

**JUDIT PLUTZER**

***Cryptosporidium* and *Giardia* as water  
contaminant pathogens in Hungary**

Ph.D. advisers:

Dr. Károly Márialigeti, C.Sc.

Eötvös Loránd University, Department of Microbiology, Budapest

Dr. Andrea Törökné, Ph.D.

National Institute of Environmental Health, Department of Water Biology, Budapest

Prof. Dr. Panagiotis Karanis

Obihiro University, National Research Center for Protozoan Diseases, Obihiro, Japan

Faculty of Medicine, University of Cologne, Department of Anatomy II, Medical and

Molecular Parasitology, Germany

Eötvös Loránd University, Biology Doctoral School

Head of Doctoral School: Prof. Dr. Anna Erdei

Zootaxonomy, Zoocology, Hydrobiology Doctoral Program

Head of Doctoral Program: Prof. Dr. Klára Dózsa-Farkas

National Institute of Environmental Health, Department of Water Biology, Budapest,  
Hungary

Obihiro University for Agriculture and Veterinary Medicine, National Research Center  
for Protozoan Diseases, Obihiro, Japan

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## PUBLICATIONS

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Plutzer J. (2001) The detection methods of *Giardia* and *Cryptosporidium* parasites in water. Duna-Tisza Medence Víz és Környezetvédelmi Nemzetközi Konferencia kiadványa, 599-603. (In Hungarian).

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Plutzer J, Takó MH, Márialigeti K, Törökné A, Karanis P. (2007) First investigations into the prevalence of *Cryptosporidium* and *Giardia* spp. in Hungarian drinking waters. J Water Health. 5: 573–584. („ISI confirmed in March 2007 that the Journal of Water and Health has been accepted for inclusion in the Science Citation Index Expanded and Current Contents/Agriculture, Biology and Environmental Sciences starting with volume 5 (1) 2007. Because of the way the impact factor is calculated (2 years of citations), they will not have a formal Figure for 2 years” IF about 1.5 – 2.5)

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Plutzer J, Törökné A, Szénási Z, Kucsra I, Farkas K, Karanis P. (2008) Epidemiological study on *Giardia* in two Hungarian villages and genotype analysis of the *Giardia* isolates detected in drinking water and in humans. Environ Res. Under review.

### Publications related to this study

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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. *IF: 1.14*

Burenbaatar B, Bakheit MA, Plutzer J, Suzuki N, Igarashi I, Ongerth J, Karanis P. (2008) Prevalence and genotyping of *Cryptosporidium* species from farm animals in Mongolia. Parasitol Res. 102: 901-905. *IF: 1.14*

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### **International conferences**

Törökné A, Plutzer J. (2002) First data on *Cryptosporidium* and *Giardia* survey in Hungary. Waterborne pathogens, AWWA conference, Cascais, Portugal. Poster. Abstract band.

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## ABSTRACT

In this thesis we investigated the prevalence of *Cryptosporidium* and *Giardia* species and analysed the genotypes from different water sources in Hungary: surface and sewage water affecting the quality of the raw water for drinking water production and the drinking water treatment plants themselves, all together 269 water samples. (Oo)cysts densities were associated with water receiving effluents of sewage treatment plants or originating from a forest environment. The detected species and genotypes are all categorized to be human pathogenic: *Giardia duodenalis* Assemblage A, Assemblage B, *Cryptosporidium parvum* and *Cryptosporidium meleagridis*. The riverbank filtrated water and the River Danube at Budapest were monitored for *Cryptosporidium* and *Giardia* (oo)cysts, which were detected regularly in the river water but never in riverbank filtrated water confirming the effectiveness of riverbank filtration as a drinking water purification method.

Monitoring and characterization of oocyst contamination sources in watersheds were also aided. 79 faecal samples from calves with diarrhoea were collected on 52 farms from different counties in Hungary. The sequence and phylogenic analysis of 21 isolates of the gp-60 PCR products showed that the most common *Cryptosporidium parvum* subtype is IIaA16G1R1. Interestingly, two isolates belonged to the *C. parvum* IId subtype group in addition to a new *C. parvum* IIa A18G1R1 subgenotype for the first time described in the *C. parvum*-complex. The findings suggest that cattle can be a source of *Cryptosporidium* infections for humans and mammals in Hungary.

The association between the consumption of *Giardia* positive drinking water and asymptomatic giardiasis has been investigated. Two hundred stool samples from inhabitants of Füzér and Mátrafüred and drinking water sources were examined by immunological and molecular methods for the presence of *Giardia* infections. One hundred stool samples have been examined from Budapest city as a control, since *Giardia* cysts have never been detected by routine examinations in the drinking water of Budapest. Individuals were asked to fill out a validated questionnaire at the time of stool collection. We found a prevalence rate of 4% of *Giardia duodenalis* infections of asymptomatic people in the village Füzér. In both water samples and human patients *Giardia* Assemblage B were detected. The results demonstrate a specific epidemiological situation, giving essential evidence about giardiasis in asymptomatic carriers.

The status of *Giardia* and *Cryptosporidium* threats to drinking water and aquatic ecosystems was uncertain in Hungary and by the outcomes of the above studies it has been clarified to some extent. The presented results will contribute a lot to better understanding the epidemiology and relevance of waterborne parasites, their surveillance and performance of future control measures to prevent waterborne infections in Hungary.

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## **SYMBOLS AND ABBREVIATIONS**

### Symbols

°C                      degrees Celsius

%                      percent

### Units of weights and measure

µg                      microgram

µL                      microlitre

µM                      micromole

g                      gram

hr                      hour

kDa                      kilo Dalton

km                      kilometre

km<sup>2</sup>                      square kilometre

L                      litre

m                      meter

min                      minute

mL                      millilitre

mM                      millimole

nmole                      nanomole

sec                      second

### Other abbreviations

BSA                      Bovine Serum Albumin

CFU                      Colony Forming Unit

Ct value	concentration × exposure time
DAPI	2-(4-amidinophenyl)-6-indolecarbamide dihydrochloride
DIC	Diffential Interference Contrast
DWI	Drinking Water Inspectorate (United Kingdom)
FITC	Fluoresceine Isothiocyanate
GDH	Glutamate Dehydrogenase
gp-60 gene	=Cp <sub>gp15/45</sub> , encodes a precursor protein that is proteolytically cleaved to yield mature cell surface glycoproteins gp45 and gp15 (also known as Cp17)
ICZN	International Code of Zoological Nomenclature
IFT	Immuno Fluorescence Test
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IMS	Immuno Magnetic Separation
LB	Luria- Bertani
USEPA	United States Environmental Protection Agency
UV	Ultraviolet light
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RBF	Riverbank Filtration
RFLP	Restriction Fragment Length Polymorphism
SSU rRNA	Small Ribosomal Subunit
TPI	Triose Phosphate Isomerase
Tween 20	Polyoxyethylene (20) sorbitan monolaurate

## 1. INTRODUCTION

### 1.1. Biology of *Cryptosporidium*

#### 1.1.1. *Cryptosporidium* taxonomy

While *Cryptosporidium* is classified within the coccidian parasites, recent investigations into the biological, morphological and phylogenetic characteristics of this parasite indicate that the genus was an early emerging lineage among the Apicomplexa and it is more closely related to the Gregarina, than to the Coccidia (Carreno et al. 1999, Hijjawi et al. 2002, Rosales et al. 2005). Therefore the taxonomic determination is more accurate by linking statements: *Cryptosporidium* spp. belongs to the Phylum Apicomplexa (=Sporozoa) (which possess an apical complex), Class Sporozoa (which reproduce by asexual and sexual cycles), Subclass Coccidia (the life cycle of which involves merogony, gametogony, and sporogony), Order Eucoccidiida (=Eucoccidiorida) (in which schizogony occurs), Suborder Eimeriina (=Eimeriorina) (in which independent micro and macrogamy develop), and Family Cryptosporidiidae (in which there are four naked sporozoites within oocysts). Unique features that distinguish *Cryptosporidium* from other coccidian include resistance to antiparasitic agents, capacity for ‘autoinfection’, and the location that it occupies within the host cell membrane.

The taxonomy of the genus *Cryptosporidium* – as it is the case for many other protozoan parasites – is still unsatisfactory and is undergoing major revisions in the light of new developmental, biochemical and genetic data, since oocyst morphology has been repeatedly shown to lack valuable species specific informative characters (Fall et al. 2003). When naming new species of *Cryptosporidium*, the following requirements should be fulfilled: to provide morphometric (i.e. size and morphology) data on oocysts; to provide genetic characterization; to demonstrate natural and, when feasible, experimental host specificity; and to comply with ICZN rules (Egyed et al. 2003, Xiao et al. 2004a). The nowadays accepted 17 *Cryptosporidium* species are shown in Table 1.

### 1.1.2. Life cycle of *Cryptosporidium* spp.

*Cryptosporidium* spp. have a complex, monoxenous life-cycle where all stages of development occur within one host. Infection begins when oocysts are ingested. As little as one oocyst can produce infection in a susceptible host (Pereira et al. 2002). Mature oocysts contain 4 sporozoites and excystation (to liberate sporozoites) is possibly triggered by a combination of environmental conditions such as pH, bile salts, carbon dioxide and temperature (Fayer and Leek 1984). The free sporozoites attach to epithelial cells and differentiate asexually to become a trophozoite. Electron microscopy has confirmed that the trophozoite has an intracellular, extracytoplasmic location within vacuoles and they contain a unique 'feeder' structure at the base of each vacuole. This organelle is thought to mediate nutrient uptake from the cell (Current and Reese 1986). Trophozoite then undergoes asexual division to produce meronts. Meronts produce 6-8 merozoites that can infect another host cell and develop into a type II meront. Type II meronts initiate sexual multiplication by differentiating into either a microgamont (male) or macrogamont (female). Upon fertilization, zygotes differentiate into mature oocysts, which sporulate in situ. The majority of oocysts are released into the gastrointestinal tract as environmentally stable, thick walled oocysts, which are excreted in faeces are capable of infecting other hosts. The remainder are thin walled oocysts capable of beginning a new cycle within the host (Clark 1999, Current and Reese 1986, Gookin et al. 2002, O'Donoghue, 1995). The life cycle of *C. parvum* is shown in Figure 1.

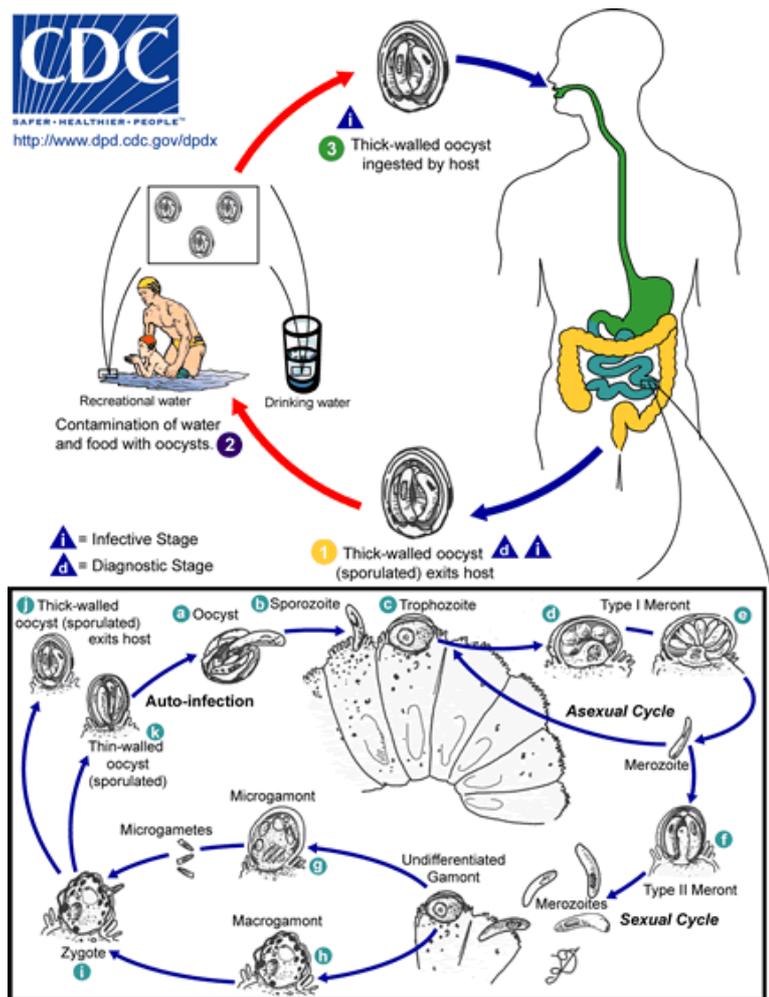


Figure 1: Life cycle of *Cryptosporidium parvum*.

### 1.1.3. *Cryptosporidium* species, genotypes and subgenotypes

#### *Cryptosporidium* species

**Mammals:** Mammals represent the largest group of animals known to be infected with *Cryptosporidium* spp., probably due to the greater number of studies as a result of the perceived importance of these animals. The taxonomy of *Cryptosporidium* in mammals has been the subject of scientific debate since 1980, and for some time only two species (*C. parvum* as the intestinal species and *C. muris* as the gastric species) were recognized. We now know that there is enormous biological and genetic diversity in mammalian *Cryptosporidium* spp., and because of a plethora of molecular studies, many new species

have been discovered and described (Xiao et al. 2004a). However, what is a species and what is a genotype within a species, regarding *Cryptosporidium* remains a future task to be clarified.

In 1907, Ernest Edward Tyzzer described firstly the *Cryptosporidium muris* that he frequently observed in the gastric glands of laboratory mice (Tyzzer 1907). Studies have shown *C. muris* to be capable of infecting a wide range of additional hosts: hamsters, squirrels, Siberian chipmunks, wood mice, bank voles, rock hyrax, Bactrian camel, ringed seal, bilby, mountain goats, cynomolgus monkeys, human, pig, cat (Chalmers et al. 1997, Torres et al. 2000, Dubey et al. 2002, Hurková et al. 2003, Palmer et al. 2003, Xiao et al. 2004a, Gatei et al. 2006, Feng et al. 2007a, Pavlasek and Ryan 2007, Zintl et al. 2007).

The most frequently reported species in mammals is *C. parvum* and it was first found in mice (Tyzzer 1912). It was differentiated from *C. muris* based on its smaller oocyst size and its location. A lot of species of mammals served as hosts of *C. parvum*. Most descriptions, however, have been based solely on microscopy, with no careful morphometric measurements, transmission experiments, genetic and/or other biological data. Recent molecular characterizations have shown that there is extensive host adaptation in *Cryptosporidium* evolution, and many mammals or groups of mammals harbour host-adapted *Cryptosporidium* genotypes, which differ from each other in both DNA sequences and infectivity (Xiao et al. 1999a, 2000a,b, 2002, Ryan et al. 2003a, 2005, Abe et al. 2004, Atwill et al. 2004, Zhou et al. 2004a, Power et al. 2004, Santin et al. 2004, Feng et al. 2007a, Gaydos et al. 2007, Karanis et al. 2007a, Nagano et al. 2007, Santin et al. 2007) . The species name of *C. parvum* has been suggested to use for the *Cryptosporidium* parasites previously known as the bovine genotype and to avoid the usage of *C. parvum* broadly for *Cryptosporidium* in mammals. Thus far, *C. parvum* is known to infect ruminants (mainly cattle), horse, mouse, wild animals (eastern grey squirrel, raccoon dog) and human (Slapeta 2006, Xiao et al. 2007a, Feng et al. 2007a).

Oocysts of *C. bovis*, previously identified as *Cryptosporidium* genotype bovine B morphologically indistinguishable from those of *C. parvum*. Multilocus analysis of 3 unlinked loci demonstrated the new species to be distinct from *C. parvum* and also demonstrated a lack of recombination, providing further evidence of species status (Fayer et al. 2005).

*C. suis* oocysts have been structurally indistinguishable from those of *C. parvum*, but genetically distinct from all known species and genotypes of *Cryptosporidium* (Ryan et al. 2004a). It primarily infects pigs, but it also has been diagnosed in human patients (Caccio 2005, Hammes et al. 2007).

*C. canis* oocysts have been observed in the faeces of dogs but they also have been found in human (Fayer et al. 2001, Pedraza-Diaz et al. 2001, Xiao et al. 2007b).

