, 20 pmol F3 and B3 each, 12.5 µL reaction buffer (1.6 mol betaine, 40 mmol Tris–HCl (pH 8.8), 20 mmol KCl, 20 mM $(NH_4)_2SO_4$, 2.8 mmol each dNTP, 0.2% Tween 20, 16 mmol $MgSO_4$), 1 µL (8 U) of *Bst* DNA polymerase (Eiken Chemicals Co, Japan), 1 µL fluorescent detection dye, 2 µL of DNA and 7.6 µL distilled water. The mixture was incubated at 65°C for 1 h and heated at 80°C for 5 min to complete the reaction. Both B1 and *Tg*OWP genes demonstrated a 210 bp ladder-like LAMP amplification product.

3.2.6 PCR specificity of LAMP outer primers (F3 and B3)

To confirm the specificity of the LAMP assay for *Toxoplasma* detection, PCR method was performed with two outer primer sets. The primer sets 1 and 2 were B3 and F3, as used in the LAMP assay for the detection of *Toxoplasma* B1 gene and *Tg*OWP (Tables 1 and 2). PCR amplification was performed in 25 µL reaction mixtures containing 5 µL PCR buffer (10 x), 5 µL (200 µmol concentration) of each dNTP, 4 µL (200 nmol concentration) of each primer B3 and F3, and 0.25 µL (2.5 U) of AmpliTaq Gold polymerase (Applied Biosystems). Initial denaturation was done at 95°C for 10 min, followed by 35 cycles of denaturation (45 sec at 95°C), annealing (1 min at 60°C), and extension (90 sec at 72°C). Both B1 and *Tg*OWP genes demonstrated the expected 210 bp amplification product.

Table 2. It demonstrates the evaluation results of LAMP and PCR specificity for the detection of *Toxoplasma* B1 and *Tg*OWP genes. The names of parasites' DNA are listed.

Parasite strains	**PCR**		**LAMP**	
	B1	***Tg*OWP**	**B1**	***Tg*OWP**
Toxoplasma RH (tachyzoites)	+	+	+	+
Toxoplasma PLK (tachyzoites)	+	+	+	+
Toxoplasma AHC1 (oocysts)	+	+	+	+
Giardia lamblia	-	-	-	-
Cryptosporidium parvum	-	-	-	-
Neospora caninum	-	-	-	-
Trypanosoma brucei	-	-	-	-
Babesia gibsoni	-	-	-	-
Negative control (dH_2O)	-	-	-	-

+ = positive result; - = negative result.

3.2.7 Electrophoresis of LAMP and PCR amplified products

Five-microliter aliquots of LAMP and 10-µL aliquots of the PCR products were subjected to electrophoresis on a 1.5% agarose gel (1 × Tris-Acetate-EDTA [TAE]) and visualized under ultraviolet (UV) light after 10 min of ethidium bromide staining.

Along with the samples, a 100-bp DNA ladder (Promega, Mannheim, Germany; G3161) was used as a pattern for comparing molecular weight. To avoid contamination between samples, we used different laboratories for DNA extraction, and the LAMP setup and all pipette tips had filters for aerosol protection.

3.2.8 Direct sequencing

Purification of the PCR products for *Tg*OWP and B1 sequence analysis was performed by the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany, 28104) using the microcentrifuge protocol according to the manufacturer's instructions. PCR products were sequenced on an ABI Prism 3100 (Applied Biosystems, Japan) Genetic Analyzer with a Big Dye Terminator V.3.1 cycle sequencing kit (Applied Biosystems, Japan, 4337454). The accuracy of the data was confirmed by two directional sequencing cycles with the forward and reverse primers (F3 and B3).

3.2.9 Nested PCR based on the 18S-rRNA gene for the detection of *Toxoplasma* DNA in spiked water pellets and in environmental water samples

18S-rRNA nested PCR was performed as described by Kourenti and Karanis (2004) after DNA extraction from spiked water final pellets (see 2.11.1) and from environmental water samples (Karanis et al. 2006, see 2.11.2).

3.2.10 Environmental water samples

Fifty-two environmental water samples, originating from different sources and countries have been included in the study and they have been primary concentrated as previously described by Karanis et al. (2006). Briefly: defined water volumes were collected and flocculated by $Al_2(SO_4)_3$. After settlement the samples were incubated with the lysis buffer and washed twice with distilled water. The samples were subjected to discontinuous Sheather's sugar gradient solution and the final pellets were transferred to Eppendorf tubes and stored at 4^oC until use for LAMP, PCR and IFT detection.

3.2.11 Application of LAMP and PCR assays in water samples collected from various sources

3.2.11.1 Application of the LAMP and nested PCR in spiked water pellets

Twenty six environmental water sample pellets have been chosen randomly from our stock samples (Karanis et al., 2006). They were derived from different areas with variable quality (Table 3, Figure 1). *Toxoplasma* oocysts were counted by haematocytometer and 10 oocysts were added in 10% aliquots of the concentrated sample pellets. After DNA extraction (see sections 2.3 and 2.4), LAMP (see section 2.5) and nested PCR (Kourenti and Karanis, 2004) assays have been applied for all 26 spiked sediments (Table 4).

3.2.11.2 Direct application of the LAMP and nested PCR in natural water sediments

The investigated environmental samples included: 26 river waters, 3 mineral, 1 spring, 3 lakes, 3 well, 9 tap and 7 sewage water samples from different geographic areas, 52 samples altogether. All samples have been subjected to LAMP (see section 2.5) detection and comparative detection by PCR (Kourenti and Karanis, 2004). Investigated aliquots are representing 10% of the final sample pellets from which DNA has been extracted. After DNA extraction the LAMP assay based on the B1 gene has been applied to all samples listed in Table 3. The PCR products were analyzed by 1.6% agarose gel electrophoresis and visualized after ethidium bromide staining.

3.2.12 Statistical methods

In order to test for a difference in the detection rates between LAMP and PCR, McNemar's test was applied (Armitage and Berry; 1994). The test was two-sided and exact probabilities were calculated.

Table 3. Demonstration of results obtained by LAMP assay based of the *Toxoplasma* B1 gene and by nested PCR of the 18S-rRNA gene in 16 environmental water samples derived from Rostov area and in 10 natural water samples from Sofia greater area in 10 L of collected water volumes.

Water quality	**LAMP-B1 assay Positive/examined (spiked samples)**	**Nested PCR Positive/examined (spiked samples)**
Origin of samples: Rostov greater area		
River water	14/14	8/14
Spring water	1/1	0/1
Lake water	1/1	1/1
Subtotal	**16/16**	**9/16**
Origin of samples: Sofia greater area		
River water	2/2	0/2
Mineral	1/1	0/1
Sewage	3/3	2/3
Well	1/1	1/1
Tap	3/3	2/3
Subtotal	**10/10**	**5/10**
Total (%)	**26/26 (100%)**	**14/26 (53.8%)**

3.2.13 IFT assay on environmental water samples for the detection of *Toxoplasma* oocysts

The IFT diagnosis for *Toxoplasma* oocysts has been established for our purposes according to Dumetre and Darde (2005) with slight modifications. The samples have been examined by 40x and 100x magnification under an epifluorescence microscope (ZEISS, Jena, Axioplan 2), filter set 09 (excitation: BP 450-490, beam splitter: FT 510, emission: LP 515). We also examined the glass slides with a filter set 1 (excitation: BP 365/12, beam splitter: FT 395, emission: LP 397) and with DIC microscopy using Nomarski polarization optics. The IFT assay as described above has been subsequently applied in 52 environmental water samples to detect *Toxoplasma* oocysts.

Figure 1. LAMP amplification of the B1 *Toxoplasma* gene in the aliquots of 10 environmental sample pellets contained 10 *Toxoplasma* oocysts. The amplification of LAMP products shows a ladder-like pattern. Lane 1, tap water; lane 2, well water; lanes 3 and 4, tap water; lanes 5 and 6, river water; lane 7, mineral water; lanes 8 to 10, sewage water (first sedimentation entry, first stage exit, exit station, respectively); P, positive control contained DNA from *Toxoplasma* oocysts (PLK strain); lane N, negative control (DDW); lane M, 100-bp DNA ladder.

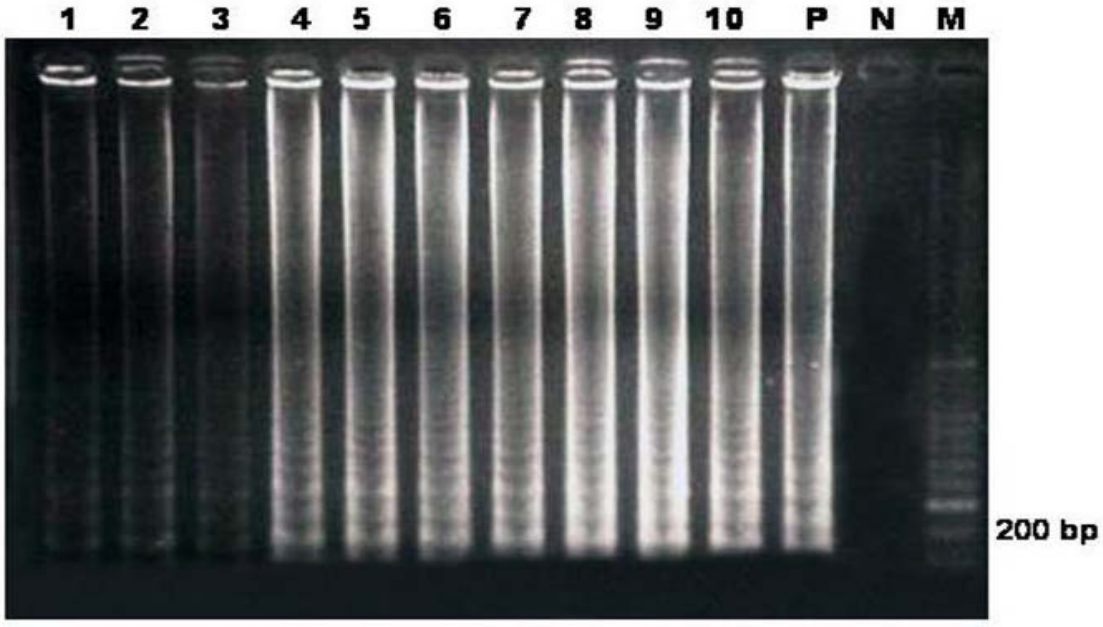


Table 4. Demonstration of results obtained by IFT, by LAMP assay based on the *Toxoplasma* B1 gene, and by nested PCR of the 18S-rRNA gene in 16 environmental water samples derived from Rostov area and in 36 natural water samples from Sofia greater area in 10 L of collected water volumes.

Water quality	Number of positive/examinned by IFT	LAMP-B1 assay Positive/examined (natural samples)	Nested PCR Positive/examined (natural samples)
Origin of samples: Rostov greater area			
River water	0/14	8/14	2/14
Spring water	0/1	1/1	0/1
Lake water	0/1	0/1	0/1
Subtotal	**0/16**	**9/16**	**2/16**
Origin of samples: Sofia greater area			
River water	0/12	7/12	0/12
Mineral	0/3	2/3	0/3
Sewage	0/7	3/7	1/7
Well	0/3	1/3	1/3
Tap	0/9	2/9	3/9
Lake	0/2	1/2	0/2
Subtotal	**0/36**	**16/36**	**5/36**
Total (%)	**0/52 (0%)**	**25/52 (48%)**	**7/52 (13.5 %)**

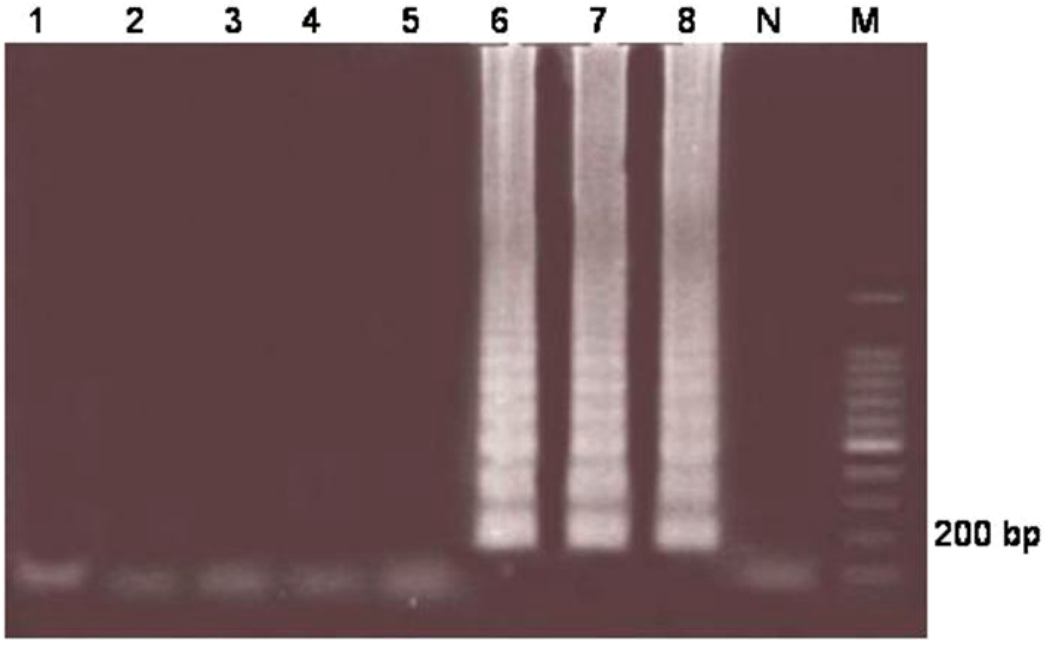
3.3 Results

Comparative results for the detection of *Toxoplasma* in water samples obtained by PCR, LAMP and IFT assays are demonstrated in Tables 2-4 and Figures 2-4.

3.3.1 LAMP specificity

The LAMP method was found to be highly specific for the *Toxoplasma* template sequences in tests with heterologous genomic DNAs (Table 2, Figure 2). Fig. 2 demonstrates the specificity of the LAMP primers for the detection of *Toxoplasma* B1 and *Tg*OWP genes. Based on this analysis and the BLAST results of the related sequences, the use of the primers listed in Table 1 can accomplish a successful amplification.

Figure 2. B1 gene agarose gel electrophoresis product of LAMP for the detection of toxoplasmic DNA. Lane 1, *Neospora* DNA; lane 2, *T. brucei* DNA; lane 3, *B. gibsoni* DNA; lane 4, *G. lamblia* DNA; lane 5, *C. parvum* DNA; lane 6, *Toxoplasma* AHC1 (oocysts) DNA; lane 7, *Toxoplasma* RH (tachyzoites); lane 8, *Toxoplasma* PLK (tachyzoites) DNA; lane N, negative control (DDW); lane M, 100-bp DNA ladder.



3.3.2 LAMP sensitivity

Both selected genes, B1 and *Tg*OWP demonstrated that they specifically amplify DNA derived from tachyzoites and oocysts of different *Toxoplasma* strains (Table 2, Figure 2). Before DNA extraction, *Toxoplasma* tachyzoites were counted and serially diluted. Application of the LAMP method using control DNA templates of *Toxoplasma* tachyzoites representing from 1×10^{-4} to 1×10^{-1} serially diluted tachyzoites is displayed in Figure 3A. The results of the application of the PCR in the same serially diluted samples by the use of the F3 and B3 primer pair strongly correlated.

Figure 3. Comparative sensitivities by LAMP (A) and PCR (B) for the specific detection of tachyzoites toxoplasmic DNA based on the B1 gene amplification. Lanes 1 through 6, dilution set (1×10^4, 1×10^3, 1×10^2, 1×10^1, 1×10^0, and 10^{-1} *Toxoplasma* tachyzoites, respectively); lane N, negative control; lane M, 100-bp DNA ladder.

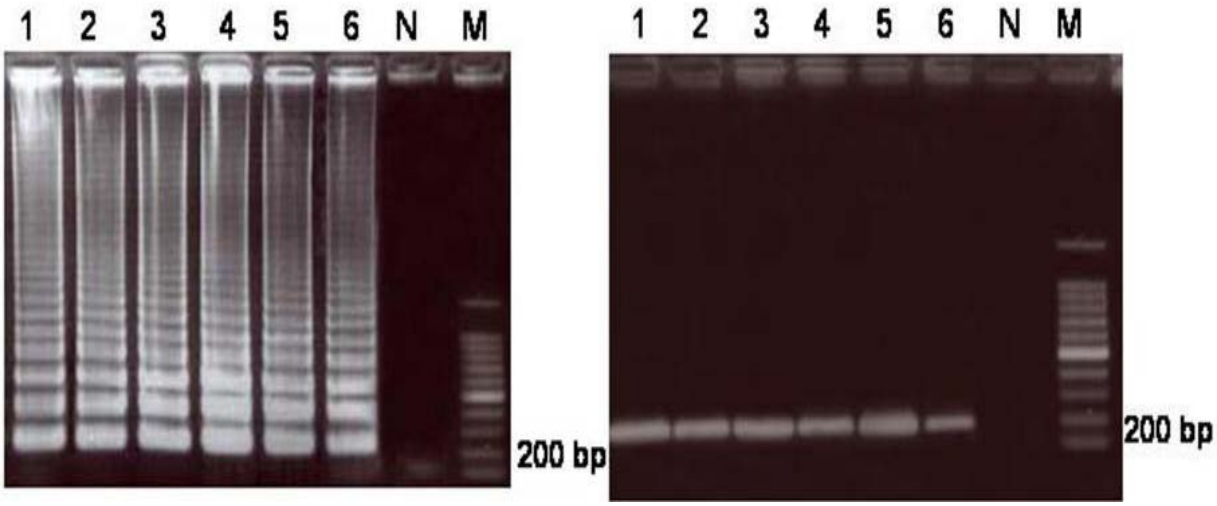
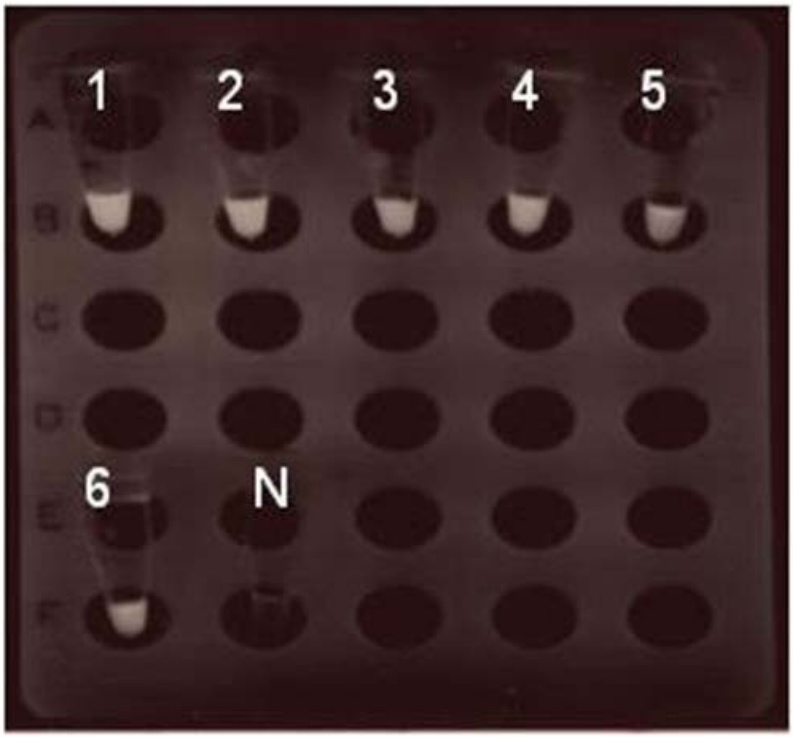


Figure 4 shows the difference in turbidity in the reaction tube due to the formation of magnesium pyrophosphate, which can be observed by the naked eye or under addition of fluorescent dyes in positive and negative samples. The reactions tubes numbered from 1 to 6 are demonstrating the turbidity under UV light in 6 serially diluted *Toxoplasma* samples containing from $1x10^{-4}$ to 10^{-1} tachyzoites. The negative sample showed no turbidity under exposure in the UV light.

Figure 4. It shows the difference in positive and negative samples due to the turbidity in the reaction tube because of the formation of magnesium pyrophosphate, which can be observed by the naked eye or in addition of fluorescent dyes. Tubes 1 through 6, dilution set (1×10^4, 1×10^3, 1×10^2, 1×10^1, 1×10^0, and 10^{-1} *Toxoplasma* tachyzoites).



3.3.3 Application of LAMP and nested PCR assays in spiked water samples

Twenty-six environmental samples were spiked with 10 *Toxoplasma* oocysts followed by DNA extraction from each sample.

3.3.3.1 LAMP

All 26 spiked pellet samples from Rostov greater area showed positive results for the presence of *Toxoplasma* DNA (Table 3). Concerning Sofia greater area we evaluated the LAMP method in a representative number of 10 spiked water samples randomly chosen (Table 3), and LAMP generated products are depicted in Fig. 1. All of them have been found positive for *Toxoplasma* DNA.

3.3.3.2 Nested PCR

The application of the nested PCR in spiked water pellets resulted in 14 (8 river and 1 lake spiked samples from Rostov grater area and 2 sewage, 1 well and 2 tap water samples from Sofia greater area) of 26 samples (Table 3) based on the amplification of the 18S-rRNA.

3.3.3.3 Statistical analysis of the spiked samples

For the samples from the Rostov area, the detection rate with LAMP was significantly higher than that with nested PCR (2-sided McNemar's test: P=0.0156). A significant difference was also found in the Sofia area samples (P=0.0625). The sensitivity of the LAMP method in spiked water samples was 100%, whereas the detection sensitivity by nested PCR was 53.8%.

3.3.4 Direct application of LAMP and PCR assays in natural water samples

3.3.4.1 LAMP

On the basis of the aforementioned LAMP analysis in spiked water sediments, we applied LAMP assays in all 52 water samples. LAMP assays rapidly detected *Toxoplasma* DNA within 60 min after starting the reaction. Twenty five out of 52 (~48%) environmental samples have been found positive for *Toxoplasma* DNA by LAMP. More detailed: nine (8 river and 1 spring) out of 16 water samples from Rostov greater area were documented as positive by the B1 gene LAMP amplification for the presence of *Toxoplasma* (Table 4) and demonstrated in the agarose electrophoresis the typical multiple loop structures formed by annealing between alternately inverted repeats of the target sequence in the same strand. Other 16 samples (7 river, 2 mineral, 3 sewage, 1 well, 2 tap and 1 lake) out of 36 water samples from Sofia area were positive by LAMP assays based on the B1 gene (Table 4).

3.3.4.2 Nested PCR

Nested PCR assay detected *Toxoplasma* DNA in 7 out of 52 samples (2 river water samples from Rostov greater area and 3 tap, 1 sewage, 1 well water samples from Sofia greater area). The overall sensitivity of the PCR was 13.5%.

3.3.4.3 Statistical analysis of the natural samples

The overall detection rate of the LAMP method in natural water samples was 48%, whereas the detection sensitivityby nested PCR was 13.5%. For the samples from the Rostov greater area samples the detection rate with LAMP was significantly higher than that with PCR (2-sided McNemar's test: *P*=0.0156). The difference in detection rates for the Sofia area samples was not significant (P=0.00342).

3.3.5 IFT

The performance of the IFT assay in positive control samples containing oocysts was successful but with poor quality. Oocyst and sporocyst wall and the inner wall of *Toxoplasma* sporulated oocysts were stained and clearly visualised under the epifluorescence microscope. However, after applying the test to the investigated pellets no *Toxoplasma* oocysts were detected in the investigated 52 natural environmental samples by IFT assay (Table 3).