*C. felis* in cats included a description of the oocyst from the faeces (Iseki 1979). *C. felis* infections have been confirmed also in cattle and in humans (Morgan et al. 1998, Bornay-Llinares et al. 1999, Pieniazek et al. 1999).

*C. wrairi* has been reported to infect only small guinea pigs weighing 200 to 300 g (Jervis et al. 1966, Vetterling et al. 1971).

*C. andersoni* infects the abomasum of cattle and produces oocysts morphologically similar to, but slightly smaller than, those of *C. muris*. Chronic infection with *C. andersoni* has been associated with gastritis, reduced milk yield and poor weight gain in adult cattle (Masuno et al. 2006, Robinson et al. 2006). *C. andersoni* invades the glandular stomach of several hosts from Artiodactyla and Rodentia (Lindsay et al. 2000, Kvác et al. 2007). The potential for the zoonotic transmission of *C. andersoni* is unknown, however *C. andersoni* infections have been detected in humans (Leoni et al. 2006).

The morphology and infectivity of the oocysts of a new species from the faeces of the red kangaroo (*Macropus rufus*) were recently described. Oocysts are structurally indistinguishable from those of *Cryptosporidium parvum*. Based on biological and molecular data, this *Cryptosporidium* infecting marsupials was proposed to be a new species *C. fayeri* (Ryan et al. 2008).

*Cryptosporidium* parasites infecting humans are well studied. Previously designated *C. parvum* human genotype, genotype 1, or genotype H has been recently delineated as a separate species, *C. hominis* based on molecular and biological differences (Morgan-Ryan et al. 2002). More studies during the last years showed not only a plethora of genetic and biological differences but also largely a lack of genetic exchange between this parasite (*C. hominis*, human genotype or genotype I) and *C. parvum* (bovine genotype or genotype 2). *C. hominis* oocysts are morphologically identical to *C. parvum*, 4.6 to 5.4 by 3.8 to 4.7  $\mu\text{m}$  (mean, 4.2  $\mu\text{m}$ ) with a length/width ratio of 1.21 to 1.15 (mean, 1.19). Unlike *C. parvum*, *C. hominis* has been previously considered non-infective for mice, rats, cats, dogs, cattle,

and immunosuppressed gerbils (Peng et al. 1997, Widmer et al. 2000, Giles et al. 2001, Akiyoshi et al. 2002, Morgan-Ryan et al. 2002). However, *C. hominis* has been reported from a dugong, lamb and cattle, the calves, lambs, and piglets can also be infected experimentally with at least some *C. hominis* isolates at high doses (Morgan et al. 2000, Akiyoshi et al. 2002, Ebeid et al. 2003, Baishanbo et al. 2005). Pathogenicity studies with gnotobiotic pigs have shown the prepatent period to be longer than for *C. parvum* (8.8 and 5.4 days, respectively) and have also shown differences in parasite-associated lesion distribution and intensity of infection (Morgan-Ryan et al. 2002). *C. hominis* and *C. parvum* showed different biological activities in cell culture (Hijjawi et al. 2001). There appear to be distinct differences in oocyst shedding patterns between *C. hominis* and *C. parvum* in humans. A study in the United Kingdom revealed that *C. hominis* was detected in a significantly greater proportion of samples with larger numbers of oocysts whereas *C. parvum* was detected in a significantly greater proportion of the samples with small numbers of oocysts (McLauchlin et al. 1999). Another study in Lima, Peru, reported that the duration of oocyst shedding in stool from humans was significantly longer and the intensity of infections was significantly higher during *C. hominis* infections (Xiao et al. 2001). A cross-sectional study to determine the epidemiology of *Cryptosporidium* in human immunodeficiency virus (HIV)-infected persons showed that only infections with *C. canis*, *C. felis*, and subtype group Id of *C. hominis* were associated with diarrhoea, and infection with *C. parvum* was associated with chronic diarrhoea and vomiting (Cama et al. 2007). There are also distinct geographical and temporal variations in the distribution of *C. parvum* and *C. hominis* infections in humans. In patients in the United Kingdom, *C. parvum* was more common during the spring time, whereas *C. hominis* was more common in late summer and autumn in those with a history of foreign travel (McLauchlin et al. 2000). Genetic characterization of *C. hominis* and *C. parvum* has consistently demonstrated distinct differences between the two species at a wide range of loci. There are also fundamental differences in ribosomal gene expression between *C. hominis* and *C. parvum*, since the latter constitutively expresses two types of rRNA genes (type A and type B) whereas more than two transcripts have been detected in *C. hominis* (Xiao et al. 2004a). In addition, despite the large number of isolates examined at multiple unlinked loci from a wide range of geographical locations, putative recombinants between *C. hominis* and *C. parvum* have never been explicitly identified (Mallon et al. 2003). Although some

interspecific recombination has suggested by several research groups (Strong et al. 2000, Feng et al. 2002), the significance or extent of any recombination is not yet clear. If recombination between species does occur, it seems to be very limited.

**Birds:** Although infections have been found in a variety of birds (Sréter and Varga 2000), only three avian *Cryptosporidium* spp. have been named: *C. meleagridis*, *C. baileyi*, and *C. galli*. These three *Cryptosporidium* spp. can each infect a broad range of birds, but they differ in predilection sites. Even though both *C. meleagridis* and *C. baileyi* are found in the small and large intestine and bursa, they differ significantly in oocyst size and only *C. baileyi* is also found in the respiratory tissues such as the conjunctiva, sinus and trachea. In contrast, *C. galli* infects only the proventriculus (Xiao et al. 2004a). *C. meleagridis* is apparently a misnomer since it infects other avian hosts (parrots, broiler chicken, red-legged partridge, cockatiels), not just turkeys and also was found in dog, deer mouse and human (Morgan et al. 2001, Abe and Iseki 2004, Feng et al. 2007a, Hung et al. 2007, Llorente et al. 2007, Pages-Mante et al. 2007, Soltane et al. 2007). *C. baileyi* originally isolated from commercial broiler chickens and became probably the most common avian *Cryptosporidium* sp. and has so far been found in chicken, turkeys, ducks, domestic geese, cockatiels, a brown quail, pullet, whooping crane, gray-bellied bulbul (Morgan et al. 2001, Abe and Iseki 2004, Hajdusek et al. 2004, Xiao et al. 2004a, Chvala et al. 2006, Ng et al. 2006). *C. galli* was first found in hens (Pavlassek 1999). The parasite has recently been redescribed on the basis of both molecular and biological differences (Ryan et al. 2003b). Confirmed hosts of *C. galli* include finches, parrots, canaries, glossters, domestic chickens, capercaille and pine grosbeaks (Ryan et al. 2003b, Ng et al. 2006). Morphologically similar oocysts have been observed in a variety of exotic and wild birds including members of the Phasianidae, Passeriformes, and Icteridae (Ryan et al. 2003b).

Based on limited biological and molecular studies, it appears that several other avian *Cryptosporidium* spp. are distinct species as well (Morgan et al. 2001, Xiao et al. 2002, Ryan et al. 2003a,b, Jellison et al. 2004, Zhou et al. 2004b, Meireles et al. 2006, Ng et al. 2006).

**Reptiles:** *Cryptosporidium* infections are common in reptiles and have been reported in several reptilian species (O'Donoghue 1995). Two main *Cryptosporidium* spp. are recognized in reptiles: *C. serpentis* is a gastric parasite mainly in snakes and *C. saurophilum* is an intestinal parasite mainly in lizards (Morgan et al. 1999a, Xiao et al.

2004b). Even though *C. saurophilum* was originally described as a lizard parasite, it has been found in two captive snakes in Missouri and in three snakes in St. Louis Zoo (Xiao et al. 2004b). Two adult corn snakes from a private snake breeding colony have been presented to the veterinarian for examination of clinical signs included diarrhoea and persistent postprandial regurgitation. PCR-RFLP analysis and sequencing of SSU rRNA and actin genes enabled a distinct molecular characterization of the infecting organism as *C. saurophilum* (Plutzer and Karanis 2007a). A group of six snakes housed together with four lizards in the same room in Maryland also had *C. saurophilum* infections, but with much lower intensity than the infection of the four lizards and all snakes were infected with multiple *Cryptosporidium* spp. (Xiao et al. 2004b). According to Pavlasek and Ryan (2008) *C. varanii* was described prior to *C. saurophilum*, it takes precedence over *C. saurophilum* and therefore *C. saurophilum* should be considered a junior synonym of *C. varanii*. Studies on isolates recovered from wild and captive animals indicated that other and new *Cryptosporidium* spp. also exist in reptiles: a tortoise genotype, snake genotypes, and another *Cryptosporidium* genotype from a lizard, which was genetically distinct but was related to *C. serpentis* (Xiao et al. 2004b, 2002, Alves et al. 2005, Traversa et al. 2008). The zoonotic implication of *Cryptosporidium* species from tortoises has been reported (Traversa et al. 2008).

**Fishes:** Little is known about the prevalence or geographic distribution of *Cryptosporidium* isolates that infect fish. Two named species of *Cryptosporidium* have been found in fish *C. molnari*, *C. scophthalmi*. *Cryptosporidium* infection in gilthead sea bream and European sea bass from the Atlantic, Cantabric, and Mediterranean coasts of Spain was studied. The species was named in honour of the Hungarian parasitologist Kálmán Molnár because of his extensive contribution to fish parasitology (Alvarez-Pellitero and Sitjà-Bobadilla 2002). Ryan et al. (2004b) presented histological, genetic, and phylogenetic analyses of a *C. molnari*-like isolate from a guppy (*Poecilia reticulata*). *C. scophthalmi* was described from the turbot (*Scophthalmus maximus*), sampled from different fish farms on the coast of Spain (Alvarez-Pellitero et al. 2004).

<b>Species</b>	<b>Major hosts</b>	<b>Site of infection</b>	<b>Size of oocysts (in <math>\mu\text{m}</math>)</b>	<b>GeneBank accession number (18S rRNA)</b>
<i>C. andersoni</i> Lindsay et al. 2000	Cattle, bactrian camel	Abomasum	$5.5 \times 7.4$	AF093496
<i>C. baileyi</i> Current et al. 1986	Poultry	Bursa	$4.6 \times 6.2$	L19068
<i>C. bovis</i> Fayer et al. 2005	Cattle	Small intestine	$4.7-5.3 \times 4.2-4.8$	AY741305
<i>C. canis</i> Fayer et al. 2001	Dogs	Small intestine	$4.5 \times 4.7$	AF112576
<i>C. fayeri</i> Ryan et al. 2008	Red kangaroo	Small intestine	$4.5 \times 5.1$	AF159112 AF112570
<i>C. felis</i> Iseki 1979	Cats	Small intestine	$4.5 \times 5.0$	AF108862
<i>C. galli</i> Pavlašek 1999	Finches, chicken	Proventriculus	$8.25 \times 6.3$	AF316624 +AY168847
<i>C. hominis</i> Morgan-Ryan et al. 2002	Human	Small intestine	$4.5 \times 5.5$	AF108865
<i>C. meleagridis</i> Slavin 1955	Turkey, humans	Small intestine	$4.5-4.0 \times 4.6-5.2$	AF112574
<i>C. molnari</i> Alvarez-Pellitero and Sitja-Bobadilla 2002	Fish	Stomach (and intestine)	$4.7 \times 4.5$	AY524773
<i>C. muris</i> Tyzzer 1907	Rodents	Stomach	$5.6 \times 7.4$	AB089284
<i>C. parvum</i> Tyzzer 1912	Cattle, livestock, humans	Small intestine	$4.5 \times 5.5$	AF112571
<i>C. saurophilum</i> Koudela and Modry 1998	Lizards, snakes	Stomach and small intestine	$4.2-5.2 \times 4.4-5.6$	AF112573
<i>C. scophthalmi</i> Alvarez-Pellitero et al. 2004	Fish	Intestine (and stomach)	$3.7-5.0 \times 3.0-4.7$	Not available
<i>C. serpentis</i> Levine 1980	Lizards, snakes	Stomach	$5.6-6.6 \times 4.8-5.6$	AF151376
<i>C. suis</i> Ryan et al. 2004a	Pigs	Small and large intestine	$4.9-4.4 \times 4.0-4.3$	AF115377
<i>C. wrairi</i> Vetterling et al. 1971	Guinea pigs	Small intestine	$4.9-5.0 \times 4.8-5.6$	AF115378

**Table 1:** Valid *Cryptosporidium* species, hosts, organ locations, morphometric characters of oocysts and reference SSU rRNA GenBank accession numbers. (Updated: May 2008.)

## ***Cryptosporidium* genotypes**

This is a time of rapid data accumulation, much of it molecular, which is impacting the genus *Cryptosporidium*. Identifying an isolate or group of organisms within this genus as a genotype exemplifies the incompleteness of knowledge about the isolate while recognizing its uniqueness. Currently, *Cryptosporidium* genotypes are named after substantial sequence differences found in the small-subunit rRNA or other genes such as actin and the 70-kDa heat shock protein. Typically, these differences are greater than or comparable to those between established genotypes that became species. This naming is done after phylogenetic analysis has eliminated the possibility that the differences are because of heterogeneity between copies of the gene or intragenotypic variations. A genotype is not a taxon; it is a partial and temporary descriptor and is the best that *Cryptosporidium* molecular taxonomy has at the present time. When more data become available, a taxon designation might be made with some assurance.

Nearly 54 *Cryptosporidium* genotypes with uncertain species status have been collectively found according to the SSUrRNA sequences, which are shown in Table 2 (Xiao et al. 2004a, Appelbee et al. 2005, Hunter and Thompson 2005). Of these the cervine, monkey and pig genotype II have already been found in humans (Cama et al. 2003, Xiao et al. 2004a, Soba et al. 2006). Limited cross-transmission studies have shown biological differences among some of the genotypes, some of which have even shown oocyst morphology different from that of *C. parvum*.

<b>Genotype name (other hosts)</b>	<b>Reference</b>	<b>GeneBank accession number (18SrRNA)</b>
Bear genotype (Black bear)	Xiao et al. 2000a	AF247535
Beaver genotype	Feng et al. 2007a	EF641022
<i>C. andersoni</i> -like genotype (Wild yak and cattle)	Karanis et al. 2007a Nagano et al. 2007	EF613341
<i>C. bovis</i> -like genotype 1 (sheep)	Santin et al. 2007	EF362478-81
<i>C. bovis</i> -like genotype 2 (yak, goat)	Feng et al. 2007b Karanis et al. 2007a	DQ871346
<i>C. muris</i> -like (Japanese field mouse)	Hikosaka and Nakai 2005	AY642591
Cervine genotype 1 (blesbok, nyala, deer, sheep, lemur, muflon, squirrel, chipmunk, beaver, woodchuck, deer)	da Silva et al. 2003	AF442484

mouse, raccoon, human )		
Cervine genotype 2 (human, ibex)	Feltus et al. 2006 Xiao et al. 2000b	DQ640639
Chipmunk genotype 1 (squirrel, deer mouse)	Feng et al. 2007a	EF641026
Chipmunk genotype 2	Feng et al. 2007a	EU096238
Coyote genotype	Xiao et al. 2002	AY120909
Deer genotype	Xiao et al. 2002	AY120910
Deer-like genotype (cattle)	Santin et al. 2004	AY587166
Deer mouse genotype 1	Xiao et al. 2002	AY120905
Deer mouse genotype 2	Feng et al. 2007a	EF641027
Deer mouse genotype 3 (squirrel)	Feng et al. 2007a	EF641014
Deer mouse genotype 4	Feng et al. 2007a	EF641019
Duck genotype 1 (Black duck)	Morgan et al. 2001	AF316630
Duck genotype 2 (Canada geese)	Zhou et al. 2004b	AY504514
Ermine genotype 1	Xiao et al. 2000b Feng et al. 2007a	AF262331
Ferret genotype	Xiao et al. 1999a	AF112572
Ferret-like genotype (Otter)	Gaydos et al. 2007	DQ288166
Fox genotype 1	Xiao et al. 2002	AY120907
Fox genotype 2	Xiao et al. 2002	AY120908
Goat genotype (Capra hircus)	Karanis et al. 2007a	EF613339
Goose genotype 1 (Canada geese)	Xiao et al. 2002	AY120912
Goose genotype 2 (Canada geese)	Zhou et al. 2004b	AY504512
Goose genotype 3 (Canada geese)	Zhou et al. 2004b	AY504513
Goose genotype 4 (Canada geese)	Jellison et al. 2004	AY324638
Goose genotype 5 (Canada geese)	Jellison et al. 2004	AY324641
Horse genotype (Prezewalski's horse)	Ryan et al. 2003a	AY273770
Lizard genotype	Xiao et al. 1999a	Not available
Marsupial genotype 1 (Koala)	Morgan et al. 1999b	AF108860
Marsupial genotype 2 (Eastern grey kangaroo)	Power et al. 2004	AF513227
Mink genotype	Feng et al. 2007a	EF641015
Mongoose genotype	Abe et al. 2004	AB102769
Monkey genotype	Xiao et al. 1999a	AF112569
Mouse genotype 1 (rat)	Xiao et al. 1999a	AF112571
Mouse genotype 2 (wild Australian mouse)	Foo et al. 2007	EF546483
Muskrat genotype 1 (muskrat, vole)	Xiao et al. 2002	AY120904
Muskrat genotype 2 (fox, vole)	Zhou et al. 2004a	AY545547
Opossum genotype 1	Xiao et al. 2002	AY120902
Opossum genotype 2	Xiao et al. 2000b	AF262334
Ostrich genotype	Meireles et al. 2006	DQ002931
Pig genotype	Ryan et al. 2003c	AY271721
Rabbit genotype	Xiao et al. 2002	AY120901
Sheep genotype	Ryan et al. 2005b	AY898790

Shrew genotype (wildebeest)	Jiang et al. 2005b Feng et al. 2007a	EF641010
Skunk genotype (raccoon, squirrel, opossum, river otter)	Xiao et al. 2002	AY120903
Snake genotype	Xiao et al. 1999b	AF093499
Squirrel genotype	Atwill et al. 2004	AY462232
Tortoise genotype (star tortoise)	Xiao et al. 2002	AY120914
Vole genotype	Jiang et al. 2005b Feng et al. 2007a	EF641020
Woodcock genotype (Eurasian woodcock)	Ryan et al. 2003a	AY273769

**Table 2:** Genotypes of *Cryptosporidium* reported in the international literature.  
(Updated: May 2008.)

### ***Cryptosporidium* subgenotypes**

The occurrence *C. hominis* and *C. parvum* in humans has provided evidence that both anthroponotic and zoonotic cycles can occur in human infections. The gp-60 gene (also known as Cpgp15/45) encodes a precursor protein that is proteolytically cleaved to yield mature cell surface glycoproteins gp45 and gp15 (also known as Cp17), both of which are implicated in zoite attachment to and invasion of enterocytes (Strong et al. 2000). An important feature of this gene is its high degree of sequence polymorphism among *C. parvum* and *C. hominis* isolates and there were identified several subtype groups in both species: Ia, Ib, Id, Ie, If subtype groups in *C. hominis*, and 2 zoonotic (IIa, IIc) and 8 anthroponotic (IIb, IId, IIe, IIg, IIh, IIi, IIj) subtype groups in *C. parvum* (Abe et al. 2006, Misic and Abe 2006, Meireles et al. 2007, Akiyoshi et al. 2006). Within each subtype groups, there are several subgenotypes based primarily on the number of trinucleotide repeats coding for the amino acid serine. Therefore, identification of the isolates at the subgenotype level is more useful for understanding of *Cryptosporidium* population structure. The use of molecular tools has also enabled identification of geographic and temporal differences in the transmission of *Cryptosporidium* species, a better appreciation of the public health significance of other *Cryptosporidium* species/genotypes, and the frequency of infection with mixed genotypes or subtypes (Alves et al. 2003). Use of sub-typing tools will improve the understanding of population genetics and of *Cryptosporidium* transmission in a variety of hosts. The supposedly zoonotic *C. parvum* subtypes are shown in Table 3.

Subgenotype detected	Author	Country of origin	Cattle (mammals)	Human
<b><i>C. parvum</i> IIa subtype group</b>				
A11G2R1	Wielinga et al. 2007	Netherlands	+	-
A12G2R1	O'Brien et al. 2008, Wielinga et al. 2007	Italy, Netherlands	+	-
A13G2R1	Wu et al. 2003, Trotz-Williams et al. 2006, Geurden et al. 2007, Wielinga et al. 2007	Italy, Canada, Belgium, Netherlands	+	-
A14G2R1	Geurden et al. 2007, Wielinga et al. 2007, Broglia et al. 2008	Belgium, Netherlands, Germany	+	-
A15G1R1	Sulaiman et al. 2005	Kuwait	-	+
A15G2R1	Abe et al. 2006, Sulaiman et al. 2005, Wu et al. 2003, Chalmers et al. 2005, Alves et al. 2003, 2006, Stantic-Pavlinic et al. 2003, Glaberman et al. 2002, Trotz-Williams et al. 2006, Blackburn et al. 2006, Peng et al. 2003, Sturbaum et al. 2003, Strong et al. 2000, O'Brien et al. 2008, Geurden et al. 2007, Meireles et al. 2007, Wielinga et al. 2007, Broglia et al. 2008	Kuwait, Japan, Australia, Slovenia, Ireland, Canada, United States, United Kingdom, Belgium, Brazil, Netherlands, Germany	+	+
A15G2R2	Strong et al. 2000, Peng et al. 2003, Trotz-Williams et al. 2006	United States, Canada	+	+
A16G2R1	Alves et al. 2006, Trotz-Williams et al. 2006, Stantic-Pavlinic et al. 2003, Peng et al. 2003, Geurden et al. 2007, Wielinga et al. 2007	Portugal, Canada, Slovenia, United States, Belgium, Netherlands	+	+
A16G1R1	Trotz-Williams et al. 2006, Peng et al. 2003, Plutzer and Karanis 2007b, Mistic and Abe 2006, Wielinga et al. 2007, Broglia et al. 2008	Canada, United States, Hungary, Serbia and Montenegro, Netherlands, Germany	+	-
A16G2R2	Peng et al. 2003, O'Brien et al. 2008	United States	+	+
A16G3R1	Trotz-Williams et al. 2006, Peng et al. 2003, Strong et al. 2000, Wielinga et al. 2007	Canada, United States, Netherlands	+	-
A16G3R2	Peng et al. 2003	United States	+	-
A17G1R1	Chalmers et al. 2005, Plutzer and Karanis 2007b, Sturbaum et al. 2003,	United Kingdom, Hungary, United States, Slovenia,	+	+

	Stantic-Pavlinic et al. 2003, Thompson et al. 2007, Wielinga et al. 2007	Ireland, Netherlands		
A17G2R1	Strong et al. 2000, Blackburn et al. 2006, Trotz- Williams et al. 2006, Glberman et al. 2002, O'Brien et al. 2008, Wielinga et al. 2007, Broglia et al. 2008	Ireland, Canada, United States, Australia, Netherlands, Germany	+	+
A17G3R1	Glberman et al. 2002	Ireland	-	+
A18G1R1	Plutzer and Karanis 2007b, Misic and Abe 2006, Wielinga et al. 2007	Hungary, Serbia and Montenegro, Netherlands	+	-
A18G2R1	Broglia et al. 2008	Germany	+	-
A18G3R1	Chalmers et al. 2005, Glberman et al. 2002, Trotz-Williams et al. 2006, O'Brien et al. 2008, Wielinga et al. 2007	Australia, Ireland, Canada, Netherlands	+	+
A18G4R1	Wielinga et al. 2007	Netherlands	+	-
A18G5R1	Strong et al. 2000	United States	+	-
A18G0R1	Wielinga et al. 2007	Netherlands	+	-
A19G1R1	Wielinga et al. 2007	Netherlands	+	-
A19G2R1	Glberman et al. 2002, O'Brien et al. 2008, Wielinga et al. 2007	Ireland, Australia, Netherlands	+	+
A19G3R1	Glberman et al. 2002, O'Brien et al. 2008	Ireland, Australia	-	+
A19G4R1	O'Brien et al. 2008	Italy	+	-
A20G1R1	Misic and Abe 2006	Serbia and Montenegro	+	-
A20G4R1	Glberman et al. 2002	Ireland	-	+
A20G3R1	Glberman et al. 2002, Chalmers et al. 2005	Ireland, United Kingdom Australia	+	+
A21G0R1	Broglia et al. 2008	Germany	+	-
A21G3R1	Wielinga et al. 2007	Netherlands	+	-
A21G6R1	Glberman et al. 2002	Ireland	-	+
A22G1R1	Broglia et al. 2008	Germany	+	-
<b><i>C. parvum</i> IId subtype group</b>				
A15G1	Wielinga et al. 2007	Netherlands	-	+
A16G1	Wielinga et al. 2007	Netherlands	-	+
A17G1	Alves et al. 2006	Portugal	+	+
A18G1	Misic and Abe 2006, Sulaiman et al. 2005, Wielinga et al. 2007	Serbia and Montenegro, Kuwait, Netherlands	+	+
A19G1	Alves et al. 2006, Plutzer and Karanis 2007b	Portugal, Hungary	+	+
A20G1	Sulaiman et al. 2005	Kuwait	-	+
A21G1	Alves et al. 2006	Portugal	-	+

A22G1	O'Brien et al. 2008, Geurden et al. 2007, Alves et al. 2006, Plutzer and Karanis 2007b, Broglia et al. 2008	Switzerland, Belgium, Portugal, Hungary, Germany	+	+
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**Table 3:** Subgenotypes of *Cryptosporidium parvum* found in the zoonotic, Ila and IId subtype groups. (Updated: May 2008.)

#### 1.1.4. Cryptosporidiosis

Profuse watery diarrhoea is the most common clinical feature of cryptosporidiosis in both immunocompetent and immunosuppressed mammals, including humans (Current and Garcia 1991). Secondary signs of infection in humans include abdominal pains, low grade fever, nausea, vomiting and associated weight loss (Current and Reese 1986, Current and Garcia 1991, O'Donoghue 1995). The severity and longevity of *Cryptosporidium* infections are directly related to the immune status of the host (Clark 1999) lasting 3-12 days in the immunocompetent and up to several month in the immunocompromised host (Tzipori et al. 1983, Current and Garcia 1991, Juranek 1995). Clinical sign are generally more chronic and severe in the immunocompromised and are not always confined to the gastrointestinal tract. Extra intestinal infection of the respiratory tract (Casemore 1990), pancreatic duct, gallbladder and biliary tree have all been documented in human immunodeficiency virus infected patients (Hunter and Nichols 2002). Most healthy hosts develop immunity after infection. In immunosuppressed hosts, however, like HIV infected humans, recovery is difficult and severe dehydration can lead to death (Hunter and Nichols 2002).

The majority of research in this disease in animals has been carried out in livestock. Cryptosporidiosis in ruminant species is asymptomatic or is typically symptomatic in the young or provided animals are under extreme stress or immunologically compromised (Skerrett and Holland 2001, Becher et al. 2004). Unlike other animals in which infection with *Cryptosporidium* spp. is usually self limiting in immunocompetent individuals, *Cryptosporidium* in reptiles is frequently chronic and sometimes lethal (Pasmans et al. 2008). *C. serpentis* infection in lizards is usually asymptomatic, whereas the infection in snakes frequently causes clinical disease (gastric hyperplasia, postprandial regurgitation and firm midbody swelling or chronic debilitating enteritis) and pathological changes (Brownstein et al. 1977, Cranfield et al. 1999, Kimbell et al. 1999, Xiao et al. 2004b). No pathological changes were found in the intestine and cloacae of adult lizards infected by *C. saurophilum*,

but weight loss, abdominal swelling, and mortality occurred in some colonies of juvenile geckos (Xiao et al. 2004b).

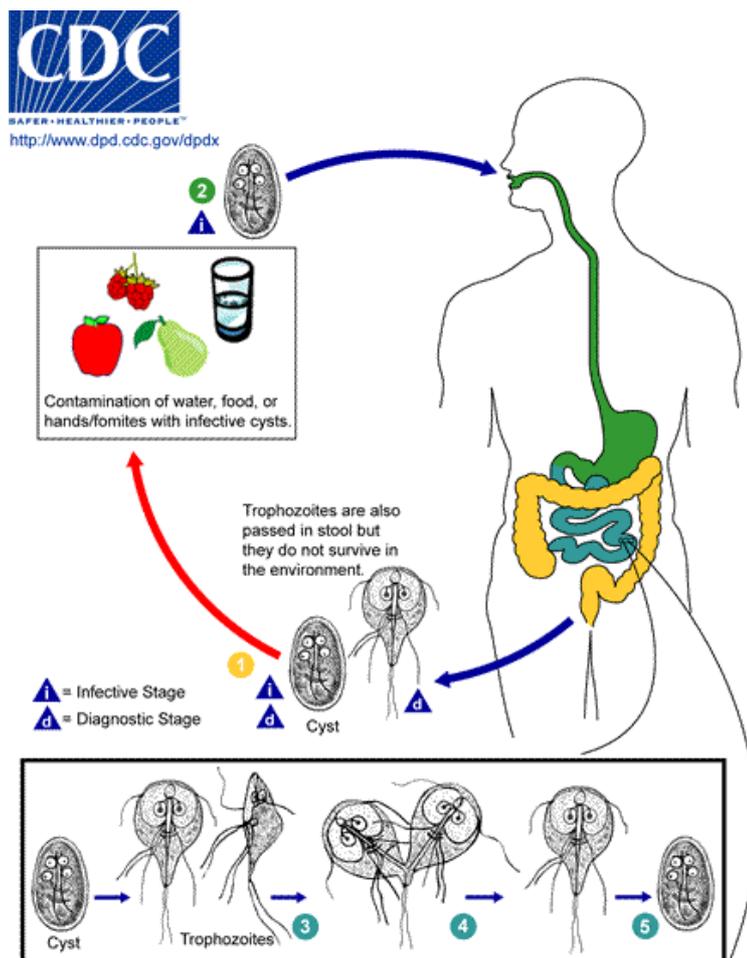
## **1.2. Biology of *Giardia***

### **1.2.1. *Giardia* taxonomy**

*Giardia* is very unusual, seemingly ancient, eukaryotic single cell organism as it shares many characteristics with anaerobic prokaryotes. This flagellated, binuclear parasite belongs to the Phylum Protozoa, Subphylum Sarcomastigophora, Superclass Mastigophora, Class Zoomastigophora, Order Diplomonadida and Family Hexamitidae.

### **1.2.2. Life cycle of *Giardia* spp.**

*Giardia* spp. have a simple, direct life cycle in which the parasite alternates between the motile feeding trophozoite stage and the infective, environmentally resistant cyst stage. Infection occurs when cysts are ingested orally by a susceptible host. Ingestion of as few as ten cysts can lead to infections in humans and animals (Rendtorff 1954). Following ingestion, the trophozoites emerge from the cysts in the duodenum, triggered by the more alkaline pH and proteolytic activity of the duodenum (Gillin et al. 1996). The trophozoites multiply by asexual binary fission and can colonize the entire small intestine. Trophozoites adhere to the intestinal epithelium using a specialized adhesive disk located on their ventral surface, but they may detach from the epithelial surface and move through the intestinal fluid using their four pairs of flagella (Gillin et al. 1996). As the trophozoites are carried downstream by the flow of the intestinal fluid, they must encyst, as they do not naturally survive outside the host. The dormant, quadrinucleate ovoid cyst form is then voided in the faeces. The life cycle of *G. duodenalis* is shown in Figure 2.



**Figure 2:** Life cycle of *Giardia duodenalis*.

### 1.2.3. *Giardia* species, genotypes, subgenotypes

Six species have been distinguished on the basis of morphological and electrokaryotypic characteristics (Adam 2001) (Table 4). Five are represented by isolates from amphibians (*G. agilis*), birds (*G. ardeae*, *G. psittaci*), mice (*G. muris*) and voles (*G. microti*) (Caccio et al. 2005). The sixth is comprised of *Giardia* strains isolated from a large range of other mammalian hosts, grouped by Filice (1952) into a single species because they share morphological features and in particular, have similar median body structures. Filice (1952) named this species *G. duodenalis*. Human-derived *Giardia* is often assigned to a separate species (*G. lamblia*) but there is no definitive evidence that they differ genetically from organisms of the “duodenalis” type isolated from various animals. The morphological similarity masks significant genetic differences. The major lineages have been defined by analysis of human derived *G. intestinalis*: Mayrhofer et al. (1995) designated Assemblage

A and B, which include all the human isolates and they corresponds respectively to groups I plus II and III plus IV of Andrews et al. (1998), Karanis and Ey (1998), to Polish and Belgian genotypes of Homan et al. (1992) and to group 1 plus 2 and group 3 of Nash et al. (1995). The animal derived *G. duodenalis* exhibit a similar genetic spectrum, although some isolates appear to be similar or identical to particular human derived genotypes within each Assemblage, others represent unique genotypes that seem likely to be host specific. All these findings bring into focus the question whether giardiasis is a zoonosis involving different *G. intestinalis* biotypes and whether animals contribute significantly to the disease in humans.