3.4. Discussion

Detection of *T. gondii* oocysts in water is difficult, and there are no reliable or standardized methods to carry out such detection. Dubey et al. (2003) and Dumetre and Darde (2003) suggested a strategy that might be used to detect *T. gondii* oocysts in the water. Advanced efforts by Dumetre and Darde (2005) described a combination of IMS and application of MAbs 4B6 and 3G4 for the specific IFT detection *Toxoplasma* oocysts. Another work by the same authors (Dumetre and Darde, 2007) involved a multi-step procedure to detect *Toxoplasma* in fresh water of various qualities by using the monoclonal antibodies 4B6. The first report for the microscopic detection of *Toxoplasma* oocysts in water was recorded by Sroka et al. (2006). A significant correlation between the consumption of unboiled water from a well and the presence of specific antibodies against *T. gondii* noted among inhabitants of farms, in combination with their results (Sroka et al., 2006) of the water examination, indicated that on neglected farms where wells could be easily contaminated, well water may be an important source of the parasite for human and animal infection. Origin and nature of the water samples significantly affect the detection of *Toxoplasma* by PCR because of the co-extracted debris (Kourenti and

Karanis, 2004; 2006; Villena et al., 2004). PCR has been established for the detection of *Toxoplasma* DNA from water samples, but the low number of *Toxoplasma* oocysts and inhibitors expected in water samples making the method non practical for routine use (Sluter et al., 1997; Kourenti and Karanis, 2004; 2006). Kourenti and Karanis (2006) made further observations and expanded the assumption that a correlation of PCR inhibition and detection sensitivity to the individual characteristics of the examined samples (origin, colour and amount of debris in the pellet) exists. Villena et al. (2004) added a plasmid insert (corresponding to the target on the B1 gene) in all examined DNA environmental samples to detect the PCR inhibitors. They found *T. gondii* DNA in 10 of 125 environmental water samples but none of the positive samples were positive by bio-assay in mice. According to our results by IFT this could not be considered valid yet for the detection of *Toxoplasma* in water samples till effective antibodies are available.

The LAMP method presented here demonstrated its effectiveness clearly in working with difficult DNA templates. Based on the sequence analysis we can exclude the cross reaction of the developed LAMP method with the aforementioned coccidian because they do not express any genes similar to B1 or *Tg*OWP genes. The DNA used in PCR and LAMP assays was extracted in all cases by the same extraction protocol described previously (Kourenti and Karanis, 2004; 2006). At first we confirmed the specificity of the applied method of screening different DNAs derived not only from different parasites which could be found in water samples (*Giardia*, *Cryptosporidium*), but also from parasites phylogenetically close to *Toxoplasma* like *Neospora caninum*. DNA has been extracted water pellets seeded and the LAMP-B1- and –*Tg*OWP-assays were applied. All spiked pellets have been proved as expected positive with both target genes B1 and TgOWP. 48% of the samples (25 out of 52) have been found positive. Statistical analysis by the 2-sided McNemar's test confirmed significant differences in the detection rates by LAMP compared to the PCR applied in spiked and in natural water samples. A major advantage of the method is that no special apparatus is needed, which renders it more economical and practical than nested PCR or, indeed, any other kind of PCR. The LAMP method is simple and easy to perform once the appropriate primers are designed. Since 4 primers are designed to recognize 6 distinct regions on the target gene, only the target gene can be specifically amplified.

We reported here a LAMP specific protocol to amplify *Toxoplasma* DNA targeting specifically the *Tg*OWP and B1 genes. Based on the results the LAMP amplification method could currently be placed among the most accurate molecular methods as a specific, sensitive, simple and rapid diagnostic tool for the detection of *Toxoplasma* in water samples. The LAMP method can be included for *Toxoplasma* detection from human samples, to the water and food analysis strategy and it can be easily adapted for use in public health service laboratories.

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3.5. References

Abbasi M, Kowalewska-Grochowska K, Bahar MA, Kilani RT, Winkler-Lowen B, Guilbert LJ (2003) Infection of placental trophoblasts by *Toxoplasma gondii. J Infect Dis* 188: 608-616.

Afonso E, Thulliez P, Gilot-Fromont E (2006) Transmission of *Toxoplasma gondii* in an urban population of domestic cats (*Felis catus*). *Int J Parasitol* 36: 1373-1382.

Alhassan A, Thekisoe OM, Yokoyama N, Inoue N, Motloang MY, Mbati PA, Yin H, Katayama Y, Anzai T, Sugimoto C, Igarashi I (2007) Development of loop-mediated isothermal amplification (LAMP) method for diagnosis of equine piroplasmosis. *Vet Parasitol* 143: 155-160.

Aramini JJ, Stephen C, Dubey JP (1998) *Toxoplasma gondii* in Vancouver Island cougars (*Felis concolor vancouverensis*): serology and oocyst shedding. *J Parasitol* 84: 438-440.

Aramini JJ, Stephen C, Dubey JP, Engelstoft C, Schwantje H, Ribble CS (1999) Potential contamination of drinking water with *Toxoplasma gondii* oocysts. *Epidemiol Infect* 122: 305-315.

Armitage P and Berry G (1994) Statistical Methods in Medical Research, 3rd edition, Blackwell Science, Oxford 125-128.

Bahia-Oliveira LM, Jones JL, Zevedo-Silva J, Alves CC, Orefice F, Addiss DG (2003) Highly endemic, waterborne toxoplasmosis in north Rio de Janeiro state, Brazil. *Emerg Infect Dis* 9: 55-62.

Benenson MW, Takafuji ET, Lemon SM, Greenup RL, Sulzer AJ (1982) Oocyst-transmitted toxoplasmosis associated with ingestion of contaminated water. *N Engl J Med* 307: 666-669.

Bowie WR, King AS, Werker DH, Isaac-Renton JL, Bell A, Eng SB, Marion SA (1997) Outbreak of toxoplasmosis associated with municipal drinking water. The BC *Toxoplasma* Investigation Team. *Lancet* 350: 173-177.

Burnett AJ, Shortt SG, Isaac-Renton J, King A, Werker D, Bowie WR (1998) Multiple cases of acquired toxoplasmosis retinitis presenting in an outbreak. *Ophthalmology* 105: 1032-1037.

Dubey JP, Sharma SP, Juranek DD, Sulzer AJ, Teutsch SM (1981) Characterization

of *Toxoplasma gondii* isolates from an outbreak of toxoplasmosis in Atlanta, Georgia. *Am J Vet Res* 42: 1007-1010.

Dubey JP, Graham DH, Blackston CR, Lehmann T, Gennari SM, Ragozo AM, Nishi SM, Shen SK, Kwok OC, Hill DE, Thulliez P (2002a) Biological and genetic characterisation of *Toxoplasma gondii* isolates from chickens (*Gallus domesticus*) from Sao Paulo, Brazil: unexpected findings. *Int J Parasitol* 32: 99-105.

Dubey JP, Saville WJ, Stanek JF, Reed SM (2002b) Prevalence of *Toxoplasma gondii* antibodies in domestic cats from rural Ohio. *J Parasitol* 88: 802-803.

Dumetre A and Darde ML (2003) How to detect *Toxoplasma gondii* oocysts in environmental samples? *FEMS Microbiol Rev* 27: 651-661.

Dumetre A and Darde ML (2005) Immunomagnetic separation of *Toxoplasma gondii* oocysts using a monoclonal antibody directed against the oocyst wall. *J Microbiol Methods* 61: 209-217.

Dumetre A and Darde ML (2007) Detection of *Toxoplasma gondii* in water by an immunomagnetic separation method targeting the sporocysts. *Parasitol Res* 101: 989-996.

Evans R, Chatterton JM, Ashburn D, Joss AW, Ho-Yen DO (1999) Cell-culture system for continuous production of *Toxoplasma gondii* tachyzoites. *Eur J Clin Microbiol Infect Dis* 18: 879-884.

Ikadai H, Tanaka H, Shibahara N, Matsuu A, Uechi M, Itoh N, Oshiro S, Kudo N, Igarashi I, Oyamada T (2004) Molecular evidence of infections with *Babesia gibsoni* parasites in Japan and evaluation of the diagnostic potential of a loop-mediated isothermal amplification method. *J Clin Microbiol* 42: 2465-2469.

Isaac-Renton J, Bowie WR, King A, Irwin GS, Ong CS, Fung CP, Shokeir MO, Dubey JP (1998) Detection of *Toxoplasma gondii* oocysts in drinking water. *Appl Environ Microbiol* 64: 2278-2280.

Karanis P, Sotiriadou I, Kartashev V, Kourenti C, Tsvetkova N, Stojanova K (2006) Occurrence of *Giardia* and *Cryptosporidium* in water supplies of Russia and Bulgaria. *Environ Res* 102: 260-271.

Karanis P, Thekisoe O, Kiouptsi K, Ongerth J, Igarashi I, Inoue N (2007) Development and preliminary evaluation of a loop-mediated isothermal amplification

procedure for sensitive detection of *Cryptosporidium* oocysts in fecal and water samples. *Appl Environ Microbiol* 73: 5660-5662.

Kasper LH and Buzoni-Gatel D (1998) Some opportunistic parasitic infections in AIDS: Candidiasis, Pneumocystosis, Cryptosporidiosis, Toxoplasmosis. *Parasitol Today* 14: 150-156.

Kourenti C, Heckeroth A, Tenter A, Karanis P (2003) Development and application of different methods for the detection of *Toxoplasma gondii* in water. *Appl Environ Microbiol* 69: 102-106.

Kourenti C and Karanis P (2004) Development of a sensitive polymerase chain reaction method for the detection of *Toxoplasma gondii* in water. *Wat Sci Technol* 50: 287-291.

Kourenti C and Karanis P (2006) Evaluation and applicability of a purification method coupled with nested PCR for the detection of *Toxoplasma* oocysts in water. *Lett Appl Microbiol* 43: 475-481.

Kuboki N, Inoue N, Sakurai T, Di CF, Grab DJ, Suzuki H, Sugimoto C, Igarashi I (2003) Loop-mediated isothermal amplification for detection of African trypanosomes. *J Clin Microbiol* 41: 5517-5524.

Miller MA, Gardner IA, Kreuder C, Paradies DM, Worcester KR, Jessup DA, Dodd E, Harris MD, Ames JA, Packham AE, Conrad PA (2002a) Coastal freshwater runoff is a risk factor for *Toxoplasma gondii* infection of southern sea otters (*Enhydra lutris nereis*). *Int J Parasitol* 32: 997-1006.

Miller MA, Gardner IA, Packham A, Mazet JK, Hanni KD, Jessup D, Estes J, Jameson R, Dodd E, Barr BC, Lowenstine LJ, Gulland FM, Conrad PA (2002b) Evaluation of an indirect fluorescent antibody test (IFAT) for demonstration of antibodies to *Toxoplasma gondii* in the sea otter (*Enhydra lutris*). *J Parasitol* 88: 594-599.

Njiru ZK, Mikosza AS, Armstrong T, Enyaru JC, Ndung'u JM, Thompson AR (2008a) Loop-Mediated Isothermal Amplification (LAMP) method for rapid detection of *Trypanosoma brucei rhodesiense*. *PLoS Negl Trop Dis* 2: e147.

Njiru ZK, Mikosza AS, Matovu E, Enyaru JC, Ouma JO, Kibona SN, Thompson RC, Ndung'u JM (2008b) African trypanosomiasis: Sensitive and rapid detection of the sub-genus Trypanozoon by loop-mediated isothermal amplification (LAMP) of

parasite DNA. *Int J Parasitol* 38: 589-599.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* 28: E63.

Pradhan S, Yadav R, Mishra VN (2007) *Toxoplasma* meningoencephalitis in HIV-seronegative patients: clinical patterns, imaging features and treatment outcome. *Trans R Soc Trop Med Hyg* 101: 25-33.

Sluter SD, Tzipori S, Widmer G (1997) Parameters affecting polymerase chain reaction detection of waterborne *Cryptosporidium parvum* oocysts. *Appl Microbiol Biotechnol* 48: 325-330.