<i>Giardia</i> species	Morphology		
	Light microscopy		Electron microscopy characteristics
	Shape of median bodies	Trophozoites	
<i>G. duodenalis (intestinalis, lamblia)</i> Leeuwenhoek 1681*, Lambl 1859*	1-2 transverse Claw hammer	Tear-drop shaped or pear shaped	-
<i>G. agilis</i> Kunstler 1882, 1883*	Club shaped or tear- drop	Long and narrow body	-
<i>G. muris</i> Grassi 1879*	Small and round	Short and rounded	-
<i>G. psittaci</i> Erlandsen and Bemrick 1987	Same as <i>G. duodenalis</i>	Same as <i>G. duodenalis</i>	Incomplete ventrolateral flange, no marginal groove
<i>G. ardeae</i> Noller 1920*, Erlandsen et al. 1990a	Same as <i>G. duodenalis</i>	Same as <i>G. duodenalis</i>	Ventral disc and caudal flagellum similar to <i>G. muris</i>
<i>G. microti</i> Feely 1988	Same as <i>G. duodenalis</i>	Same as <i>G. duodenalis</i>	Cysts contain two trophozoites with mature ventral discs

**Table 4:** Morphological features of *Giardia* species.  
(\* See references McRoberts et al. 1996 and Adam 2001).

The levels of genetic diversity of the genotypes in Assemblage B are greater than those found in Assemblage A, with many of the genotypes representing single isolates from specific hosts (Thompson et al. 2000, van der Giessen et al. 2006). A number of other distinct genotypes have also been identified within the *G. duodenalis* morphological group, and they all appear to be confined to individual species of mammalian hosts (Hunter and Thompson 2005, Caccio et al. 2005). The non-human, host-specific genotypes found in

dogs, cats, rats and voles/muskrats, are quite distinct from those found in Assemblages A and B. In contrast, the genotypes identified in hoofed livestock and cats appear to be closely related to isolates in the major Assemblages, suggesting a much more recent divergence. The genetic distance separating Assemblages A and B is greater than that used to discriminate between other species of protozoa (Thompson et al. 2000). Therefore, we may have to consider giving separate species names to these Assemblages, although at present, there are few characters of epidemiological or biological significance which can consistently distinguish between them. However, differences in metabolism, in vitro growth rates and susceptibility to infection with a dsRNA *Giardia* virus have been found to correlate with the genetic division between the Assemblages. In contrast, the apparent host specificity of genotypes in dogs, cats, livestock and rats may justify their recognition as distinct species in the future. Fortunately, finding appropriate species names for a future taxonomic revision of the genus *Giardia* should not be problematic, since numerous species have been described based on host occurrence (Thompson et al. 2000). *Giardia* species, genotypes and hosts are shown in Table 5.

<b><i>Giardia</i> species</b>	<b>Genotypes</b>	<b>Subtypes</b>	<b>GenBank accession number (18S rRNA)</b>	<b>Major hosts</b>
<i>G. duodenalis</i> ( <i>intestinalis</i> , <i>lamblia</i> ) Leeuwenhoek 1681*, Lambl 1859*	Assemblage A (Polish) Homan et al. 1992, Mayrhofer et al. 1995 Karanis and Ey 1998	A1-2 (Nash et al. 1995) A I-II (Andrews et al. 1998)	AF199446 (A2) AB159796 (A1) X52949 (A1) M54878 (A1)	Humans and other primates, dogs, cats, livestock, rodents and other wild mammals
	Assemblage B (Belgan) Homan et al. 1992, Mayrhofer et al. 1995	B 3 (Nash et al. 1995) B III-IV (Andrews et al. 1998)	U09491 (B) AF199447 (B) AF113898 (BIV) AF113897 (BIII)	Humans and other primates, dogs, livestock, wild mammals
	Assemblage C-D Meloni and Thompson 1987, Monis et al. 1998	-	AF113899, AF199443 (C) AF113900, AF199449 (D)	Dogs

	Assemblage E Ey et al. 1997	-	AF199448	Cattle and other hoofed livestock
	Assemblage F Monis et al. 1999	-	AF113901	Cats
	Assemblage G Monis et al. 1999	-	AF199450 AF113896	Rats
<i>G. agilis</i> Kunstler 1882, 1883*	-	-	Not available	Amphibians
<i>G. muris</i> Grassi 1879*	-	-	X65063 AF113895	Rodents
<i>G. psittaci</i> Erlandsen and Bemrick 1987	-	-	AF473853	Birds
<i>G. ardeae</i> Noller 1920*, Erlandsen et al. 1990a	-	-	Z17210 U20351	Birds
<i>G. microti</i> Feely 1988	-	-	AF006676 AF006677 AF473852	Vole Muskrat Deer mouse

**Table 5:** *Giardia* species, genotypes, hosts and reference SSU rRNA GenBank accession numbers. (Updated: May 2008.)  
(\* See references McRoberts et al. 1996 and Adam 2001).

#### 1.2.4. Giardiasis

The clinical features of giardiasis in humans are similar to cryptosporidiosis, including severe diarrhoea, abdominal cramps, nausea and weight loss (Wolfe 1992, Adam 2001, Lebwohl et al. 2003). These symptoms may persist for a few weeks, in the case of acute giardiasis or evolve into chronic reoccurring disease (Farthing 1996).

*Giardia* infection in ruminants causes intestinal malabsorption thereby reducing rate of body weight gain and affecting feed efficiency in growing animals by as much as 10%. Although mortality is not common, fatal *Giardia* infections have been reported in chinchillas and birds (Shelton 1954, Upcroft et al. 1997). It is thought that the pathology of disease is related to the asexual phase, the trophozoites, whilst attached to the intestinal epithelium will disrupt host secretory and excretory functions either by the sheer number

of its presence, causing electrolyte transport abnormalities, enterocyte apoptosis and loss of epithelial barrier function or by as yet unidentified specific mechanisms (Buret 2007).

### **1.3. *Cryptosporidium* oocysts and *Giardia* cysts in water supplies**

*Giardia* cysts and *Cryptosporidium* oocysts are transmitted by the faecal-oral route. Potential sources of transmission include person to person through direct or indirect contact, animal to animal, animal to human, human to animal, waterborne from humans or animals through drinking water or recreational water and food-borne from contamination of water used in food preparation and manufacture or from food handlers. Indirect person-to person or zoonotic transmission may occur by contamination of water used for recreation or swimming pools, drinking or food (Karanis et al. 2007b, Yoshida et al. 2007, Xiao and Feng 2008).

Surface water becomes contaminated through the discharge of untreated and treated sewage and run-off of manure (Hunter and Thompson 2005). The relative significance of these sources may differ between watersheds. Large rivers and lakes often receive both agricultural run-off and treated and untreated domestic wastewater. Many groundwater supplies in the United States were contaminated with *Cryptosporidium* and/or *Giardia*, mostly in infiltration galleries and horizontal wells (impacted by surface water or sewage contamination) (Dworkin et al. 1996).

Several characteristics of *Cryptosporidium* and *Giardia* (oo)cysts facilitate the waterborne transmission: Compared to other protozoan parasites, the oocysts of *Cryptosporidium* are relatively small (4-6  $\mu\text{m}$ ). Due to their small size, they are less efficiently removed during soil passage in bank filtration and in rapid or slow sand filtration. Filtration processes are important barriers for (oo)cysts in water treatment. Before the discovery of the sensitivity of the (oo)cysts to UV, the attention of the water industry was focused on (oo)cysts removal by filtration process and especially upgrading filter design and operations to optimise (oo)cyst removal. Full scale conventional treatment with coagulation, floc removal and rapid granular filtration removes >2.3 logs. Other filtration processes such as slow sand filtration and diatomaceous earth filtration give similar removal efficiencies for (oo)cysts. Pressure driven membrane processes (microfiltration, ultrafiltration, nanofiltration and reverse osmosis) are playing an important role in drinking water

production in the United States and in Europe. These processes are being employed in water treatment for multiple purposes including control of disinfection by products, pathogen removal, clarification, and removal of inorganic and synthetic organic chemicals. It was demonstrated, that different microfiltration and ultrafiltration membranes provide >4-6 log removals of *Cryptosporidium* oocysts and *Giardia* cysts (Betancourt and Rose 2004). The log credits are originating from the United States, where the data from different studies are combined using mathematical or statistical approaches and are an approximation for removal by well designed, maintained and operated treatment process (Medema et al. 2006).

The state in which (oo)cysts occur in water (suspended or attached to particles) is relevant for water treatment (sedimentation, filtration) and (oo)cysts readily attach to particles in sewage effluent (Medema et al. 2006). Assavasilavasukul et al. (2008) showed that *Cryptosporidium* oocyst and *Giardia* cyst removal across conventional treatment were dependent on initial pathogen concentrations, with lower pathogen removals observed when lower initial pathogen spike doses were used. In addition, higher raw water turbidity appeared to result in higher log removal. Disinfection with chlorine and chloramines has always been an important barrier for waterborne pathogens, but they are less effective against *Giardia* cysts and not effective against the *Cryptosporidium* oocysts. Chlorine-dioxide treatment may result in inactivation, but the required product of concentration and contact time is still high especially at low temperatures (Medema et al. 2006). Ozone is the most potent chemical against (oo)cysts, although the effectiveness of ozone also reduces at lower temperatures and the Ct values required are high (Bukhari et al. 2000). Chemical disinfectants cannot be dosed too high concentrations, because toxic by products are formed by the reaction with compounds in the water, such as trihalomethanes by chlorine, nitrite by monochloramine, chlorite by chlorine dioxide and bromate by ozone.

Exposure of (oo)cysts to multiple disinfectants has been shown to be more effective than was to be expected from both disinfectants alone. The multiple stresses that oocysts encounter in the environment and during treatment might limit the infectivity of the oocysts (Belosevic et al. 1997, Medema et al. 2006). Using animal infectivity, experiments showed that oocysts are sensitive to UV and short-duration pulsed-UV light is an effective disinfection measure for *C. parvum* (Bukhari et al. 2004, Rochelle et al. 2004, Lee et al. 2008).

Post treatment contamination is a significant hazard. When the water in the distribution system or in storage containers is contaminated, no barriers are in place to prevent ingestion of infectious (oo)cysts. Post treatment contamination may occur through infiltration of contaminants in the distribution system through leaks (during surges), in open distribution reservoirs or other open connections and during construction and repair. Cross connection and back siphonage may draw water from toilets or sewers into the network. Storage tanks used in houses (i.e. in sand pipe systems) may also become contaminated. Several outbreaks of cryptosporidiosis have been caused by post treatment contamination (Medema et al. 2006, Smith et al. 1989).

(Oo)cysts can survive for months or years in surface water and in soil (Robertson et al. 1992, Kato et al. 2004). The infectivity of these parasites is high, and varies between isolates. The high density of cattle and sheep in watersheds and the excretion of high numbers of oocysts make the infected animal important sources of water contamination. This is essential for the management of animal farming in watersheds ensuring that the newborns and their manure are kept away from water sources may reduce the waterborne transmission considerably (Graczyk et al. 2000, Collick et al. 2006). Unlike coccidian parasites and helminths, *Cryptosporidium* and *Giardia* does not require a period of maturation of (oo)cysts after shedding with faeces. They are immediately able to infect a new host.

#### 1.4. Aims and objectives of the present work

Risk management requires a system approach and the actions that are most frequently employed for controlling the *Cryptosporidium* and *Giardia* risk to water supply are the monitoring, enhanced catchment protection, optimization of filtration in water treatment, the installation of additional water treatment process, protection of treated water during transport and distribution to the customer's home.

The aims of the present work were:

- to apply the modern detection techniques for *Giardia* and *Cryptosporidium* from water sources, such as different water sample concentration methods, separation of (oo)cysts from debris (IMS) and detection by IFT, PCR and real-time PCR
- to use the established methods for regular monitoring of the River Danube, the water after riverbank filtration and all other surface water sources (raw waters) in different seasons taking into consideration the peak events, such as heavy rainfalls and contamination sources
- to estimate the efficacy of the water removal efficiency of the current water treatment technologies in the Hungarian water supplies
- to identify the parasites and to perform sequence analysis of the PCR products and get data about the species, genotypes and subtypes present in the water sources in Hungary
- to use the sequence data and subtype analysis information for the identification of water contamination sources
- to investigate Hungarian cattle farms whether they are risky to water supplies by contaminating the raw water with human pathogenic *Cryptosporidium* and *Giardia*
- to investigate the correlation between the consumption of *Giardia* contaminated drinking water and giardiasis in the relevant settlements
- to keep relation with the relevant waterworks in Hungary to overcome the protozoa contamination problems

## 2. MATERIALS AND METHODS

### 2.1. Buffers and solutions

PBS: 1.07 g  $\text{Na}_2\text{HPO}_4$  (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and 0.39 g  $\text{NaH}_2\text{PO}_4 \times 2\text{H}_2\text{O}$  (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was dissolved in 1 litre of aqueous sodium chloride solution containing 8.5 g of NaCl (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The pH of the resulting solution should be 7.2.

TFN solution for preparation of competent *Escherichia coli* DH5a cells: 3.68 g  $\text{CaCl}_2 \times 2\text{H}_2\text{O}$  (Takara Shuzo Co. Ltd. Kyoto, Japan), 75 mL glycerol (Takara Shuzo Co. Ltd. Kyoto, Japan), 5 mL 1 M PIPES (Takara Shuzo Co. Ltd. Kyoto, Japan) (pH=6.5) filled up to 500 mL with distilled water.

DAPI staining solution: 2 mg DAPI (Fluka Chemi GmbH, Buchs, Germany) was solved in 1 mL methanol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and 10  $\mu\text{L}$  of this solution was filled up until 1 mL with PBS.

Sucrose solution: 500 g sucrose (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and 6.5 g phenol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was solved in 320 mL distilled water.