Sroka J, Wojcik-Fatla A, Dutkiewicz J (2006) Occurrence of *Toxoplasma gondii* in water from wells located on farms. *Ann Agric Environ Med* 13: 169-175.

Thekisoe OM, Inoue N, Kuboki N, Tuntasuvan D, Bunnoy W, Borisutsuwan S, Igarashi I, Sugimoto C (2005) Evaluation of loop-mediated isothermal amplification (LAMP), PCR and parasitological tests for detection of *Trypanosoma evansi* in experimentally infected pigs. *Vet Parasitol* 130: 327-330.

Villena I, Aubert D, Gomis P, Ferte H, Inglard JC, Denis-Bisiaux H, Dondon JM, Pisano E, Ortis N, Pinon JM (2004) Evaluation of a strategy for *Toxoplasma gondii* oocyst detection in water. *Appl Environ Microbiol* 70: 4035-4039.

Weintraub JM (2006) Improving *Cryptosporidium* testing methods: a public health perspective. *J Water Health* 4 Suppl 1: 23-26.

Zarlenga DS and Trout JM (2004) Concentrating, purifying and detecting waterborne parasites. *Vet Parasitol* 126: 195-217.

4. Molecular identification of *Giardia* and *Cryptosporidium* from dogs and cats

Abstract

The aim of the present study was to diagnose the presence of *Giardia* cysts and *Cryptosporidium* oocysts in household animals using nested polymerase chain reaction (PCR) and sequence analysis. One-hundred faecal samples obtained from 81 dogs and 19 cats were investigated for the presence of *Giardia* and *Cryptosporidium*. The *Cryptosporidium* genotypes were determined by sequencing a fragment of the small subunit (SSU) rRNA gene, while the *Giardia* Assemblages were determined through analysis of the glutamate dehydrogenase (GDH) locus. Isolates from five dogs and two cats were positive by PCR for the presence of *Giardia*, and their sequences matched the zoonotic Assemblage A of *Giardia*. *Cryptosporidium* spp. isolated from one dog and one cat were both found to be *C. parvum*. One dog isolate harboured a mixed infection of *C. parvum* and *Giardia* Assemblage A. These findings support the growing evidence that the anthropozoonotic pathogens *Giardia* spp. and *Cryptosporidium* spp. in household animals are potential reservoirs for infections in humans.

4.1. Introduction

Cryptosporidium and *Giardia* are protozoan pathogens that cause diarrheal illness when they colonise and reproduce in the intestines of humans or domestic animals, particularly dogs, cats, and livestock respectively. Both *Giardia* and *Cryptosporidium* are capable of completing their life cycle within a single host, resulting in cyst or oocyst stages that are excreted in the faeces. Epidemiological studies have focused on the transmission route of *Giardia* and *Cryptosporidium* (Robertson, 2009; Pozio, 2008) and have sought to determine their zoonotic potential (Xiao and Fayer, 2008; Thompson et al., 2009). Animals kept as household and/or pets play a significant role in the zoonotic and/or antropozoonotic transmission routes of the parasites due to their close association with their owners and the abundance of parasite cysts/oocysts excreted in large quantities (Eligio-Garcia et al., 2005; Berrilli et al., 2012). Household animals may also come into contact with free-living and/or domestic animals and can contract infections from them (Berilli et al., 2012). Animals can harbour infections of either zoonotic or host-specific *Giardia* Assemblages (Traub et al., 2004; Thompson et al., 2008; Covacin et al., 2011). In general, cats and dogs may be affected by the host specific *C. felis* and *C. canis,* respectively, and dogs can be infected with *C. parvum* due to the broader host range of this species (Lucio-Forster et al., 2010). Genotyping of oocysts recovered from the faeces of infected cats has shown that cats can also be infected with *C. muris* (Santin et al., 2006; Pavlasek and Ryan, 2007). *Giardia* host-adapted Assemblages in cats are usually A and F, whereas for dogs, the Assemblages include A, C and D (Hopkins et al., 1997; Sprong et al., 2009; Ballweber et al., 2010).

The objective of the present study was to examine household animals for *Cryptosporidium* and *Giardia* infections.

4.2 Materials and methods

4.2.1 Sample collection

Faecal samples from domestic dogs and cats with clinical suspicion for giardiasis or cryptosporidiosis (predominantly diarrhoea of variable duration) were submitted by veterinary clinics from Germany and other European countries to a private veterinary laboratory in Germany (Idexx Vet Med Lab, Ludwigsburg). Fresh faecal samples from 81 dogs and 19 cats were collected during 2007 and were labelled and stored immediately at -20 °C. The samples were shipped to the laboratory at Cologne University for purification and processing and were kept frozen until used.

4.2.2 Sample purification

Purification of the faecal samples and isolation of the oocysts/cysts was performed using the diethyl ether sedimentation technique combined with the saturated salt flotation technique, as described by Joachim et al. (2003a). Briefly, each faeces sample was suspended in a 50 ml polypropylene tube with phosphate buffered saline (PBS, pH 4) and homogenised by shaking vigorously. The excess faecal debris and lipids were removed by mixing the suspension with a one-fourth volume of diethyl ether until the sample became a homogenised emulsion; the suspension was then centrifuged at 2500 g for 10 min. The supernatant was discarded and the remaining sediment was resuspended in distilled water and centrifuged twice as described above to remove other residues. The resulting pellet was resuspended in 5 M cold saturated sodium chloride solution and carefully overlaid with cold distilled water so that a visible gradient was obtained. The samples were centrifuged at 2300 g for 10 min, and oocysts/cysts were recovered from the interphase were washed twice with distilled water and stored in PBS (pH 7.4) with streptomycin (200 mg/ml) and amphotericin B (5 mg/ml) at 4 °C.

4.2.3 DNA extraction

DNA was extracted from the purified faecal suspension and used for molecular analysis and sequencing. DNA extraction was performed using the modified method described by Karanis et al. (2007) followed by the use of the QIAamp Stool Kit

(Qiagen GmbH, Hilden, Germany). In brief, the oocysts/cysts were ruptured using ten freeze-thaw cycles in the presence of lysis buffer in a Dry Thermo device (DTU-2B, Taitec, Japan) and were further processes according to the Qiagen manufacturer's instructions. DNA was eluted in 100 µl AE buffer and stored at –20 °C.

4.2.4 Molecular assays for the detection of *Giardia* and *Cryptosporidium* in faecal samples

To characterise the *Giardia* and *Cryptosporidium* species isolated from each positive sample, the DNA of all samples was extracted from the oocysts/cysts and was concequently tested by nested PCR. Positive faecal specimens were sequenced to identify the *Cryptosporidium* species and the *Giardia* Assemblages of the parasites found in the positive samples of the infected animals.

4.2.4.1 *Giardia* spp.

A fragment of the GDH gene of approximately 220 bp in length was amplified using previously published primers and conditions (Abe et al., 2003). All PCR amplifications were performed in an ABI 2720 Thermal cycler (Applied Biosystems, Foster, CA) in standard mixtures of 50 µl containing 1x PCR buffer, 200 nmol of each primer, 200 µM of dNTPs, 1.5 mM of $MgCl_2$, 2.5 U of HotstarTaq DNA polymerase (Qiagen GmbH, Hilden, Germany), 2 µl of bovine serum albumin (BSA, acetylated, 10 mg/mL) (Promega, Madison, WI), 2 µl DNA template and distilled water. The PCR program included one incubation at 96 °C for 15 min and 40 amplification cycles (94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 60 sec), followed by one final extension incubation of 7 min at 72 °C. The PCR products were separated on a 1.6% agarose gel, stained with ethidium bromide and visualised on a UV transilluminator. PCR negative-control samples omitted template DNA, which was replaced by distilled water, and PCR positive-controls, containing DNA extracted from 10 *Giardia* cysts were always included for each test.

4.2.4.2 *Cryptosporidium* spp.

Cryptosporidium DNA were amplified by nested PCR using primers and conditions as described by Nichols et al. (2003) and Karanis et al. (2007) to produce a DNA fragment of approximately 440 bp in length of the SSU rRNA gene. PCR reactions

were performed in 50 µl volumes containing 200 nmoles of each primer, 1x PCR buffer, 200 µM dNTP, 3 mM $MgCl_2$, 2.5 U GoTaq DNA polymerase (Promega, Wisconsin, USA), 2 µl BSA (10 mg/ml), 2 µl DNA template and distilled water. PCR consisted of one initial denaturation cycle at 96 °C for 15 min and 35 cycles (94 °C for 30 s, primary PCR 68 °C and secondary PCR 60 °C for 60 sec, 72 °C for 30 s), followed by one final extension at 72 °C for 10 min. The PCR products were analysed as described above. Positive controls containing DNA extracted from 10 *C. parvum* oocysts and negative controls containing distilled water were included for each test.

4.2.5 PCR product purification and sequencing

All PCR products from the molecular assays for both *Giardia* cysts and *Cryptosporidium* oocysts were cut out of agarose gels and purified with QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The purified PCR products were directly sequenced in both directions on an ABI Prism 3100 (Applied Biosystems, Japan) Genetic Analyser using a Big Dye Terminator V.3.1 cycle sequencing kit (Applied Biosystems, Japan). The accuracy of the data was confirmed by two directional sequencing of the obtained sequences and by the alignment of the nucleotide sequences of the GDH gene for *Giardia* cysts and the SSU rRNA for *Cryptosporidium* oocysts against reference sequences retrieved from GenBank using the program ClustalW. Sequence similarity was also determined using the Basic Local Alignment Search Tool (BLAST) and the phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) and the respective evolutionary analyses were conducted using MEGA 5 (Tamura et al., 2011). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches Figure 1 (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site.

The nucleotide sequences of the positive samples have been submitted to the GenBank and they are available in the GenBank database under the accession numbers EU583462-EU583467 and EU591705 (93%-97% homology) for *Giardia* and EU606193 (94% homology) and EU606194 (99% homology) for *Cryptosporidium*.

4.3 Results and discussion

A total of 100 faecal samples from household animals, 19 cats and 81 dogs, were investigated by nested PCR for the presence of *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts. To test for *Giardia,* all samples were amplified with the primers GDF3 and GDB5 and the PCR products were sequenced. Five dog and two cat faecal samples were found to be positive for *Giardia*. The results are presented in Table 1. In all cases, the samples found positive by PCR were confirmed by sequence analysis and no sequences were identified other than those of the typical *Giardia* Assemblage A in the dog and cat samples. In faecal samples of two dogs (6 year old, mix-breed with diarrhoea and vomiting and 1 year old with persistant diarrhoea) additional laboratory examinations identified the presence of *Escherichia coli* and *Campylobacter jejuni* (diagnosed by aerobe cultivation), respectively, but no other infections with yeasts and parasites have been found.

Table 1. Positive household animals for *Giardia* infections by PCR of the GDH locus, sequencing analysis results and accession numbers (n=19 cats and n=81 dogs).

Sample name	Species	*Giardia* PCR result of the investigation of the GDH locus	*Giardia* Assemblage by sequencing analysis	GenBank Accession number
G7	Dog	+	A	EU583462
G9§	Cat	+	A	EU583463
G10	Dog	+	A	EU583464
G14	Dog	+	A	EU583465
G16	Cat	+	A	EU583466
G41	Dog	+	A	EU583467
G39*	Dog	+	A	EU591705

+ = positive.

§ = cat from Denmark.

* = dog with mixed infection (*Giardia* and *Cryptosporidium*).

SSU rDNA PCR and sequencing detected *Cryptosporidium* in one dog and one cat sample. Interestingly, both samples had high identity values to *C. parvum* (Table 2). Direct sequencing of these PCR products for both *Giardia* and *Cryptosporidium* showed that one dog carried a mixed infection with both *C. parvum* and *Giardia* Assemblage A (Tables 1 and 2). According to the study of Nichols et al. (2010) their developed outer amplification primers (N18SF2/R2) showed nonspecific amplification of DNA from the dinoflagellate *Gymnodinium*, but in both positive cases of our study, the results of nested PCR were confirmed by sequence analysis and no sequences were identified other than those of the typical *C. parvum*.