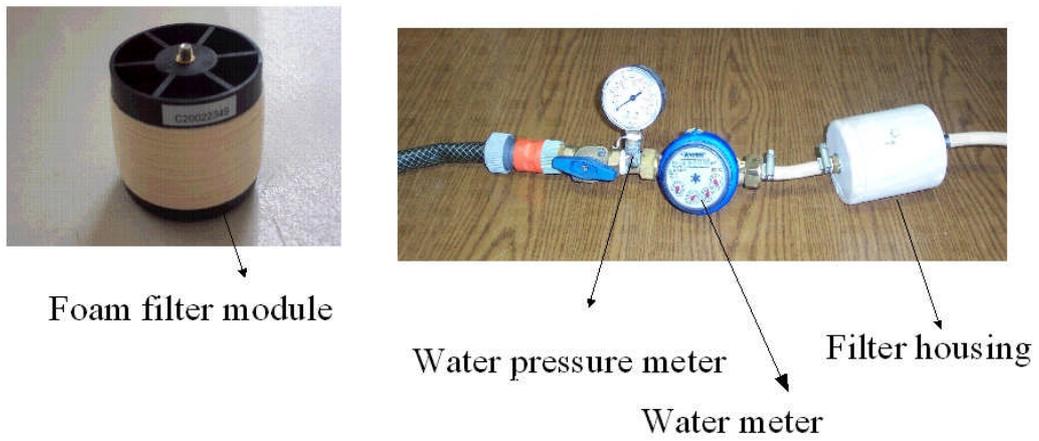
Ethidium bromide solution: 10  $\mu\text{L}$  ethidium-bromide (0.625 mg/ml) (Pierce, Rockford, IL) was dissolved in 100 mL TAE buffer (Tris-acetate-EDTA) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

## 2.2. *Cryptosporidium* and *Giardia* (oo)cysts concentration from water

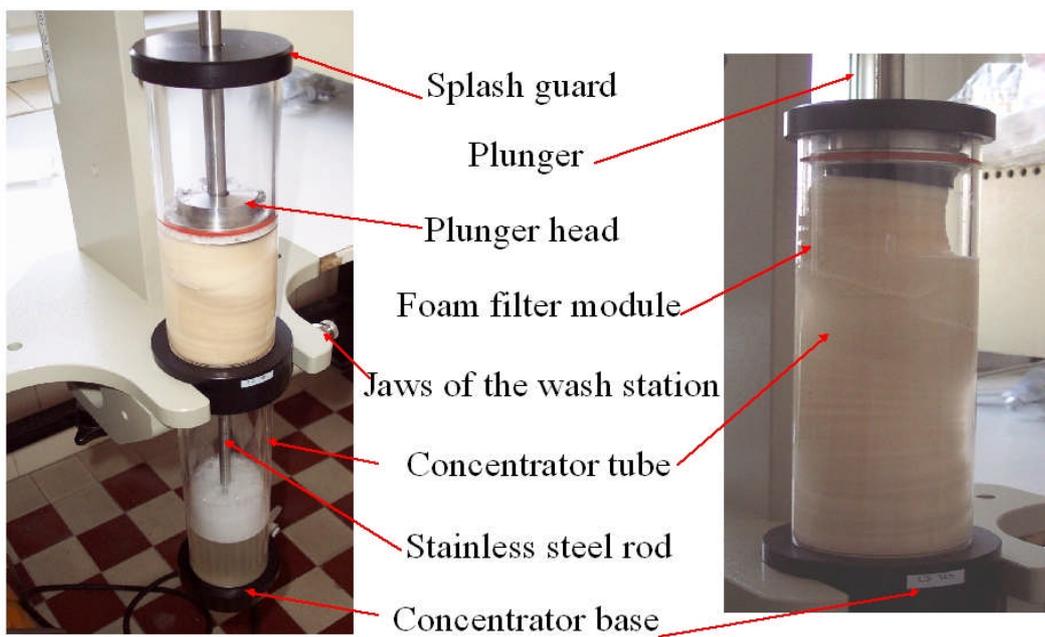
### 2.2.1. Filtration

**Filta-Max filtration:** For primary concentration of 400-1000 L of drinking water, Filta-Max (IDEXX-Genera Technologies Ltd, Newmarket, UK) compressed-foam depth filters with an equivalent pore size of 1  $\mu\text{m}$  were used (USEPA 2001). The foam filter module was placed into the filter housing and the lid was secured using the tools provided. The filter housing was then attached to the desired water supply after the water and pressure meters. During filtration, the pressure was adjusted to 1-2 bars, and the flow rate to 1-2 L/min. The equipment for filtering is shown on the Figure 3. Following filtration the foam filters were removed, immersed in 50 mL water and placed in container for shipping to the laboratory for further analysis. Filters were prevented from drying out, as this can impair their ability to expand when decompressed. In the laboratory, the wash station was prepared for washing the foam filter. The Figure 4 shows the parts of the wash station. The removable plunger head was detached using the tool provided and the splash guard was also removed. The concentrator base was located in the jaws of the wash station and was screwed on the concentrator tube, the larger of the two tubes, creating a tight seal. The assembled concentrator then was taken out of the jaws and was placed on the bench. The splash guard was then replaced and temporarily secured at least 15 cm above the end of the rack. The plunger head was secured with the tool provided ensuring that the lever is fully locked down. The filter module was then removed from the transportation container. The excess liquid was poured into the assembled concentrator, the container was rinsed and the rinse was added to the concentrator tube. The filter module was screwed onto the base of the plunger. The second elution tube base was located in the jaws of the wash station and the elution tube was screwed firmly in place, the smaller of the two tubes. The plunger was pulled down until the filter module sits at the bottom of the elution tube. The locking pin at the top left of the wash station was clicked to lock the plunger in position. The filter module bolt was then removed by turning the adapted allen key provided in the clockwise direction. Then the stainless steel rod was attached to the elution tube base. For the first wash, 600 mL of PBS containing 2 drops of Tween 20 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added to the assembled concentrator and the concentrator tube

was screwed onto the base beneath the elution tube. The locking pin was released. The foam filter was washed by moving the plunger up and down 20 times. Gentle movements of the plunger were applied to avoid generating excess foam. The plunger has an upper movement limit during the wash process to prevent it popping out of the top of the chamber. Finally, the concentrator was detached and held such that the stainless steel tube is just above the level of the liquid. The remaining liquid was purged from the elution tube by moving the plunger up and down 5 times and the plunger was locked in place. To prevent dips, the bung provided was placed in the end of the stainless steel rod. The washing liquid was collected in a 2 litres glass container. For the second wash step, further 600 mL of PBS containing 2 drops of Tween 20 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added to the concentrator module. The bung was removed from the end of the steel rod and the concentrator tube was screwed back onto the elution module base. The locking pin was released. The foams were washed by moving the plunger up and down 10 times. The concentrate was added to the 2 litres glass container containing the first wash. The washing puffer was dispensed in plastic 500 mL centrifuge tubes and (oo)cysts were further concentrated by centrifugation at 2,500 rpm for 20 min in a S70D (MLW, Germany) centrifuge. After centrifugation the supernatant was aspirated above the pellet and the pellet was resuspended in the small volume of liquid remaining. Then (oo)cysts were further concentrated by centrifugation in 50 mL Falcon tubes at 2,500 rpm for 20 min (S70D centrifuge). The supernatant was aspirated again above the pellet and the pellet was resuspended in the remaining supernatant. This pellet (the amount was always different according to the type of the water) was used for IMS as described in chapter 2.2.3.



**Figure 3:** Filta-Max filtration.

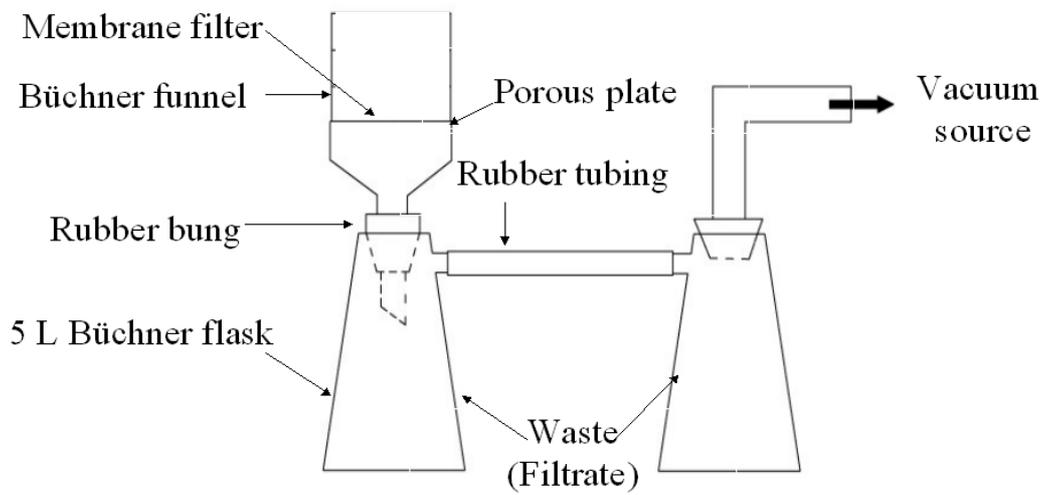


**Figure 4:** Washing of the Filta-Max foam filter.

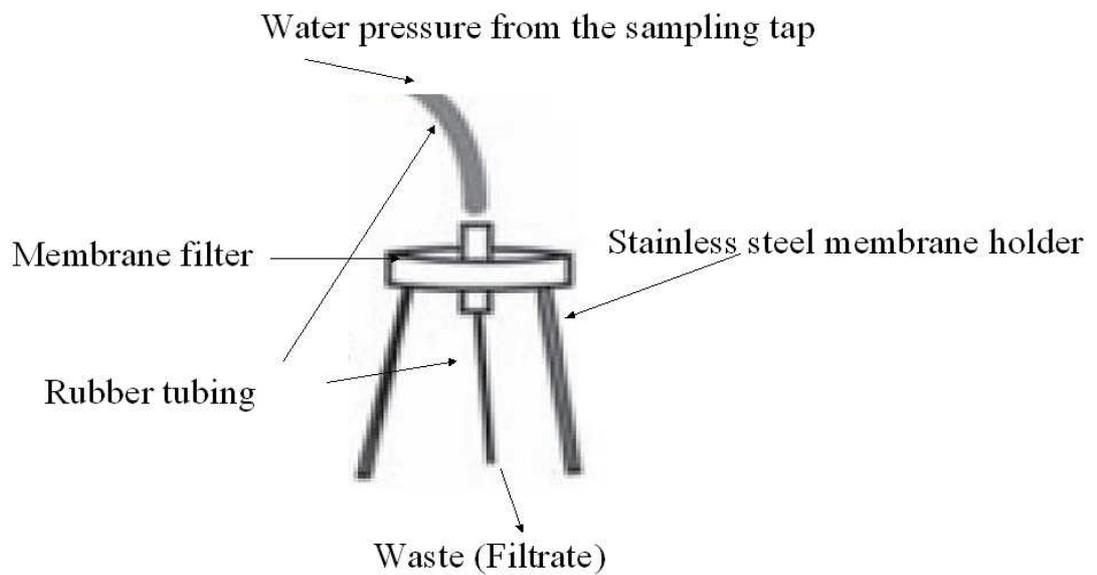
**Membrane filtration:** Volumes less than 400 L of water were concentrated using membrane filtration (USEPA 2001). We used 5-20 L of raw water and 20-400 L of drinking water depending on the water turbidity. During vacuum filtration the Büchner flasks were connected to the Büchner funnel, which contains a porous membrane holder. The 47 mm diameter, 3 µm pore size cellulose mixed esters, fabric-reinforced membrane filter (PORAFIL<sup>®</sup> MV, Macherey-Nagel, Düren, Germany) was placed onto the membrane holder. During the filtration the assembled filtering system was connected to the vacuum source and the collected water sample, which was collected in a clean 10-20 L can, was poured manually in the Büchner funnel. This equipment is shown in Figure 5.

During pressure membrane filtration the filtration equipment including the special stainless steel membrane holder, was connected to the tap of system pipes and filtration was achieved through 142 mm diameter, 3 µm pore size cellulose nitrate membrane filters (Millipore Co., Bedford, MA) as shown in Figure 6. During filtration, the pressure never exceeded the 2 bar, and the flow rate was 1-2 L/min.

For high turbidity water, multiple membrane filters were used for each sample before final filtered sediments were combined. The sediment from the surface of the membrane filters were washed with 10-50 mL PBS using brush and (oo)cysts were further concentrated by centrifugation in 50 mL Falcon tubes at 2,500 rpm for 20 min in a S70D (MLW, Germany) centrifuge. The supernatant was aspirated just above the pellet and the pellet was resuspended in the remaining supernatant. This pellet (the amount was always different according to the type of the water) was used for IMS as described in chapter 2.2.3.



**Figure 5:** Vacuum membrane filtration.



**Figure 6:** Pressure membrane filtration.

### 2.2.2. Chemical flocculation

Raw water samples with high turbidity were collected into 10-20 L cans from sampling taps inside of the water treatment plants and the samples were transferred immediately to the laboratory in order to perform chemical flocculation for recovery of *Cryptosporidium* oocysts and *Giardia* cysts according to Vesey et al. (1993). 100 mL 1 M CaCl<sub>2</sub> (Reanal Finomvegyszergyár ZRt, Budapest, Hungary) and 100 mL 1 M NaHCO<sub>3</sub> (Reanal Finomvegyszergyár ZRt, Budapest, Hungary) was added to 10 litres of water sample. The water was mixed and the pH was adjusted to 10 using approximately 10 mL 1 M NaOH (Reanal Finomvegyszergyár ZRt, Budapest, Hungary). During flocculation, the formation of a calcium carbonate precipitate adsorbs and pulls water particulates and protozoa to the bottom of vessel, giving a dense, stable form within 4 hours. After discarding the supernatant, the calcium carbonate floc residue was dissolved in 350 mL 10% sulphamic acid (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), giving a concentrate containing the (oo)cysts. The floc is fully dissolved if the effervescing stopped. Then the pH was adjusted to 6.5 using approximately 100 mL 1 M NaOH (Reanal Finomvegyszergyár ZRt, Budapest, Hungary). The sample was decanted into 0.5 L plastic centrifuge bottles and the can was rinsed with 0.1 % Tween 20 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The liquid from the rinsing was added to the centrifuge bottle. The bottles were centrifuged at 2,500 rpm for 20 min in a S70D (MLW, Germany) centrifuge, then the supernatant was aspirated just above the pellet. The pellet was resuspended in the remaining supernatant and was pooled into a 50 mL Falcon centrifuge tube. The first centrifuge tube was rinsed into the second with 0.1% Tween 20 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and the sample was centrifuged at 2,500 rpm for 20 min (S70D centrifuge). The supernatant was aspirated just above the pellet and the pellet was resuspended in the remaining supernatant. This pellet (the amount was always different according to the type of the water) was used for IMS as described in chapter 2.2.3.

### 2.2.3. Immunomagnetic separation (IMS)

After filtration or flocculation, IMS (Dynabeads GC-Combo kit, Dynal Biotech ASA, Oslo, Norway) was performed to separate the (oo)cysts from the debris. Firstly 1× dilution of SL-buffer –A was prepared: 100 µL of 10× SL-buffer-A supplied was mixed with 0.9 mL double distilled, sterilized water. Then 1 mL 10× SL-buffer-A and 10× SL-buffer-B was added to a flat-sided 20 mL glass tube and using a graduated 10 mL pipette, 8 mL sample concentrate from the filtration and flocculation steps was transferred to the flat-sided tubes containing the SL-buffers. The Falcon centrifuge tubes previously containing the samples were rinsed with 2 mL PBS and the rinsate was added to the flat-sided tube containing the sample. Then the Dynabeads Crypto-Combo and Dynabeads Giardia-Combo vial was vortexed approximately 10 sec to suspend the beads. By inverting the sample tube should not be any residual pellet at the bottom. Then 100 µL of the resuspended Dynabeads Crypto-Combo and Dynabeads Giardia-Combo was added to the flat-sided tube containing the water sample concentrate and SL-buffers. The sample tubes were affixed to a rotating mixer and were rotated at 18 rpm for 1 hr at room temperature (Figure 7A). After rotation the sample tube was removed and was placed in the magnetic particle concentrator (MPC-1, Dynal Biotech ASA, Oslo, Norway) with flat side of the tube toward the magnet (Figure 7B). Without removing the sample tube from the MPC-1, the magnet side of the MPC-1 was placed downwards, so the tube is horizontal and the flat side of the tube is facing down. The sample tube was gently rocked by hand end-to-end thorough 90°, tilting the cap end and base end of the tube up and down in turn. This tilting action was continued for 2 min with approximately one tilt per second. It is important to ensure that the tilting action is continued thorough this period to prevent binding of low mass, magnetic or magnetizable material. Finally the MPC-1 was returned to the upright position and the sample tube vertical with cap at the top. The cap was immediately removed keeping the tube on top and all of the supernatant was poured off from the tube held in the MPC-1 into a suitable container. It is important not to shake the tube and all the supernatant have to be poured out. Finally the sample tube was removed from the MPC-1 and the sample was gently resuspended in 0.5 mL 1× SL-buffer-A prepared at the beginning of the procedure. The sample was transferred to a 1.5 mL Eppendorf microcentrifuge tube. The flat-sided tube was rinsed twice with 0.5 mL 1× SL-buffer-A and

the rinsate was also transferred in the Eppendorf tube. The microcentrifuge tubes were placed into the second magnetic particle concentrator (MPC-M, Dynal Biotech ASA, Oslo, Norway) with its magnetic strip in place (Figure 7C). Without removing the microcentrifuge tube from the MPC-M the tube was gently rocked/rolled thorough 180° by hand and was continued for 1 min with one rock/roll per second. At the end of this procedure the beads should produce a distinct brown dot at the back of the tube. The supernatant was aspirated from the tube and the cap of the tube held in the MPC-M magnet. The material attached to the wall of the tube adjacent to the magnet should not be disturbed. Finally, the magnetic strip from the MPC-M was removed and 50  $\mu$ L of 0.1 N HCl (Reanal Finomvegyszergyár ZRt, Budapest, Hungary) was added and vortexed at the highest setting for 50 sec. The tube was placed back in the MPC-M without magnetic strip and was allowed to stand in a vertical position for at least 10 min at room temperature. Then the tube containing the sample was vortexed vigorously for 30 sec and placed in the MPC-M with magnetic strip and was allowed to stand undisturbed for 10 sec. Finally, the sample was added to a clean PCR tube containing 5  $\mu$ L of 1 N NaOH (Reanal Finomvegyszergyár ZRt, Budapest, Hungary) for neutralization. This 55  $\mu$ L final concentrate was used for staining with FITC and DAPI or for DNA extraction.



A.

B.

C.

**Figure 7:** Steps of the immunomagnetic separation.

### **2.3. *Cryptosporidium* and *Giardia* (oo)cysts purification from faeces**

#### **2.3.1. Ether-phosphate-buffered saline sedimentation**

The stool specimens as sources of *Cryptosporidium* oocysts were treated with organic solvent, diethyl-ether before further processing in order to remove the fats. Faecal suspension in PBS was mixed with diethyl-ether (Reanal Finomvegyszergyár ZRt, Budapest, Hungary) in the ratio 2:1, which means, for example, that 30 mL faecal suspension was transferred into 50 mL Falcon-tubes, 15 mL diethyl-ether (Reanal Finomvegyszergyár ZRt, Budapest, Hungary) was added and mixed by vortexing. The mix was centrifuged at 2,500 rpm for 10 min in a S70D (MLW, Germany) centrifuge, the supernatant was discarded and the pellet with oocysts was washed twice with 40 mL of PBS centrifuged each time at 2,500 rpm for 10 min (Waldman et al. 1986).

#### **2.3.2. Discontinuous density gradient centrifugation**

The faecal samples after ether-phosphate-buffered saline sedimentation were treated by discontinuous sucrose gradient centrifugation in order to enhance oocyst recovery and purity (Arrowood and Sterling 1987). For this procedure the sucrose solution was diluted in the ratio 1:2 and 1:4 with PBS. Then 20 mL of the 1:2 solution was transferred into a 50 mL centrifuge tube and overlaid with 20 mL of the 1:4 solution. The contents of each tube were overlaid with 10 mL PBS-faecal suspension. The tubes were centrifuged at 2,500 rpm for 30 min in a S70D (MLW, Germany) centrifuge. Following the centrifugation, the upper layer was discarded (approximately 15 mL), while the second layer, the interface between two layers and upper 5-10 mL of the lower layer were transferred to 50 mL tubes. The tubes were filled up with sterile double distilled water and centrifuged at 2,500 g for 10 min. The supernatant was aspirated and the tubes were filled up again with sterilized double distilled water and centrifuged at 2,500 rpm for 10 min (S70D centrifuge). The pellet (approximately 1.2 mL) was used for molecular analysis or sample staining.

### **2.3.3. Immunomagnetic separation (IMS)**

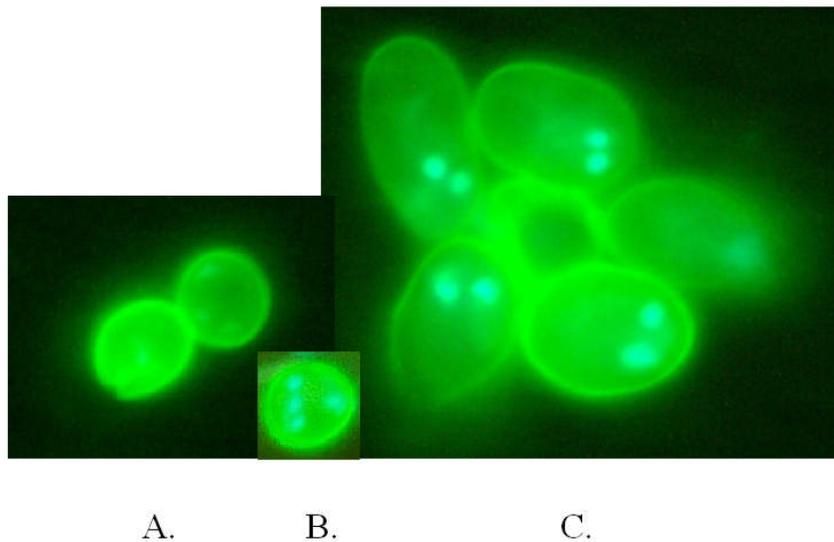
In order to concentrate the *Giardia* cysts from faecal samples the immunomagnetic separation was performed on the same way as described in the chapter 2.2.3. for water samples. The 55  $\mu$ L final concentrate was used for staining with FITC and DAPI or for DNA extraction.

### **2.4. Staining of (oo)cysts**

The concentrated 55  $\mu$ L pellet was transferred into the well of the slide, were air-dried and fixed with 20  $\mu$ L methanol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Then 1 $\times$  fluorescently labelled monoclonal antibody solution (FITC) (Waterborne, Inc, New Orleans, LA) was prepared from the supplied buffer (dilution buffer) and stock solution (A100FLR-20 $\times$  Aqua-Glo G/C): 5  $\mu$ L stock solution was diluted in 95  $\mu$ L buffer and 50  $\mu$ L of this 1 $\times$  fluorescently labelled monoclonal antibody solution was added to the well containing the (oo)cysts. The slides were placed in a humid chamber in the dark and incubated at 37 °C for 1 hr. The humid chamber consists of a tightly sealed plastic container containing damp paper towels on top of which the slides are placed. After incubation, the slides were removed from the humid chamber and the condensation was allowed to evaporate. One drop of PBS was applied to each well and each slide was tilted on a clean paper towel, long edge down. The excess reagent from the well was absorbed by a paper towel placed at the end of the slide. It is important to take care not to touch the sample. Then 50  $\mu$ L DAPI staining solution was applied to each well and the sample was allowed to stand at room temperature for 5 min. After this DAPI staining the washing step with PBS described above was repeated twice and once with sterilized double distilled water. Finally one drop mounting medium (M101 No-Fade Mounting Medium, Waterborne, Inc, New Orleans, LA) was added to each well. The cover slip was applied to the slide. In order to remove the excess mounting fluid from the edges of the coverslip tissue was used. The edges of the coverslip were sealed onto the slide using clear nail polish. The slides were read immediately.

## 2.5. Microscopy (FITC, DAPI, DIC)

Using epifluorescence microscopy (DM IRB inverted microscope, Leica Microsystems GmbH, Wetzlar, Germany) the entire slide well was scanned for apple green fluorescence of oocysts and cysts shapes. When brilliant apple green fluorescing ovoid or spherical objects 4-6  $\mu\text{m}$  in diameter or 8-18  $\mu\text{m}$  long by 5-15  $\mu\text{m}$  wide were observed with brightly highlighted edges, this presumptive organisms were further examined to detect the DAPI stained nuclei. If light blue internal staining is seen, this means no distinct nuclei. If intense blue internal staining or up to four distinct, sky-blue nuclei are seen, nuclei are present. DIC microscopy was also performed at 1000 $\times$  magnification for the confirmation of characteristic surface and internal structures of the (oo)cysts (USEPA 2001). DIC examination was carried out with the same microscope using Nomarski polarisation optics. *Giardia* cysts were confirmed by observing the cyst wall, internal cytoplasm, peritrophic space and nuclei. *Cryptosporidium* oocysts were confirmed by the oocyst wall and the observed sporozoites, nuclei or densely packed cytoplasm. The structure of the oocyst surface was visible many times. Only samples in which (oo)cysts fulfilling defined and published morphological criteria and according to the USEPA protocol were enumerated and samples deemed positive. All in all, a positive result is a *Cryptosporidium* oocyst or *Giardia* cyst which exhibits typical fluorescence, size and shape as indicated above and nothing atypical on IFT, DAPI fluorescence or DIC microscopy. Each object was categorized as one of the following: empty *Cryptosporidium* oocyst, *Cryptosporidium* oocyst with amorphous structure, *Cryptosporidium* oocyst with internal structure (sporozoites) and empty *Giardia* cyst, *Giardia* cyst with amorphous structure, *Giardia* cysts with one type of internal structure or *Giardia* cyst with more than one type of internal structure. Pictures about the FITC and DAPI stained *Cryptosporidium* oocysts are shown in Figure 8A,B and picture about the FITC and DAPI stained *Giardia* cysts are shown in Figure 8C.



**Figure 8:** *Cryptosporidium* oocysts and *Giardia* cysts stained by FITC and DAPI.

## 2.6. Protein analysis

### 2.6.1. *Giardia* microplate assay

All collected faecal samples were prepared within 24 hours after collection, directly for *Giardia* protein analysis using ProSpecT *Giardia* microplate assay (Remel Inc. Lenexa, KS) to detect *Giardia* specific antigen. GSA 65 is a 65-kDa molecular weight glycoprotein that is produced in large quantities by *G. duodenalis* as they multiply in the intestinal tract of the host organism. The antigen is present in stool only when *Giardia* trophozoites and cysts exist in the gastrointestinal tract of the affected individual. It is also possible to detect GSA 65 in stool specimens without identification of cysts and/or trophozoites. In this solid phase sandwich immunoassay diluted stool specimens were added to the microplate wells coated with anti-GSA 65 antibodies. If GSA 65 is present, it is captured by the bound antibody. Monoclonal anti-GSA antibody labelled with horseradish peroxidase enzyme is used to detect the captured antigens. In a positive reaction, the enzyme bound to the well by GSA 65 converts the chromogenic substrate to a coloured reaction product.

During the detection procedure firstly the foil pouch was opened and the required number of microplate strips were removed and placed into a microplate strip holder. The faecal

specimens were diluted in Eppendorf tubes before adding into the wells: 1 mL SDB buffer was added to the Eppendorf tube and was coated with 1 swab with fresh faecal specimen and virgously stirred into the SDB buffer. As much fluid as possible was expressed and the swab was discarded. 0.2 mL of each specimen was added to a well. In order to avoid the splashing into the adjacent wells the transfer pipettes were placed just inside the wells. Then the microplate was incubated at room temperature for 1 hr. In the meantime 1× wash buffer was prepared from the supplied 110 ml wash buffer stock by adding 990 ml double distilled sterilized water. After incubation the contents of the wells were aspirated and 350 µl 1× wash buffer was added and aspirated. This wash step was performed 3 times. It is necessary to remove as much wash buffer as possible, but it is not advisable to dry out the wells. Then 200 µl enzyme conjugate was added to each well and the microplate was incubated for 30 min. Each well was washed again 5 times as described above. Then 200 µl colour substrate was added to each well and the microplate was incubated at room temperature for 10 min. Finally 50 µl stop solution was added to each well and the wells were gently vortexed until the yellow colour is uniform. The reactions were read within 10 min using spectrophotometer (Model 6300, Jenway, UK) at 450 nm. Positive and negative control was included each time the test was performed. During spectrophotometry the negative control was used as a blank, which means that the negative control optical density (OD) was automatically subtracted from all of the other readings. Based on this, if the OD is equal to or greater than 0.050 in the test well, the sample is positive; if the reading is less than 0.050 the sample is negative.

## **2.7. DNA analysis**

### **2.7.1. DNA extraction**

DNA was extracted from (oo)cysts following IMS separation step using the QIAmp Mini Kit (Qiagen GmbH, Hilden, Germany) or directly from faecal samples using the QIAmp Stool Kit (Qiagen GmbH, Hilden, Germany) using the supplied buffers. In case of QIAmp Mini Kit 50-100 µL water pellet was mixed, well homogenized with 180 µL buffer ATL (lysis solution) and freezing-thawing was performed 10-15 times in liquid nitrogen and on 65 °C in water bath (BM 302, Nüve, Turkey) in order to rupture the (oo)cysts. After the

freeze-thaw cycles 20  $\mu$ L proteinase K was added to the sample, mixed by vortexing and it was incubated on 56 °C for 3 hours in shaking water bath (BM 302, Nüve, Turkey). Then 200  $\mu$ L Buffer AL was added, mixed by pulse-vortexing and incubated for 10 min on 70 °C in water bath (BM 302, Nüve, Turkey). The DNA was precipitated by pulse-vortexing with 200  $\mu$ L 99% ethanol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and the entire sample were transferred into the Spin column and centrifuged at 8,000 rpm for 1 min. The column (and the DNA) was washed with 500  $\mu$ L buffer AW1 and centrifuged at 8,000 rpm for 1 min. Then the column was washed with 500  $\mu$ L buffer AW2 and centrifuged at 14,000 rpm for 3 min. Since residual buffers in the eluate may cause problems in the downstream applications, the Spin column was centrifuged additionally at 14,000 rpm for 1 min. After each centrifugation step the collection tubes (containing the centrifugate) were discarded and for the next centrifugation new provided collection tube were used. Finally the Spin column was loaded with 100  $\mu$ L buffer AE and was centrifuged at 8,000 rpm for 1 min in order to eluate the DNA from the column. Subsequently 5 min incubation at room temperature before centrifugation increases the DNA yield.

In case of QIAmp Stool Kit 200  $\mu$ L faecal suspension was well homogenized by vortexing with 1.4 mL buffer ASL (lysis solution) and freezing-thawing was performed 10-15 times in liquid nitrogen and on 90 °C in water bath (BM 302, Nüve, Turkey) in order to rupture the (oo)cysts. After the freeze-thaw cycles the sample was centrifuged at 14,000 rpm for 1 min in order to pellet the stool particles. 1.2 mL supernatant was aspirated into a new Eppendorf tube, one InhibitEX tablet was completely suspended in the supernatant and the suspension was incubated for 1 min at room temperature to allow inhibitors to absorb. After centrifugation of the suspension at 14,000 rpm for 3 min, 15  $\mu$ L proteinase K was added to 200  $\mu$ L supernatant of the suspension. Then 200  $\mu$ L Buffer AL was added, thoroughly vortexed and incubated for 10 min on 70 °C in water bath (BM 302, Nüve, Turkey). The DNA was precipitated by mixing with 200  $\mu$ L 99% ethanol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and the sample was transferred into the Spin column and centrifuged at 14,000 rpm for 1 min. The column (and the DNA) was washed with 500  $\mu$ L buffer AW1 and centrifuged at 14,000 rpm for 1 min. Then the column was washed with 500  $\mu$ L buffer AW2 and centrifuged at 14,000 rpm for 3 min. Since residual buffers in the eluate may cause problems in the downstream applications, the Spin column was

centrifuged additionally at 14,000 rpm for 1 min. After each centrifugation step the collection tubes containing the centrifugate were discarded and for the next centrifugation new provided collection tubes were used. Finally, the Spin column was loaded with 100  $\mu$ L buffer AE and was centrifuged at 14,000 rpm for 1 min in order to eluate the DNA from the column. A subsequently 5 min incubation at room temperature before centrifugation increases the DNA yield. All centrifugation steps were carried out in Himac CF15RXE High Speed microcentrifuge, Hitachi, Japan or in Microcentrifuge 1-14, Sigma, Germany.

## 2.7.2. DNA amplification

### 2.7.2.1. *Cryptosporidium* PCR assays

**SSU rRNA PCR -1:** This PCR amplifies a 826-864 bp long polymorphous region of the gene, which is suitable for *Cryptosporidium* species or genotype identification.

The nested PCR was performed as described by Xiao et al. (1999a, 2001) in standard mixtures of 50  $\mu$ L containing 1  $\mu$ L of each primer (10  $\mu$ M), 1  $\mu$ L dNTP (10 mM) (Finnzymes, Espoo, Finland), 5  $\mu$ L 10  $\times$ PCR buffer containing 1.5 mM MgCl<sub>2</sub> (Qiagen GmbH, Hilden, Germany), 3  $\mu$ L MgCl<sub>2</sub> (25 mM) (Qiagen GmbH, Hilden, Germany), 0.5  $\mu$ L HotStarTaq DNA polymerase (5 U/ $\mu$ L) (Qiagen GmbH, Hilden, Germany) and 2  $\mu$ L BSA (Acetylated, 10 mg/mL) (Promega, Madison, WI) using the following primers: 5'-CCC ATT TCC TTC GAA ACA GGA-3' and 5'-TTC TAG AGC TAA TAC ATG CG-3' for primary PCR and 5'-GGA AGG GTT GTA TTT ATT AGA TAA AG-3' and 5'-AAG GAG TAA GGA ACA ACC TCC A-3' for secondary PCR. The templates were subjected to 35 amplification cycles (94 °C for 45 s, 55 °C at primary PCR and 58 °C at secondary PCR for 45 s, 72 °C for 60 s) followed by one cycle of 10 min at 72 °C.

**SSU rRNA PCR -2:** This PCR amplifies a 435 bp long polymorphous region of the gene, which is suitable for *Cryptosporidium* species or genotype identification.