Table 2. Positive household animals for the presence of *Cryptosporidium* genotypes by PCR of the SSU rRNA and sequencing analysis results (n=19 cats and n=81 dogs).

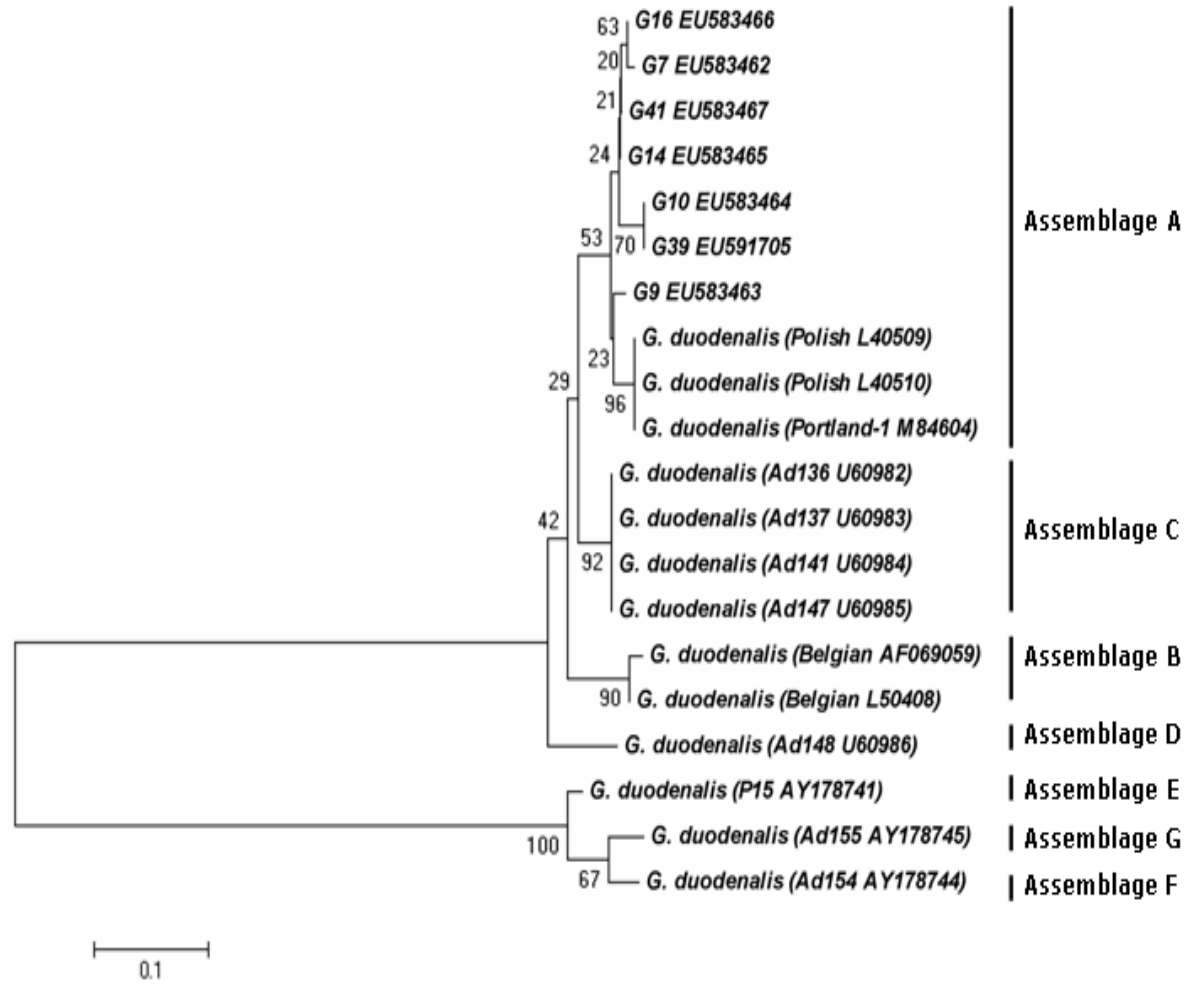
Sample name	Species	*Cryptosporidium* PCR result of the investigation of the SSU rRNA gene	*Cryptosporidium* genotype by sequencing analysis	GenBank Accession number
G39*	Dog	+	*C. parvum*	EU606193
G37	Cat	+	*C. parvum*	EU606194

+ = positive.

* = dog with mixed infection (*Giardia* and *Cryptosporidium*).

The phylogenetic relation of *Giardia* species is shown in Figure 1 using sequences of thirteen *Giardia* Assemblages downloaded from GenBank as reference sequences. The phylogenetic tree indicates that the sequences obtained in our study cluster to *Giardia* Assemblage A. Even though the number of positive samples was relativey low, these results clearly demonstrate the presence of *Giardia* and *Cryptosporidium* among household animals from different regions in Germany.

Figure 1. Phylogenetic relationship between the 7 *Giardia* Assemblages (G7, G9, G10, G14, G16, G41 and G49) obtained in this study and other previously published *Giardia* Assemblages. Nucleotide sequences of the GDH gene obtained in this study were aligned against reference sequences retrieved from GenBank using ClustalW and MEGA 5. The evolutionary history was inferred using the Neighbor-Joining method and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method with the pairwise deletion option and are in the units of the number of base substitutions per site.



According to a 5 year survey (1993-1997) of dairy herds in five German state veterinary laboratories, *Cryptosporidium* was diagnosed in 19 to 34% of the examined faecal samples and 20 to 36% of the post mortem cases (Joachim et al., 2003b) by conventional techniques. The primary cause of diarrhoea was acknowledged to be *Cryptosporidium* by only 1/5 of the investigators, indicating that the role of *Cryptosporidium* in Germany was underestimated. The recorded mixed infections were mostly associated with the presence of rotavirus and *E. coli*.

Only few studies have been carried out to evaluate the occurrence of different *Giardia* genotypes and *Cryptosporidium* species/genotypes in household animals in Germany and the actual risk of transmission to their owners. Broglia et al. (2008) genotyped *Cryptosporidium* isolates and defined the subtypes of *C. parvum* that originated from neonatal calves in Germany. All of the calf isolates in their study were identified as *C. parvum*. Other reports have also indicated that *C. parvum* is the most frequently found species of *Cryptosporidium* in pre-weaned calves (Alves et al., 2003; Plutzer and Karanis, 2007). *Erinaceus europaeus L* (European hedgehogs) in Germany were found to be naturally infected with *Cryptosporidium* in 29.8% by coproantigen analysis, while molecular analysis revealed IIa, IIc and VIIa subtype families of *C. parvum (*Dyachenko et al., 2010).

The retrospective study of Barutzki and Schaper (2003) reported a prevalence rate of 16.6% and 12.6% for *Giardia* infections in dogs and cats, respectively in Germany between 1999 and 2002. The same authors reported that *Giardia* spp. were the most commonly found parasites using coprological examinations (coproantigen ELISA or SAF technique) with prevalence rates of 18.6 % and 12.6% in dogs and cats, respectively (Barutzki and Schaper, 2011). *Giardia* human isolates belong to Assemblages A and B (Karanis and Ey, 1998; Caccio et al., 2005). Each Assemblage consists of two distinct subgroups; Assemblage A can be divided into into A-I and A-II and Assemblage B can be divided into subgroups B-III and B-IV (Homan, 1992; Andrews et al., 1998). Zoonotic genotypes of *Giardia* Assemblage A that are specific for hosts other than humans and Assemblages C and D were specifically assigned for dogs (Hopkins et al., 1997; Monis et al., 1998; Thompson et al., 2000; Plutzer et al., 2010). In contrast, Assemblages A and B are not human-specific and infect a wider host range including dogs, cats, livestock and wildlife and are considered to have zoonotic potential. Barutzki et al. (2007) investigated two different groups of dogs; one group presented clinical symptoms of gastrointestinal disorders, and the

other group was randomly selected. They concluded that 7% of the randomly selected *Giardia* positive dogs carried zoonotic species of *Giardia* belonging to Assemblage A. Interestingly, Leonhard et al. (2007) examined asymptomatic dogs in southern Germany that were kept isolated or in groups and found that Assemblage A was most prevalent. They also identified Assemblages A and C, but Assemblages C and D were very rarely found.

Only one case was recorded as an outbreak of gastroenteritis caused by *C. parvum* after diagnosis by serological tests, such as ELISA for specific IgG, analysis of stool specimens, genotyping of the isolates, and epidemiological analysis in Germany. In an August 2001 field training session, half of the recruits (n=201) of the German armed forces became ill with acute gastroenteritis (Brockmann et al., 2008). The zoonotic transmission of the disease was excluded after analysis of faecal droppings of the sheep that grazed in the area. Although the investigators were not able to identify the source of infection, the analysis of the risk factors correlated between drinking of tap water during the field exercise or the consumption of various meals at the beginning of the field training and gastroenteritis.

Giardia and *Cryptosporidium* infections in household and domestic animals have been described also in other european countries highlighting the role of animals, and in particular of cats and dogs, in transmission of the infection to humans (Hamnes et al., 2007; Overgaauw et al., 2009; Beck et al., 2011; Lebbad et al., 2011; Paoletti et al., 2011).

Thus the small number of the indicated positive samples, the present study enhance the knowledge of the occurrence of *Giardia* spp. and *Cryptosporidium* spp. in privately owned household animals and genetically characterise the isolates found in the infected animals.

In summary, both *Giardia* and *Cryptosporidium* parasites are capable of infecting a variety of vertebrate species, from humans to animals, demonstrate a worldwide distribution, and are pathogens of veterinary and public health concern because of their ability to cause gastrointestinal disease, their ubiquitous presence in the environment, and the propensity for waterborne and foodborne outbreaks of these parasites. The absence of clinical symptoms results in the true prevalence of these parasites being underestimated, not only in cats and dogs but also in humans, and should be of major concern to veterinarians and physicians alike. Factors that may be affecting the underestimation of the risk could be related to poor and out-dated

information, improper interpretation of the published data and lack of sensitive methods. Infected household animals may especially pose a risk to immunocompromised people because they can be more susceptible to infection with *Giardia* and *Cryptosporidium.*

4.4 References

Abe N, Kimata I. Iseki M (2003) Identification of genotypes of *Giardia intestinalis* isolates from dogs in Japan by direct sequencing of the PCR amplified glutamate dehydrogenase gene. *J Vet Med Sci* 65: 29-33.

Alves M, Xiao L, Sulaiman I, Lal AA, Matos O, Antunes F (2003) Sub genotype analysis of *Cryptosporidium* isolates from humans, cattle, and zoo ruminants in Portugal. *J Clin Microbiol* 41: 2744–2747.

Andrews RH, Monis PT, Ey PL, Mayrhofer G (1998) Comparison of the levels of intra-specific genetic variation within *Giardia muris* and *Giardia intestinalis*. *Int J Parasitol* 28: 1179–1185.

Ballweber LR, Xiao L, Bowman DD, Kahn G, Cama VA (2010) Giardiasis in dogs and cats: update on epidemiology and public health significance. *Trends Parasitol* 26: 180–189.

Barutzki D, Schaper R (2003) Endoparasites in dogs and cats in Germany 1999-2002. *Parasitol Res* 90: 148-150.

Barutzki D, Schaper R (2011) Results of parasitological examinations of faecal samples from cats and dogs in Germany between 2003 and 2010. *Parasitol Res* 109: 45-60.

Barutzki D, Thompson RCA, Wielinga C, Parker U, Schaper R (2007) Observations on *Giardia* infection in dogs from veterinary clinics in Germany. *Parasitol Res* 101: 153–156.

Beck R, Sprong H, Pozio E, Cacciò SM (2011) Genotyping *Giardia duodenalis* isolates from dogs: lessons from a multilocus sequence typing study. *Vector Borne Zoonotic Dis* DOI: 10.1089/vbz.2011.0751

Brockmann SO, Dreweck C, Wagner-Wiening C, Hagen RM, Kimmig P, Petry F, Jakobi V (2008) Serological and epidemiological analysis of an outbreak of gastroenteritis among military recruits in Germany caused by *Cryptosporidium parvum*. *Infect* 36: 450-457.