The nested PCR was performed in 50  $\mu$ L volume containing in standard mixtures of 50  $\mu$ L containing 1  $\mu$ L of each primer (10  $\mu$ M), 1  $\mu$ L dNTP (10 mM) (Finnzymes, Espoo, Finland), 5  $\mu$ L 10  $\times$ PCR buffer containing 1.5 mM MgCl<sub>2</sub> (Qiagen GmbH, Hilden, Germany), 3  $\mu$ L MgCl<sub>2</sub> (25 mM) (Qiagen GmbH, Hilden, Germany), 0.5  $\mu$ L HotStarTaq

DNA polymerase (5 U/ $\mu$ L) (Qiagen GmbH, Hilden, Germany) and 2  $\mu$ L BSA (Acetylated, 10 mg/mL) (Promega, Madison, WI) according to Nichols et al. (2003). Primers: CPB-DIAGF 5`-AAG CTC GTA GTT GGA TTT CTG-3` and CPB-DIAGR 5`-TAA GGT GCT GAA GGA GTA AGG-3`, N-DIAGF2 5`-CAA TTG GAG GGC AAG TCT GGT GCC AGC-3`, N-DIAGR2 5`-CCT TCC TAT GTC TGG ACC TGG TGA GT-3`. Primary PCR consisted of 35 cycles at 94°C for 30 s, 68°C for 60 s, 72°C for 30 s; and extension at 72°C for 10 min. Secondary PCR consisted of 35 cycles at 94°C for 30 s, 60°C for 60 s, 72°C for 30 s; and extension at 72°C for 10 min.

**Gp-60 PCR:** The gp-60 gene was reported to display a high degree of polymorphism in *C. parvum*, which allows differentiation at subgenotype level. A fragment of the gp-60 gene (800-850 bp) was amplified with primers AL3531 (5' ATA GTC TCC GCT GTA TTC 3'), AL3535 (5' GGA AGG AAC GAT GTA TCT 3') and AL3532 (5' TCC GCT GTA TTC TCA GCC 3'), AL3534 (5' GCA GAG GAA CCA GCA TC 3') in standard mixtures of 50  $\mu$ L containing 1  $\mu$ L of each primer (10  $\mu$ M), 1  $\mu$ L dNTP (10 mM) (Finnzymes, Espoo, Finland), 5  $\mu$ L 10  $\times$ PCR buffer containing 1.5 mM MgCl<sub>2</sub> (Qiagen GmbH, Hilden, Germany), 0.5  $\mu$ L HotStarTaq DNA polymerase (5 U/ $\mu$ L) (Qiagen GmbH, Hilden, Germany) and 2  $\mu$ L BSA (Acetylated, 10 mg/mL) (Promega, Madison, WI). The templates were subjected to 35 amplification cycles (94 °C for 45 s, 50 °C for 45 s, 72 °C for 60 s) followed by one cycle of 10 min. at 72 °C.

In cases, when the nested PCR described above did not give any yield of product the primer set AL3532 (5' TCC GCT GTA TTC TCA GCC 3') and LX0029 (5' CGA ACC ACA TTA CAA ATG AAG T 3') was used for secondary PCR and 400 bp of PCR product was amplified under the same conditions (Alves et al. 2003, Sulaiman et al. 2005).

#### **2.7.2.2. *Giardia* PCR assays**

**18S rRNA PCR:** A nested PCR was performed to amplify a 292 bp fragment of *Giardia* 18S rRNA locus according to Appelbee et al. (2003). This *Giardia* PCR assay is well applicable and sensitive, however, according to Sulaiman et al. (2004) it has limited ability to detect mixed genotypes in environmental samples.

Primers: GiaF: 5`-AAG TGT GGT GCA GAC GGA CTC-3`, GiaR 5`-CTG CTG CCG TCC TTG GAT GT-3` and RH11 5`-CAT CCG GTC GAT CCT GCC-3`, RH4 5`-AGT CGA ACC CTG ATT CTC CGC CAG G-3`. The PCR was performed in standard mixtures of 50 µL containing 1 µL of each primer (10 µM), 1 µL dNTP (10 mM) (Finnzymes, Espoo, Finland), 5 µL 10 ×PCR buffer containing 1.5 mM MgCl<sub>2</sub> (Qiagen GmbH, Hilden, Germany), 10 µL of Q-solution (Qiagen GmbH, Hilden, Germany), 0.5 µL HotStarTaq DNA polymerase (5 U/µL) (Qiagen GmbH, Hilden, Germany) and 2 µL BSA (Acetylated, 10 mg/mL) (Promega, Madison, WI). The templates were subjected to 35 amplification cycles (96 °C for 45 s, 55 °C for 30 s, 72 °C for 45 s) followed by one cycle of 4 min at 72 °C at the primary PCR, then 35 amplification cycles (96 °C for 45 s, 59 °C for 30 s, 72 °C for 30 s) followed by one cycle of 4 min at 72 °C at the secondary PCR.

**GDH PCR:** A semi-nested PCR was performed using the primers and PCR conditions published by Read et al. (2004) to amplify a 432 bp fragment of *Giardia* GDH gene. The GDH gene was reported to display a high degree of polymorphism in *G. duodenalis*, which allows differentiation at both genotype and intra-genotype level. Primers: GDHeF: 5`-TCA ACG TYA AYC GYG GYT TCC GT-3`, GDHiF: 5`-CAG TACAAC TCY GCT CTC GG-3`, GDHiR: 5`-GTT RTC CTT GCA CAT CTC C-3`. The PCR was performed in standard mixtures of 50 µL containing 1 µL of each primer (10 µM), 1 µL dNTP (10 mM) (Finnzymes, Espoo, Finland), 5 µL 10 ×PCR buffer containing 1.5 mM MgCl<sub>2</sub> (Qiagen GmbH, Hilden, Germany), 0.5 µL HotStarTaq DNA polymerase (5 U/µL) (Qiagen GmbH, Hilden, Germany) and 2 µL BSA (Acetylated, 10 mg/mL) (Promega, Madison, WI). The templates were subjected to one cycle of 94 °C for 2 min, 56 °C for 1 min, 72 °C for 2 min and 55 amplification cycles (94 °C for 30 s, 56 °C for 20 s, 72 °C for 45 s), followed by one cycle of 7 min at 72 °C.

All PCR reactions were carried out in ABI 2720 Thermal cycler (Applied Biosystems, Foster, CA).

### 2.7.3. Real-time PCR

The primary advantages of real-time PCR over conventional PCR are that it provides high-throughput analysis in a closed tube format (no post-PCR handling is required), that it can be used for quantitation over a broad dynamic range and that it can be used to differentiate DNA fragments by analysing the melting curve of DNA. Quantitation exploits the proportional relationship between the threshold cycle at which exponential amplification is detected (Ct) and the starting number of copies of the target nucleic acid fragment. The amplification of appropriate DNA strands enables the construction of a standard curve and estimation of gene copy number from the Ct of an unknown sample. Amplified fragments can be characterized further by analysis of the DNA melting curve, which measures the dissociation kinetics of the amplified fragment in the case of intercalating dyes e.g. SYBR Green I, which we used. Plotting the first derivative of the melting curve versus temperature enables the melting temperature (T<sub>m</sub>) of the product to be determined, which is affected by the GC content of the fragment and the absolute order of the bases in the sequence. This detects genetic variation in products in which the number of base differences relative to the size of the fragment is sufficient to affect the melting temperature. The amplification of the correct fragment could be confirmed by DNA melting curve analysis and non-specific products excluded from analysis by raising the acquisition temperature above the melting temperature of the non-specific products (Monis et al. 2005).

***Giardia* real-time PCR:** For *G. duodenalis* Assemblage B specific real-time PCR the primers targeting the 141 bp fragment of the TPI gene (TPIB4IF 5`-GCA CAG AAC GTG TAT CTG G-3`, TPIB4IR 5`- CTC TGC TCA TTG GTC TCG C-3`) were used as described by Amar et al. (2004) with some modifications: reactions were performed using ABI 7300 Real Time PCR System (Applied Biosystems, Foster, CA). The PCR was achieved in standard mixtures of 50 µL containing 1 µL of each primer (10 µM) and 25 µL Power SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA). The templates after incubation for 10 min at 95 °C were subjected to 50 amplification cycles (96 °C for 10 s, 60 °C for 30 s). Fluorescent readings were taken after each annealing step. For the melting curve analysis, after the completion of the last PCR cycle, a quick denaturation

was done at 95 °C (0 s holding time), followed by 30 s annealing step at 60 °C with slow ramp (0.1 °C /s) up to 95 °C with continuous detection thorough the ramp. Following PCR melting peaks with a T<sub>m</sub> of 81.4-81.9 were generated from DNA recovered from *G. duodenalis* H3 isolate (Assemblage B).

This real-time PCR method is a quick and sensitive tool for the first screening of the samples, whether they are contain *Giardia duodenalis* Assemble B, and it was applied during our investigations for the routine water monitoring.

#### 2.7.4. Restriction Fragment Length Polymorphism (RFLP) analysis

**RFLP-1 assay:** After SSU rRNA PCR-1 reaction DNA was cut into restriction fragments using endonucleases SspI and MboII, which only cut the DNA molecule where there are species specific DNA sequences, that are recognized by the enzymes (Xiao et al. 1999a, 2001, Feng et al. 2006). Recognition sites of **SspI: AAT<sup>^</sup>ATT** and **MboII: GAAGA(8/7)** (Figure 9).

```
GGAAGGGTTGTATTTATTAGATAAAGAACCAATATAATTGGTGACTCATAATAACTTTACGGATCACATTAATGTGACA
TATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCTACCGTGGCAATGACGGGTAACGGGGAATTA
GGGTTTCGATTCCGGAGAGGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCC
TAATACAGGGAGGTAGTGACAAGAAATAACAATACAGGACTTTTTGGTTTTGTAATTGGAATGAGTTAAGTATAAACCCC
TTTACAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAGTTGT
TGCAGTTAAAAAGCTCGTAGTTGGATTTCTGTTAATAATTATATAAAAATATTTTGATGAAATATTTATATAATATTAACAT
AATTCATATTACTATATATTTTTAGTATATGAAATTTTACTTTGAGAAAATTAGAGTGCTTAAAGCAGGCATATGCCTTGAA
TACTCCAGCATGGAATAATATTAAAGATTTTTATCTTTCTTATTGGTTCTAAGATAAGAATAATGATTAATAGGGACAGTT
GGGGGCATTGTATTTAACAGTCAGAGGTGAAATCTTAGATTTGTTAAAGACAAAATAATGCGAAAGCATTTGCCAAGG
ATGTTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCC
AACTAGAGATTGGAGGTTGTTTCCTT
```

**Figure 9:** Recognition sites of the restriction enzymes Ssp I (lilac) and MboII (green) on *C. parvum* SSU rRNA PCR-1 products.

The master mix used for restriction digestion contained 2 µL of reaction buffer (Promega, Madison, WI), 7.8 µL of nuclease free water and 0.5 µL of SspI (10 U/µL) (Promega, Madison, WI) or 0.5 µL MboII (10 U /µL) (Promega, Madison, WI) per reaction. Each reaction mixture contained 10 µL of master mix and 10 µL of secondary PCR product. Restriction digestion was carried out on 37 °C for 3 h in water bath (BM 302, Nüve, Turkey).

The restriction fragments are then separated according to length by agarose gel electrophoresis as described in chapter 2.7.6.

The main *Cryptosporidium* parasites of cattle, *C. andersoni* can be differentiated from *C. parvum*, *C. bovis* and deer-like genotype by the SspI pattern and the latter three can be differentiated from each other by the MboII pattern. The predicted sizes of the diagnostic RFLP fragments (Feng et al. 2006) are shown in Table 6.

<b><i>Cryptosporidium</i> species and genotypes</b>	<b>SspI restriction fragments</b>	<b>MboII restriction fragments</b>
<i>C. andersoni</i>	448 bp, 397 bp	769 bp, 76 bp
<i>C. parvum</i>	449 bp, 267 bp, 108 bp	771 bp, 76 bp
<i>C. bovis</i>	432 (413) bp, 267 bp, 103 bp	412 bp, 162 bp, 76 bp
<i>C. deer-like genotype</i>		574 bp, 76 bp

**Table 6:** The predicted sizes of the RFLP fragments of *Cryptosporidium* SSU rRNA PCR-1 products.

**RFLP-2 assay:** RFLP analysis of secondary *Giardia* GDH PCR products were performed using RsaI and NlaIV restriction enzymes to differentiate the Assemblages (Read et al. 2004). Recognition sites of **Rsa I: GT<sup>^</sup>AC** and **NlaIV: GGN<sup>^</sup>NCC** (Figure 10).

CA **GTAC**AACTCCGCTCTC**GGGCC**TACAAGGGTGGTCTCCGCTTCCACCCCTCTGTCAACCTCTCGATCCTTAAGTTCCTC  
GGCTTTGAGCAGATCCTGAAGAACTCCCTTACCACGCTTCCGATGGGCGGTGGTAAGGGC**GGCTCC**GACTTCGATCCTAA  
GGGCAAGTCGGACAACGAGGTCATGCGCTTCTGCCAGTCCTTATGACCGAGCTCCAGAGGCACGTCGGGGCTGACACCG  
ACGTTCTGCTGGCGATATTGGCGTCGGCGGTCCGAGATCGGTTATCTGTTTGGACAGTATAAGCGCCTCAGGAACGAG  
TTTACGGGCGTCTCACGGGCAAGAACATCAAGTGGGCGGGTCTCTCATCAGACCAGAGGCCACAGGGTATGGAGCTG  
TCTACTTCTGGAGGAGATGTGCAAGG

**Figure 10:** Recognition sites of the restriction enzymes NlaIV (blue) and RsaI (lilac) on *G. duodenalis* Assemblage BIV GDH PCR products.

The master mix used for restriction digestion contained 2 µL of reaction buffer supplied with the enzyme, 7.8/6.3 µL water and 0.5/2 µL of restriction enzymes RsaI (10 U/µL) (Promega, Madison, WI) or NlaIV (1 U/µL) (New England Biolabs Inc. Ipswich, MA) per reaction. Each reaction mixture contained 10 µL of master mix and 10 µL of secondary PCR product. Restriction digestion was carried out at 37 °C for 3 h in water bath (BM 302, Nüve, Turkey).

The restriction fragments were then separated according to length by on chip gel electrophoresis as described in chapter 2.7.5.

*Giardia duodenalis* Assemblages AI-II, B, C, D, E can be differentiated from each other by the *NlaIV* pattern and *G. duodenalis* Assemblage BIII and BIV can be differentiated from each other by the *RsaI* pattern. The predicted sizes of the diagnosable RFLP fragments (Read et al. 2004) are shown in Table 7.