Broglia A, Reckinger S, Cacció SM, Nöckler K (2008) Distribution of *Cryptosporidium parvum* subtypes in calves in Germany. *Vet Parasitol* 154: 8-13.

Cacciò SM, Thompson RC, McLauchlin J, Smith HV (2005) Unravelling

Cryptosporidium and *Giardia* epidemiology. *Trends Parasitol* 21: 430-437.

Dyachenko V, Kuhnert Y, Schmaeschke R, Etzold M, Pantchev N, Daugschies A (2010) Occurrence and molecular characterization of *Cryptosporidium* spp. genotypes in European hedgehogs (*Erinaceus europaeus L*.) in Germany. *Parasitol* 137: 205-216.

Eligio-Garcia L, Cortes-Campos A, Jimenez-Cardoso E (2005) Genotype of *Giardia intestinalis* isolates from children and dogs and its relationship to host origin. *Parasitol Res* 97: 1–6.

Hamnes IS, Gjerde BK, Robertson LJ (2007) A longitudinal study on the occurrence of *Cryptosporidium* and *Giardia* in dogs during their first year of life. *Acta Vet Scand* 11: 49: 22.

Homan WL (1992) Comparison of *Giardia* isolates from different laboratories by isoenzyme analysis and recombinant DNA probes. *Parasitol Res* 78: 316–323.

Hopkins RM, Meloni BP, Groth DM, Wetherall JD, Reynoldson JD, Thompson RCA (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

Joachim A, Eckert E, Petry F, Bialek R, Daugschies A (2003a) Comparison of viability assays for *Cryptosporidium parvum* oocysts after disinfection. *Vet Parasitol* 11: 47–57.

Joachim A, Krull T, Schwarzkopf J, Daugschies A (2003b) Prevalence and control of bovine cryptosporidiosis in German dairy herds. *Vet Parasitol* 112: 277-288.

Karanis P, Ey P (1998) Characterization of axenic isolates of *Giardia intestinalis* established from humans and animals in Germany. *Parasitol Res* 84: 442-449.

Karanis P, Plutzer J, Halim NA, Igori K, Nagasawa H, Ongerth J, Liqing M (2007) Molecular characterization of *Cryptosporidium* from animal sources in Qinghai province of China. *Parasitol Res* 101: 1575-1580.

Lebbad M, Petersson I, Karlsson L, Botero-Kleiven S, Andersson JO, Svenungsson B, Svärd SG (2011) Multilocus genotyping of human *Giardia* isolates suggests limited zoonotic transmission and association between assemblage B and flatulence in children. *PLoS Negl Trop Dis* 5: e1262.

Leonhard S, Pfister K, Beelitz P, Wielinga C, Thompson RC (2007) The molecular characterisation of *Giardia* from dogs in southern Germany. *Vet Parasitol* 150: 33-38.

Lucio-Forster A, Griffiths JK, Cama VA, Xiao L, Bowman DD (2010) Minimal zoonotic risk of cryptosporidiosis from pet dogs and cats. *Trends Parasitol* 26: 174-179.

Monis PT, Andrews RH, Mayrhofer G, Mackrill J, Kulda J, Isaac-Renton JL, Ey PL (1998) Novel lineages of *Giardia intestinalis* identified by genetic analysis of organisms isolated from dogs in Australia. *Parasitol* 16: 7-19.

Nichols RA, Campbell BM, Smith HV (2003) Identification of *Cryptosporidium* spp. oocysts in United Kingdom noncarbonated natural mineral waters and drinking waters by using a modified nested PCR-restriction fragment length polymorphism assay. *Appl Environ Microbiol* 69: 4183-4189.

Overgaauw PA, van Zutphen L, Hoek D, Yaya FO, Roelfsema J, Pinelli E, van Knapen F, Kortbeek LM (2009) Zoonotic parasites in fecal samples and fur from dogs and cats in The Netherlands. *Vet Parasitol* 163: 115-122.

Paoletti B, Otranto D, Weigl S, Giangaspero A, Cesare AD, Traversa D (2011) Prevalence and genetic characterization of *Giardia* and *Cryptosporidium* in cats from Italy. *Res Vet Sci* 91: 397-399.

Pavlasek I, Ryan U (2007) The first finding of a natural infection of *Cryptosporidium muris* in a cat. *Vet Parasitol* 144: 349–352.

Plutzer J, Karanis P (2007) Genotype and subtype analyses of *Cryptosporidium* isolates from cattle in Hungary. *Vet Parasitol* 146: 357–362.

Plutzer J, Ongerth J, Karanis P (2010) *Giardia* taxonomy, phylogeny and epidemiology: Facts and open questions. *Int J Hyg Environ Health* 213: 321-333.

Pozio E (2008) Epidemiology and control prospects of food-borne parasitic zoonoses in the European Union. *Parassitologia* 50: 17-24.

Robertson LJ (2009) *Giardia* and *Cryptosporidium* infections in sheep and goats: a review of the potential for transmission to humans via environmental contamination. *Epidemiol Infect* 10: 1-9.

Santin M, Trout J.M, Vecino JA, Dubey JP, Fayer R (2006) *Cryptosporidium*, *Giardia* and *Enterocytozoon bieneusi* in cats from Bogota (Colombia) and genotyping of isolates. *Vet Parasitol* 141: 334–339.

Sprong H, Caccio SM, van der Giessen JWB (2009) Identification of zoonotic genotypes of *Giardia duodenalis*. *PLoS Negl Trop Dis* 3: e558.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731-2739.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22: 4673-4680.

Thompson RCA, Hopkins RM, Homan WL (2000) Nomenclature and genetic groupings of *Giardia* infecting mammals. *Parasitol Today* 16: 210–213.

Thompson RCA, Palmer CS, O'Handley R (2007) The public health and clinical significance of *Giardia* and *Cryptosporidium* in domestic animals. *Vet J* 177: 18-25.

Thompson RC, Colwell DD, Shury T, Appelbee AJ, Read C, Njiru Z, Olson ME (2009) The molecular epidemiology of *Cryptosporidium* and *Giardia* infections in coyotes from Alberta, Canada, and observations on some cohabiting parasites. *Vet Parasitol* 159: 167-170.

Traub RJ, Monis PT, Robertson I, Irwin P, Mencke N, Thompson RCA (2004) Epidemiological and molecular evidence supports the zoonotic transmission of *Giardia* among humans and dogs living in the same community. *Parasitol* 128: 253–262.

van der Giessen JW, de Vries A, Roos M, Wielinga P, Kortbeek LM, Mank TG (2006) Genotyping of *Giardia* in Dutch patients and animals: a phylogenetic analysis of human and animal isolates. *Int J Parasitol* 36:849-858.

Xiao L, Fayer R (2008) Molecular characterization of species and genotypes of *Cryptosporidium* and *Giardia* and assessment of zoonotic transmission. *Int J Parasitol* 38: 1239-1255.

5. Summary

There is an ongoing demand for development of different molecular tools toward achieving a better fundamental method that overcomes the limitation barriers and the drawbacks of other methods for an effective detection of protozan *Giardia, Cryptosporidium* and *Toxoplasma*.
Within the present thesis, a method has been developed for detection of the human pathogenic species, *Cryptosporidium* and *Toxoplasma*, applying the Loop mediated isothermal amlification (LAMP) technique. Beyond this, the phylogenetic analysis of *Giardia* and *Cryptosporidium* from houshold animals has been performed.

SNP based SAM-LAMP assay for *Cryptosporidium*
The single nucleotide polymorphism (SNP)-LAMP assay based on the SAM gene amplification was developed to detect specifically the most important human pathogenic species of *Cryptosporidium* (*C. parvum*, *C. hominis* and *C. meleagridis*), according to the available nucleotide sequences, and it was applied in water, clinical and animal samples. The assay was compared with two nested PCR-assays based on the SSU rRNA gene amplifying a 435 bp and 850 bp long polymorphous region of the gene for the species characterisation of the samples. The SNP based SAM-LAMP assay was additionally compared with the results from the IFT-test for *Cryptosporidium* species and with the previously developed GP60-LAMP assay able to detect only *C. parvum* infections.
The sensitivity detection limit was 800 fg/µl which corresponds to 0.1 *Cryptosporidium* oocysts. The developed assay was initially tested in 53 water sample pellets spiked with known numbers of *Cryptosporidium* oocysts. The detection sensitivity for the spiked pellets was 100% for both LAMP assays (GP60 and SNP based SAM-LAMP assays), while both nPCRs showed sensitivities of only 3% and 7%. In the same natural water samples 50% were found to be positive for *Cryptosporidium* DNA by the SNP based SAM-LAMP assay and 24% were positive by the GP60-LAMP assay; the nPCR assays generated products only in 5% and 7% of the samples. When the method was applied in water samples from Thailand 12 out of 15 (80%) samples were found positive, in 4 out of 8 mussel samples (50%), in 11 of 44 HIV/AIDS patient fecal samples (25%) and in 12 out of 22 (55%) animal fecal samples were found positive. The GP60-LAMP and the two nPCR assays detected lower numbers of positive samples than the SNP based LAMP assay.
Among the species identified from the investigated samples by sequencing analysis, the most commonly detected species was *C. parvum*, followed by *C. hominis* in one mussel sample, *C. andersoni* in one animal fecal and *C. fragile* in one water sample. The present work reports for first time the presence of *C. fragile* in water samples.

B1 and *Tg*OWP LAMP assays for *Toxoplasma* detection in water samples

The LAMP assay was developed and evaluated for the rapid detection of *Toxoplasma gondii* in water samples based on the amplification of B1 and *Tg*OWP *Toxoplasma* genes. LAMP demonstrated a sensitivity detection limit of 0.1 tachyzoites' DNA for both genes and subsequently the LAMP detection technique was evaluated and compared with the nested PCR in 26 water samples pellets spiked with *Toxoplasma* oocysts. LAMP showed a sensitivity of 100%, whereas PCR only 53.8% when applied in the same spiked environmental water samples. The comparison of the IFT, LAMP and PCR in the 52 natural water samples demonstrated that 25 (48%) of the samples were positive for *Toxoplasma*-DNA by LAMP, none by IFT, while nested PCR products were generated in 7 (13.5%) water samples.

Infections of household animals and phylogenetic analysis of *Giardia* and *Cryptosporidium* from Germany

The molecular diagnosis of *Giardia* cysts and *Cryptosporidium* oocysts in household animals mainly with origin from Germany using nPCRs and sequence analysis was the subject of this part of this work. The animal fecal samples were investigated after the amplification of the glutamate dehydrogenase locus (GDH) locus for the presence of *Giardia* species and the SSU rRNA for the presence of *Cryptosporidium* species. Of the 81 dogs and 19 cats investigated, the zoonotic Assemblage A of *Giardia* was identified in isolates from five dogs and two cats. *Cryptosporidium* spp. were isolated from one dog and one cat, where both found to be *C. parvum*. One dog isolate harboured a mixed infection of *C. parvum* and *Giardia* Assemblage A. These findings support the growing evidence that the anthropozoonotic pathogens *Giardia* spp. and *Cryptosporidium* spp. in household animals are potential reservoirs for infections in human and animal populations.

Comparatively to other molecular methods LAMP is ranked among the most accuarate molecular tools through its high diagnostic sensitivity and specificity for the accurate detection of the human pathogenic protozoan *Cryptosporidium* and *Toxoplasma* in environmental, clinical and food material. The application of the LAMP technique, coupled with the PCR-sequencing approach, would contribute for the rapid detection and differentiation of species of public health significance and the identification of the sources of contamination. The stand-alone application of the LAMP assay has the potential to become a rapid diagnostic tool for the control and prevention of protozoan-related diseases and will contribute for a better assement of the risk to public health from food- and waterborne (oo)cysts.

6. Zusammenfassung

Es besteht ein enormer Bedarf an der Entwicklung effektiver molekularer Methoden, die die Nachteile bisheriger Nachweisverfahren für parasitäre Protozoen wie *Giardia*, *Cryptosporidium* und *Toxoplasma* überwinden.
Die vorliegende Arbeit beschreibt die Entwicklung und Anwendung der Loop mediated-isothermal-amplification (LAMP) Technik zum Nachweis von *Cryptosporidium* und *Toxoplasma*. Darüber hinaus wurde eine phylogenetische Analyse von aus Haustieren isolierten *Giardia* und *Cryptosporidium* durchgeführt.

SNP basierende SAM-LAMP-Methode für *Cryptosporidium*
Das auf der Amplifizierung des SAM-Gens basierende SNP- (single nucleotid polymorphism-) LAMP-Verfahren wurde anhand der entsprechenden Nukleotid-sequenzen für den spezifischen Nachweis der wichtigsten humanpathogenen *Cryprosporidium*-Arten (*C. parvum*, *C. hominis* und *C. meleagridis*) entwickelt, anschließend in Wasser- sowie klinischen und Tierproben angewendet. Für die Speziescharakterisierung der Proben wurde die Methode mit zwei nPCR-Methoden verglichen, die auf dem Nachweis des SSU rRNA-Gens mit der Amplifizierung von 435 bp und einer 850 bp langen polymorphen Regionen des Gens beruhen. Das auf SNP basierende SAM-LAMP-Verfahren wurde mit den Ergebnissen des Immunofluoreszenz-Tests (IFT) für *Cryptosporidium* und mit dem zuvor entwickelten GP60-LAMP-Verfahren zum ausschließlichen Nachweis von *C. parvum* verglichen.
Die ermittelte Nachweisgrenze von 800 fg/µl entspricht 0,1 *Cryptosporidium*-Oozysten. Das entwickelte Verfahren wurde zunächst an 53 Wasserproben, die mit einer bekannten Anzahl an *Cryptosporidium*-Oozysten kontaminiert wurden, getestet. Die Sensitivität bei dem LAMP-Verfahren betrug 100% für die aufgestockten Proben, während die Sensitivität beider PCRs bei nur 3% und 7% lag. Bei den gleichen ursprünglichen (natürlichen) Proben wurden mittels auf SNP basierendem SAM-LAMP-Verfahren 50% und mittels GP60-Verfahren 24% *Cryptosporidium* DNA-Positive ermittelt; mit den nPCR Verfahren wurden hingegen nur 5% bzw. 7% Positivergebnisse generiert. Von 15 Wasserproben aus Thailand waren 12 (80%) positiv; ebenso positiv waren 4 von 8 (50%) Muschelproben, 11 von 44 (25%) Stuhlproben von HIV/AIDS Patienten und 12 von 22 (55%) tierischen Fäkalproben. Mit dem GP60-LAMP-Test und den beiden nPCR-Verfahren konnte vergleichsweise eine geringere Anzahl an positiven Proben ermittelt werden als durch SNP-basierende LAMP-Verfahren.
Unter den durch Sequenzanalyse identifizierten Arten aus den untersuchten Proben war *C. parvum* am häufigsten, gefolgt von *C. hominis* in einer Muschelprobe, *C. andersoni* in einer tierischen Fäkalprobe und *C. fragile* in einer Wasserprobe.

B1 und TgOWP LAMP-Methode für den Nachweis von *Toxoplasma* in Wasserproben

Das auf der Amplifizierung der B1 und TgOWP-Gene basierende LAMP-Verfahren wurde für den schnellen Nachweis von *Toxoplasma gondii* in Wasserproben entwickelt und evaluiert. Die Nachweisgrenze der LAMP lag für beide Gene bei 0,1 Tachyzoiten-DNA. Das LAMP-Verfahren wurde vorher evaluiert und an 26 mit *Toxoplasma*-Oozysten aufgestockten Wasserproben mit der nested PCR verglichen. Die LAMP-Sensitivität betrug 100%, hingegen erreichte die nPCR der gleichen Proben nur 53.8% bei den gleichen aufgestockten ursprünglichen Wasserproben. Der Vergleich von IFT, LAMP und nPCR anhand von 52 ursprünglichen Wasserproben zeigte, dass 25 (48%) der mittels LAMP untersuchten Proben und keine mit IFT–Verfahren untersuchten Proben positiv waren, während nPCR-untersuchte in 7 (13,5%) der Proben generiert wurden.

Haustierinfektionen und phylogenetische Analyse von *Giardia* und *Cryptosporidium*

Die molekulare Diagnose von *Giardia*-Zysten und *Cryptosporidium*-Oozysten von Haustieren mit Ursprung aus Deutschland mittels nPCR und Sequenzanalyse war ein Teilgegenstand dieser Arbeit. Die Tierkotproben wurden im Anschluss an die Amplifizierung des Glutamat-Dehydrogenase-Locus (GDH Locus) auf das Vorhandensein von *Giardia* und die SSU-rRNA auf das Vorhandensein von *Cryptosporidium* untersucht. Von den 81 untersuchten Hunden und 19 Katzen wurde *Giardia* Assemblage A in Isolaten von fünf Hunden und zwei Katzen identifiziert. Bei *Cryptosporidium*-Isolaten aus einem Hund und einer Katze handelte es sich jeweils um *C. parvum*. In einem Hundeisolat lag eine Mischinfektion von *C. parvum* und *Giardia* Assemblage A vor. Diese Ergebnisse liefern einen deutlichen Hinweis darauf, dass die anthropozoonotischen Krankheitserreger *Giardia* spp. und *Cryptosporidium* spp. in Haustieren ein mögliches Infektionsreservoir für die Populationen von Mensch und Tier darstellen.

Im Vergleich zu anderen molekularbiologischen Methoden ist das LAMP-Verfahren wegen seiner hohen Sensitivität und Spezifität eines der besten molekulardiagnostischen Verfahren zum Nachweis der humanpathogenen Protozoen *Cryptosporium* und *Toxoplasma* in Umweltproben, klinischem Material und Nahrungsmitteln. Die Anwendung der LAMP-Technik, kombiniert mit der PCR-Sequenzierung, kann zu einem schnellen Speziesnachweis, zur Differenzierung von für die Bevölkerung gesundheitsrelevanten Spezies sowie zur Identifizierung von Kontaminationsquellen dienen.

Die LAMP-Anwendung hat das Potenzial, ein Werkzeug für den schnellen Nachweis dieser wichtigen anthropozoonotischen Spezies, für die menschliche und tierische Wirte anfällig sind, zu sein. Diese Methode kann dazu beitragen, der Kontrolle und Prävention von Krankheiten zu dienen und das Gesundheitsrisiko für die Bevölkerung durch nahrungsmittel- und wasserbürtige (Oo)zysten einzuschätzen.

Teilpublikationen

1. Sotiriadou I, Karanis P. (2008) Evaluation of loop-mediated isothermal amplification for detection of *Toxoplasma gondii* in water samples and comparative findings by polymerase chain reaction and immunofluorescence test (IFT). *Diagn Microbiol Infect Dis* 62: 357-65.

2. Sotiriadou I, Pantchev N, Gassmann D, Karanis P. (2012) Molecular identification of *Giardia* and *Cryptosporidium* from dogs and cats in Germany and other European countries. (Parasite - under review Parasite).

3. Sotiriadou I, Srisuphanunt M, Wila, Karanis P. (2012) Single nucleotide polymorphism (SNP)-based loop-mediated isothermal amplification (LAMP) assay for the detection of human pathogenic *Cryptosporidium* species – clinical and environmental verification and first detection of *C. fragile* in water (ready to submit in Applied and Environmental Microbiology).

Declaration

The research work presented herewith was carried out by me under the supervision of Prof. Dr. Panagiotis Karanis at the Anatomy Institute II, Medical School, University of Cologne and Prof. Dr. Hartmut Arndt at Cologne Biocenter, Zoological Institute, University of Cologne.

Erklärung

Diese Arbeit wurde vom November 2005 bis Juni 2012 in Institut für Anatomie II der Medizinischen Fakultät der Universität zu Köln unter der Leitung von Prof. Dr. Panagiotis Karanis und von Prof. Dr. Hartmut Arndt im Biozentrum Köln der Universität zu Köln, angefertigt.

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen-, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat, dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde.
Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Panagiotis Karanis und von Prof. Dr. Hartmut Arndt betreut worden.

Köln, den 28.04.2012 **Isaia Sotiriadou**

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Education

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Visit of elementary school and high school in Kozani/Greece.

1995-1996

Studies of Bulgarian language at the University of Sofia “St. Kliment Oxridski”.

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Work Experience

2001-2003

Research Fellow at the University of Bonn/Germany; Institute for Medical Parasitology (Laboratory of Professor P. Karanis).

Research Master Thesis: Studies on the infectivity, occurrence and molecular diagnosis of *Giardia lamblia* (Supervisor: Prof. P. Karanis).

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Participation in Seminars for PhD students, Institute for Medical Parasitology, University of Bonn.

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Awarded the Degree in Biotechnology and the Degree of Master in Gene and Cell Engineering.

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2004 – Today:

Research associate at the project „Functional Genomics of Mouse Embryonic Stem (ES) cells“ (FunGenES). Institute of Neurophysiology University of Cologne. Leader: Professor J. Hescheler and Professor A. Sachinidis; http://www.fungenes.org/ as a member of the knowledge management and optimising synergy group.

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Analytical biologist and research associate and supervisor of the S2 standard laboratory of Operative Dentistry and Periodontology, University of Cologne. Director: Professor M. J. Noack.

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Japan: 11 September – 10 December 2006: National Research Center for Protozoan Parasite, University of Obihiro, Obihiro, Hokkaido, Japan.

Greece: 21 Juni -11 July, 2005: Department of Microbiology, Medical School University of Ioannina, Ioannina, Greece.

Russia: 20 - 31 May 2005: Department of Infectious Diseases, Medical School, Rostov University, Rostov, Russia.

China: 2 – 16 September 2011: Academy of Animal and Veterinary Sciences, Quinghai, Xining, China.

List of published abstracts and participations in international conferences

Abstracts:

1. Sotiriadou I, Karanis P. (2001). The course of giardiasis in mongolian gerbils. 6th National Conference of Parasitology, Sofia, 5-7 October 2001.
2. Karanis P, Karentzou E, Sotiriadou I. (2003). Histopathology of giardiasis. 28th Annual Panhellenic Medical Congress, Athens 21-25 May 2002.
3. Sotiriadou I, Kourenti C, Karanis P. (2003). Improvement of the polymerase chain reaction method (PCR) for the diagnosis of lambliasis. 29th Annual Panhellenic Medical Congress, Athens 20-24 May 2003.
4. Karanis P, Mitov R, Kourenti C, Sotiriadou I, Kartashev V, Gusarev S, Ponomarenko Y, Kushnir L, Ambalov Y. (2003). *Cryptosporidium parvum* among HIV patients in Rostov region (Southern Russia)-possible association with contaminated tap water. IWA Symposium on Health-Related Water Microbiology, Cape Town 14-19 September 2003.
5. Karanis P, Sotiriadou I, Kostova D, Mitov R. (2002). Diagnostic procedure and inactivation of *Cryptosporidium parvum* oocysts by UV light: A project summary results. International symposium of waterborne pathogens, Cascais/Lisbon, 22-25 September 2002.
6. Karanis P, Kartashev V, Sotiriadou I, Kourenti C. (2004). Investigations on *Giardia* and *Cryptosporidium* in drinking water in Rostov region (Southern Russia). Improving Research for a Common Future. 3rd German-Russian Cooperative Symposium, 13-14 Mai 2004, Cologne, Germany.
7. Karanis P, Sotiriadou I, Kostova D, Kourenti C. (2004). Detection of *Giardia* cysts and *Cryptosporidium* oocysts in water samples in Bulgaria. Improving Research for a Common Future. 3rd German-Russian Co-operative Symposium, 13-14 May 2004, Cologne, Germany.
8. Sotiriadou I, Karanis P. (2004). Development of molecular diagnostic tools for the detection of *Giardia lamblia* in water sources. 14th Japanese-German Symposium on Protozoan Diseases. 20-24.09.2004, Duesseldorf, Germany.
9. Karanis P, Kartashev V, Sotiriadou I, Kourenti C, Kostova D. (2004). *Giardia* and *Cryptosporidium* in drinking water supplies of Rostov region (Southern Russia) and Sofia (Bulgaria). 4th International *Giardia* Conference and first

combined *Giardia*/*Crypto* meeting in Amsterdam, 20-24 September, Amsterdam, Netherlands.

10. Sotiriadou I, Kostova D, Karanis P. (2005). PCR detection on *Toxoplasma*, *Giardia*, *Cryptosporidium* in common environmental and drinking water samples. 8th National Conference of Parasitology, 21-25 September 2005, Sofia, Bulgaria.
11. Karanis P, Sotiriadou I. (2006). New approaches on molecular detection of waterborne parasites. 15th European SOVE Meeting, 10-14 April, Sérres, Greece.
12. Karanis P, Dangendorf F, Sotiriadou I. (2006). An evaluation on geographical distribution of Leishmaniasis and sandflies in Greece. 15th European SOVE meeting, 10-14 April, Sérres, Greece.
13. Sotiriadou I, Dumetre A, Karanis P. (2006). Detection of *Toxoplasma* in environmental water samples by IFT and nested PCR. ICOPA XI, 11th International Congress of Parasitology, 6-11 August 2006, SECC, Glasgow, Scotland.
14. Sotiriadou I, Leetz S, Karanis P. (2006). Design of new specific *Cryptosporidium* primers and optimization of PCR conditions for oocysts detection. ICOPA XI, 11th International Congress of Parasitology, 6-11 August 2006, SECC, Glasgow, Scotland.
15. Sotiriadou I, Karanis P. (2006). Water supplies monitoring and distribution of *Giardia lamblia* in southern Russia and Bulgaria. ICOPA XI, 11th International Congress of Parasitology, 6-11 August 2006, SECC, Glasgow, Scotland.
16. Sotiriadou I, Karanis P. (2009). Detection of *Toxoplasma* by LAMP and comparative findings by Polymerase Chain Reaction and immunofluorescence test (IFT) in water samples. 35th Annual Panhellenic Medical Congress, 5-9 May 2009, Athens, Greece.
17. Sotiriadou I, Karanis P. (2006). Application of conventional and molecular tools on environmental waters for the common detection of *Cryptosporidium*, *Giardia*, and *Toxoplasma*. 15th Japanese-German Symposium on Protozoan Diseases. 16 - 24.09.2006, Obihiro, Japan.
18. Sotiriadou I, Karanis P. (2008). Comparative detection of *Toxoplasma gondii* in by LAMP, PCR and IFT. 16th Japanese-German Co-operative Symposium on Protozoan Diseases 24-29.09.2008, Göttingen, Germany.

19. Sotiriadou I, Karanis P. (2009). Detection of T*oxoplasma* by LAMP and comparative findings by polymerase chain reaction and immunofluorescence test (IFT) in water samples / Διάγνωση της τοξοπλάσμωσης με την μέθοδο της LAMP και σύγκριση της μεθόδου με την αλυσιδωτή αντίδραση της πολυμεράσης (PCR) και το τεστ ανοσοφθορισμού (IFT) σε δειγματοληψίες απο υδάτινους πόρους. 35th Annual Medical Pan-Hellenic Conference, 5-9 May, Athens, Greece.
20. Karani M, Sotiriadou I, Karanis P. (2010). New diagnostic method of the cutaneous and visceral leishmaniasis by LAMP. Νεα διαγνωστικη μεθοδος της δερματικης και σπλαχνικης λεισμανιασης με την LAMP (loop-mediated isothermal amplification). 36th Annual Medical Pan-Hellenic Conference, 4-8 May, Athens, Greece.
21. Sotiriadou I, Karanis P. (2010). Diagnosis of cryptosporidiosis by LAMP assay based on the SAM gene. Διάγνωση της κρυπτοσποριδίωσης με την LAMP (loop-mediated isothermal amplification) βασισμένη στο γονίδιο SAM (s-adenosylmethionine synthetase). 36th Annual Medical Pan-Hellenic Conference, 4-8 May, Athens, Greece.
22. Sotiriadou I, Pantchev, N. Karanis, P. (2010). Molecular characterization of *Giardia* and *Cryptosporidium* from dogs and cats in Germany. Joint Meeting of the German Societies of Parasitology and Protozoology, 17-20 March, 2010, Düsseldorf, Germany.
23. Gallas-Lindemann C, Plutzer J, Sotiriadou I, Felgenhauer N, Kosmac U, Karanis P. (2010*) Cryptosporidium* in Trinkwasserressourcen am Linken Niederrhrein: Spezies-Nachweis und Charakterisierung. 26. Jahrestagung der Deutschen Gesellschaft für Limnologie 2010. Universität Bayreuth, 27.09-01.10.2010, Germany.
24. Karani M, Sotiriadou I, Karanis P. (2011). Diagnosis of coutaneous, visceral and muco-cutaneous leishmaniasis by LAMP. 37th Annual Medical Pan-Hellenic Conference, 17-22 May, Athens, Greece, 2011.
25. Sotiriadou I, Karanis P. (2011). New improved diagnostic method for the detection of *Giardia* based on the EF1a (Elongation-factor-1-alfa) LAMP (loop-mediated isothermal amplification). 37th Annual Medical Pan-Hellenic Conference, 17-22 May, Athens, Greece 2011.

Original papers

1. Sotiriadou I, Karanis P. (2004). The course of Giardiasis in Mongolian gerbils and SCID mice infected with new strains of *Giardia lamblia.* Water Science and Technology: *Water Supply* 4: 63–72.
2. Karanis P, Chronis I, Zakas G, Kourenti C, Sotiriadou I. Papadopoulou C. (2005). A preliminary survey of the level of microbiological pollution of major rivers in northern Greece. *Acta Hydroch Hydrobiol* 33: 1-4.
3. Karanis P, Sotiriadou I, Kartashev V, Kourenti C, Tsvetkova N, Stojanova K. (2006). Occurrence of *Giardia* and *Cryptosporidium* in water supplies of Russia and Bulgaria. *Environ Res* 102: 260-271.
4. Doss MX, Winkler J, Chen S, Hippler-Altenburg R, Sotiriadou I, Halbach M, Pfannkuche K, Liang H, Schulz H, Hummel O, Hübner N, Rottscheidt R, Hescheler J, Sachinidis A. (2007). Global transcriptome analysis of murine embryonic stem cell-derived cardiomyocytes. *Genome Biol* 8: R56.
5. Leetz AS, Sotiriadou I, Ongerth J, Karanis P. (2007). An evaluation of primers amplifying DNA targets for the detection of *Cryptosporidium* spp. using *C. parvum* HNJ-1 Japanese isolate in water samples. *Parasitol Res* 101: 951-962.
6. Doss MX, Chen S, Winkler J, Hippler-Altenburg R, Odenthal M, Wickenhauser C, Balaraman S, Schulz H, Hummel O, Hübner N, Ghosh-Choudhury N, Sotiriadou I, Hescheler J, Sachinidis A. (2007). Transcriptomic and phenotypic analysis of murine embryonic stem cell derived BMP2+ lineage cells: an insight into mesodermal patterning. *Genome Biol* 8: R184.
7. Sachinidis A, Sotiriadou I, Seelig B, Berkessel A, Hescheler J. (2008). A chemical genetics approach for specific differentiation of stem cells to somatic cells: a new promising therapeutical approach. *Comb Chem High Throughput Screen* 11: 70-82.
8. Sotiriadou I, Karanis P. (2008) Evaluation of loop-mediated isothermal amplification for detection of *Toxoplasma gondii* in water samples and comparative findings by polymerase chain reaction and immunofluorescence test (IFT). *Diagn Microbiol Infect Dis* 62: 357-65.
9. Akasha AA, Sotiriadou I, Doss MX, Halbach M, Winkler J, Baunach JJ, Katsen-Globa A, Zimmermann H, Choo Y, Hescheler J, Sachinidis A. (2008) Entrapment of embryonic stem cells-derived cardiomyocytes in macroporous

biodegradable microspheres: preparation and characterization. *Cell Physiol Biochem* 22: 665-72.

10. Koloren Z, Sotiriadou I, Karanis P. (2011) Investigations and comparative detection of *Cryptosporidium* species by microscopy, nested PCR and LAMP in water supplies of Ordu, Middle Black Sea, Turkey. *Ann Trop Med Parasitol* 105: 607–615.
11. Sotiriadou I, Pantchev N, Gassmann D, Karanis P. (2011) Molecular identification of *Giardia* and *Cryptosporidium* from dogs and cats in Germany and other European countries. Parasite (under review).
12. Gallas-Lindemann C, Sotiriadou I, Mahmoodi Mohammad R, Karanis P. (2012) Detection of *Toxoplasma gondii* oocysts in sewage water by Loop Mediated Isothermal Amplification (LAMP). Acta Tropica (in press).
13. Karani M, Sotiriadou I, Plutzer J, Karanis P. (2012) Unified Loop-mediated isothermal amplification assay for the detection of *Leishmania* promastigotes. Pathogens and Global Health (submitted).
14. Gallas-Lindemann C, Sotiriadou I, Plutzer J, Karanis P. (2012) Circulation of *Cryptosporidium* and *Giardia* in wastewater and the surface, drinking and ground waters in the Lower Rhine, Germany. Epidemiology and Infection (in press).
15. Gallas-Lindemann C, Sotiriadou I, Plutzer J. Karanis P. (2012) *Giardia* and *Cryptosporidium* species dissemination during wastewater treatment and comparative detection by IFT, nPCR and LAMP. Zoonoses and Public Health (in press).

Nucleotide sequences

78 Nucleotide sequences registered at the GenBank.

Oral Presentations

1. Sotiriadou I, Karanis P. (2005) Third Expert Working Group-Microbial Quality Indicators. Maisons Laffitte. 1-14 March 2005, Web-based European Knowledge Network on Water. Paris, France.
2. Sotiriadou I, Karanis P. (2006). Application of conventional and molecular tools on environmental waters for the common detection of *Cryptosporidium*, *Giardia* and *Toxoplasma*. 15th Japanese-German Symposium on Protozoan Diseases. 16. - 24.09.2006, Obihiro, Japan.
3. Sotiriadou I, Karanis P. (2008). Comparative detection of *Toxoplasma gondii* by LAMP, PCR and IFT. 16th Japanese-German Cooperative Symposium on Protozoan Diseases. 24-29.09.2008, Göttingen, Germany.
4. Sotiriadou I, Karanis P. (2009) Detection of T*oxoplasma* by LAMP and comparative findings by polymerase chain reaction and immunofluorescence test (IFT) in water samples. 35th Annual Medical Pan-Hellenic Conference, 5-9 May, Athens, Greece.
5. Sotiriadou I, Karanis P. (2011) A new method for *Toxoplasma* oocysts in water. Xining China. 2 - 16September 2006.

Honors and Awards

2009 **Price for excellence in pure or applied research at the annual medical conference in Greece:** Sotiriadou I, Karanis P. (2009). Detection of T*oxoplasma* by LAMP and comparative findings by polymerase chain reaction and immunofluorescence test (IFT) in water samples / Διαγνωση της τοξοπλάσμωσης με την μέθοδο της LAMP και σύγκριση της μεθόδου με την αλυσιδωτή αντίδραση της πολυμεράσης (PCR) και το τεστ ανοσοφθορισμού (IFT) σε δειγματοληψίες απο υδάτινους πόρους. 35th Annual Medical Pan-Hellenic Conference, 5-9 May, Athens, Greece.

2010 **Price for excellence in pure or applied research at the annual medical conference in Greece:** Karani, M., Sotiriadou I, Karanis P. (2010). New diagnostic method of the cutaneous and visceral leishmaniasis by LAMP. Νεα διαγνωστική μέθοδος της δερματικής και σπλαχνικής λεισμανίασης με την LAMP (loop-mediated isothermal amplification). 36th Annual Medical Pan-Hellenic Conference, 4-8 May, Athens, Greece.