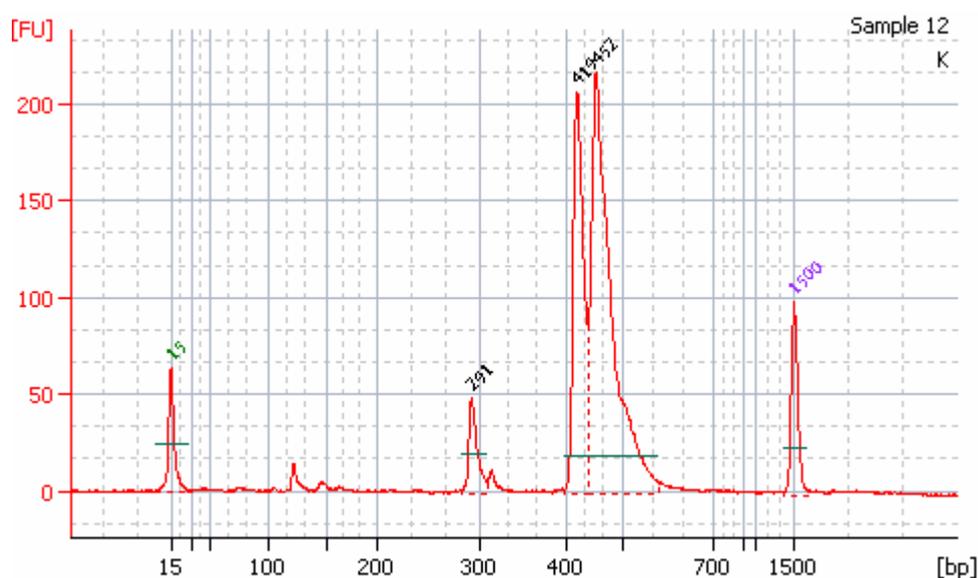
<i>Giardia duodenalis</i> Assemblage	<i>NlaIV</i> restriction fragments	<i>RsaI</i> restriction fragments
AI	87 bp, 123 bp and 149 bp	-
AII	72 bp, 77 bp, 87 bp, 123 bp	-
BIII	123 bp and 291 bp	131 bp and 297 bp
BIV		423 bp
C	72 bp, 123 bp and 187 bp	-
D	126 bp and 249 bp	-
E	72 bp, 106 bp and 218 bp	-

**Table 7:** The predicted sizes of the RFLP fragments of *Giardia duodenalis* GDH PCR products.

#### 2.7.4. DNA analysis by on chip electrophoresis after RFLP assays

This method allows automatically size and quantitate the PCR fragments and restriction digests accurately and reproducibly. While in some cases, it is sufficient to detect the presence or absence of a PCR product, in many cases quantitation of this product and detection of unspecific amplification is critical. Therefore DNA analysis was performed using Agilent's Chip DNA assays using Bioanalyser 2100 and the DNA 1000 Kit (5067-1504, Agilent). Firstly the gel-dye mix was prepared: 25  $\mu$ L DNA Dye concentrate was pipetted into the gel matrix tube and was vortexed for 5 sec. Then the mixture was transferred into the spin filter and centrifuged at 1,500 rpm for 15 min (Microcentrifuge 1-14, Sigma, Germany). This filtrate was used later. The chip was loaded with the necessary chemicals as follows: The chip was placed into the Chip Priming Station. 9  $\mu$ L gel-dye mix was pipetted in the "white G" well. The tip of the pipette was inserted to the bottom of the well when dispensing, which prevents large air bubbles forming under the gel-dye mix. The plunger of the Priming Station was placed at 1 mL position and the base of the plate at position C. Then the Chip Priming Station was closed until the latch is secure. The plunger was pressed until it is held by the syringe clip and after exactly 30 sec the plunger was

released with the clip release mechanism. The plunger automatically returns to 6-8 mL if a proper seal was achieved. Then the plunger was pulled back to the 1 ml position to release the pressure. After opening the Chip Priming Station, the chip was checked for air bubbles. It is not advisable to touch underside the chip. Then 9  $\mu$ L gel-dye mix was pipetted in both of the wells marked “black G”, 5  $\mu$ L DNA marker was dispensed in each well 1-12 and in the well marked as “L”. Then 1  $\mu$ L DNA ladder was dispensed in the well marked as “L” and 1  $\mu$ L of the samples were dispensed in wells 1-12. The chip was placed in the adapter of the vortex mixer (Agilent) and was vortexed for 1 min at the IKA vortexer set-point. Finally, the chip was placed in the Agilent Bioanalyser 2100 and the run was started within 5 min. An example of electropherogram of the DNA on chip analysis is shown in Figure 11. There are shown the sizes of the DNA fragments (bp) in the sample and the relative amount of each DNA fragment after RFLP-2, NlaIV digestion (chapter 2.7.4).



**Figure 11:** An example of electropherogram of a DNA on chip analysis. Peaks at 19 bp and at 1500 bp are DNA markers, peaks at 419, 452, ~291 bp and a small peak around 125 bp are DNA fragments in the sample after RFLP-2, NlaIV digestion.

### 2.7.6. Agarose gelelectrophoresis

Firstly to pour a gel, 2 g agarose powder (Promega, Madison, WI) was mixed with 100 mL TAE buffer (Tris-acetate-EDTA) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), and heated in a microwave oven under maximum settings until completely melted

(approximately 5 min). After cooling the solution to about 60 °C, it was poured into a casting tray containing a sample comb and allowed to solidify at room temperature. After the gel has solidified, the comb was removed, using care not to rip the bottom of the wells. The gel, still in its plastic tray, was inserted horizontally into the electrophoresis chamber (Minigel 2 Horizontal Gel Tank, Apelex, France) and just covered with TAE buffer (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). 5 µL of the samples containing DNA was mixed with 1 µL 6× loading dye solution (Fermantas Life Sciences, Hannover, MD) and then the mix was pipeted into the sample wells. The lid and power leads were placed on the apparatus, and a current was applied (100V). Bubbles coming off the electrodes confirm that current is flowing. DNA migrated towards the positive electrode (red coloured). After 30 min, when adequate migration has occurred, the gel was stained after electrophoresis by soaking in a dilute solution of ethidium bromide. This fluorescent dye intercalates between bases of DNA. To visualize DNA the gel was placed on a ultraviolet transilluminator (MiniBis, DNR Bio-Imaging Systems Ltd., Germany). Fragments of linear DNA migrate through agarose gels with a mobility that is inversely proportional to the log<sub>10</sub> of their molecular weight. For the estimation of the size of our DNA molecules a 100 bp DNA ladder (New England Biolabs Inc., Ipswich, MA) was used, which contains DNA fragments of various known sizes.

### **2.7.7. Cloning of the PCR products**

#### **2.7.7.1. PCR product purification**

For sequencing and ligation, the purification of all the PCR products is essential in order to eliminate other by products. The DNA fragment from the agarose gel was excised with a scalpel and purified with QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany) using the supplied buffers: the approximately 300 mg gel slice was put into Eppendorf tube, 900 µL buffer QG was added to the gel slice and was incubated on 50 °C for 15 min in water bath (BM 302, Nüve, Turkey). The tube every 2-3 min was vortexed to help the gel solubilization. After the gel slice has dissolved completely, 300 µL isopropanol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added to the sample and mixed. Then the entire sample was applied to the QIAquick column and centrifuged at 13,000 rpm

for 1 min. The column was washed firstly with 500  $\mu$ L buffer QG and secondly with 750  $\mu$ L buffer PE. For better salt elimination the column, containing 750  $\mu$ L buffer PE, was incubated for 5 min on room temperature before centrifugation. After each centrifugation step the centrifugate in the collection tubes was discarded. Finally the column was centrifuged again at 13,000 rpm for 1 min. To elute the DNA from the column, 30  $\mu$ L buffer EB was added to the center of the membrane, incubated at room temperature for 2 min and centrifuged at 13,000 rpm for 1 min.

All centrifugation steps were carried out in Himac CF15RXE High Speed microcentrifuge, Hitachi, Japan or in Microcentrifuge 1-14, Sigma, Germany.

#### **2.7.7.2. Competent *Escherichia coli* cell preparation for cloning of the PCR products**

*Escherichia coli* DH5 $\alpha$  cells from the National Research Center of the Obihiro University stock were used for competent cell preparation. 5  $\mu$ L of the stock were inoculated in 5 mL LB broth (Takara Shuzo Co. Ltd. Kyoto, Japan) and cultured overnight on 37 °C with shaking (Bioshaker BR-180LF, Taitec, Japan). Then the cells were further cultured: firstly 200  $\mu$ L in 20 mL LB broth (Takara Shuzo Co. Ltd. Kyoto, Japan) for 2 hr, then 2 mL in 200 mL LB broth (Takara Shuzo Co. Ltd. Kyoto, Japan) for 3 hr on 37 °C with shaking (Bioshaker BR-180LF, Taitec, Japan). After culturing, the broth was centrifuged at 3,000 rpm for 30 min (Himac CF7D2, Hitachi, Japan) and all the supernatant was aspirated. 100 mL cold TFN was added to the pellet and the cells were incubated for 20 min on ice. The mixture was centrifuged again at 3,000 rpm for 30 min (Himac CF7D2, Hitachi, Japan) and the supernatant was removed. Finally 15 mL cold TFN was added to the cells and stored in aliquots 90  $\mu$ L on -80 °C. All steps were performed under laminar sterile conditions.

#### **2.7.7.3. Ligation of the *Giardia* GDH PCR product into plasmid and transformation of plasmid into *Escherichia coli* DH5 $\alpha$ competent cells**

Ligation of PCR products is necessary to pick out a particular gene sequence of a mixed population, which is not possible by direct sequencing. In our case it was necessary to

ligate some *Giardia* secondary GDH PCR products in order to confirm the uniqueness of the sequence.

For this procedure firstly LB agar plates were prepared supplemented with 50 µg/mL of ampicillin: 16 g LB agar (Takara Shuzo Co. Ltd. Kyoto, Japan) was added to a 1 L bottle and filled upto 500 mL of deionised water. It was mixed, shaken and finally autoclaved at 121 °C for 15 min. After autoclaving the medium was cooled down until it is cool enough to be hold by hands (about 40 °C) and the appropriate amount of antibiotic, 500 µL of ampicillin solution (50 mg/ml) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added to the medium and swirled to mix. Then the LB agar was poured from the flask into sterile plastic plates under laminar box. The flask mouth was sterilized by flame, the lid of the top plate was opened, and then the LB agar was poured onto the plate until about half-way full. If there were any bubbles formed, the bubble was bursted by passing the flame on the LB agar quickly. The plates were wrapped with saran rap.

Purified products from *Giardia* GDH PCR assay were ligated into pGEM-T plasmid vector (Promega, Madison, WI) and used to transform competent *Escherichia coli* DH5α cells.

The 10 µL ligation reaction mix: 1 µL ligase (Promega, Madison, WI), 1 µL vector (Promega, Madison, WI), 5 µL buffer (Promega, Madison, WI), 3 µL purified PCR product was kept on ice for 12 hr and this mix was added to the 90 µL competent cells (chapter 2.7.7.2.). After heat shock (20 min on ice, 50 sec on 42 °C, then 2 min on ice), which helps the transformation of the plasmids into the competent cells, 900 µL LB broth (Takara Shuzo Co. Ltd. Kyoto, Japan) was added to the cells and were cultured on 37 °C for 1.5 hr with shaking (Bioshaker BR-180LF, Taitec, Japan). The culture was centrifuged (Himac CF7D2, Hitachi, Japan) at 2,500 rpm for 10 min, the supernatant was aspirated and the 100 µL pellet was spread on the surface of the prepared LB-ampicillin agar. Plates were incubated on 37 °C for 12 hr in incubator (BNR-110, Espec, Japan) and 3 clones per sample were further cultured in 50 mL LB broth supplemented with 50 µg/mL of ampicillin (Takara Shuzo Co. Ltd. Kyoto, Japan) on 37 °C for 12 hr with shaking (Bioshaker BR-180LF, Taitec, Japan). Finally the broth was centrifuged at 2,500 rpm for 10 min (Himac CF7D2, Hitachi, Japan) and the supernatant was aspirated remaining 1 mL pellet.

100 µL of PBS was added to 10 µL of the pellet and was centrifuged (Himac CF15RXE High Speed Micro Centrifuge, Hitachi, Japan) at 15,000 rpm for 30 sec. The supernatant

was discarded and 10  $\mu$ L distilled water was added to the pellet. Then the suspension was boiled for 5 min to release the plasmids from the cells. The presence of the insert in the vector was checked by insert specific PCR using the inner primers as described in chapter 2.7.2.2.

All steps were performed under laminar sterile conditions.

#### **2.7.7.4. Plasmid isolation**

In order to sequence the inserted PCR product it is necessary to isolate the plasmid DNA (containing the insert) of the *E. coli* clones. For this procedure we used the Plasmid Mini Kit (Qiagen GmbH, Hilden, Germany) along with the supplied buffers: the bacterial pellet was resuspended in 0.3 mL buffer P1, then 0.3 mL of buffer P2 was added, mixed gently and incubated at room temperature for 5 min. Then 0.3 mL of chilled buffer P3 was added, mixed immediately and gently and incubated on ice for 5 min. Then the mixture was centrifuged at 15,000 rpm for 10 min and the supernatant was aspirated. In the meantime the Qiagen tip was equilibrated with 1 mL buffer QBT and later the aspirated supernatant was applied to this Qiagen tip. The tip (column) was allowed to empty by gravity flow and it was washed four times with 1 mL buffer QC. The DNA was eluted with 0.8 mL buffer QF. Then 560  $\mu$ L of room temperature isopropanol (Wako Pure Chemical Industries Ltd. Osaka, Japan) was added to the eluate, centrifuged at 15,000 rpm for 30 min and the supernatant was aspirated. 1 mL of 70% ethanol (Wako Pure Chemical Industries Ltd. Osaka, Japan) was added to the pellet, centrifuged at 15,000 rpm for 10 min, the supernatant was aspirated and the pellet was dried. Finally the plasmid was dissolved in 30  $\mu$ L TE buffer.

All centrifugation steps were carried out in Himac CF15RXE High Speed Micro Centrifuge, Hitachi, Japan.

#### **2.7.8. Sequencing, sequence and phylogenetic analysis**

The purified secondary PCR products and plasmids were sequenced on an ABI Prism 3100 Genetic Analyzer by using a BigDye Terminator V.3.1 cycle sequencing kit (Applied Biosystems, Foster, CA). Sequencing PCR reaction was carried out in 10  $\mu$ L final volume

containing 4 µL Big Dye 3.1 master mix (Applied Biosystems, Foster, CA), 5 µL purified PCR product or plasmid, 1 µL primer used in secondary PCR or T7 promoter primer or M13 primers designed for the plasmid (10 µM). The templates after incubation on 96 °C for 2 min were subjected to 35 cycles of 96 °C for 10 sec, 50°C for 5 sec, 60°C for 4 min. After the sequencing reaction ethanol precipitation of the product was performed. Firstly 40 µL of 80% ethanol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added to the sample; it was incubated in dark for 15 min and centrifuged at 15,000 rpm for 10 min. All the supernatant was aspirated. Then 125 µL of 70% ethanol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added to the pellet, centrifuged at 15,000 rpm for 10 min, all the supernatant was aspirated and the pellet was dried. All centrifugation steps were carried out in Himac CF15RXE High Speed microcentrifuge, Hitachi, Japan or in Microcentrifuge 1-14, Sigma, Germany. Before dispensing 20 µL of the samples per well into the 96-well sequencing plate, 20 µL formamide was added to the pellet and vortexed for 10 min. The accuracy of data was confirmed by two directional sequencing. Chromatograms and sequences were edited using Chromas: <http://www.technelysium.com.au/chromas.html>.

In case of *Giardia* 18S rRNA products, this region was previously shown to be reliable in differentiating between genotypes (Hopkins et al. 1997). Isolates of genetic Assemblage A have bases GCG at positions 22-24 and G, T and C at positions 44, 62, and 72, respectively, compared to isolates of genetic Assemblage B that have bases ATC at positions 22-24 and C, G and G at positions 44, 62 and 72, respectively and an insertion, A, at position 43 (Hopkins et al. 1997, Monis et al. 1999). Additionally the *Giardia* 18S rRNA sequences obtained were compared to reference sequences downloaded from the GenBank using ClustalW. Reference sequences are shown in Table 5.

*Giardia* GDH nucleotide sequences obtained from the isolates were aligned with each other and published human and animal GDH sequences from different countries by using ClustalW. Tree was constructed using the neighbour joining algorithm based on evolutionary distances calculated by the Kimura two-parameter model with 1,000 bootstrap sampling: [www.clustalw.ddbj.nig.ac.jp/top-e.html](http://www.clustalw.ddbj.nig.ac.jp/top-e.html). The constructed tree and used reference sequences are shown in Figure 14. Representative or unique sequences were submitted in the GenBank under accession numbers: EU375521-EU375522, EU350516.

In case of *Cryptosporidium* SSUrRNA or gp-60 nucleotide sequences obtained from the isolates were aligned with each other and published human and animal SSUrRNA or gp-60 sequences from different countries by using ClustalW. Tree was constructed using the neighbour joining algorithm based on evolutionary distances calculated by the Kimura two-parameter model with 1,000 bootstrap sampling: [www.clustalw.ddbj.nig.ac.jp/top-e.html](http://www.clustalw.ddbj.nig.ac.jp/top-e.html). Reference sequences used are indicated in Table 1, 2, 3 and in Figure 15. Representative or unique sequences were submitted in the GenBank under accession numbers: EF073047-EF073051.

### 3. FIRST INVESTIGATIONS INTO THE PREVALENCE OF *CRYPTOSPORIDIUM* AND *GIARDIA* SPP. IN HUNGARIAN DRINKING WATERS

#### 3.1. Introduction

With a land area of 93,030 km<sup>2</sup>, Hungary is a country in Central Europe and it measures about 250 km from north to south and 524 km from east to west. It has 2,258 km of boundaries, shared with Austria to the west, Serbia, Croatia and Slovenia to the south and southwest, Romania to the southeast, the Ukraine to the northeast, and Slovakia to the north (Map 1).

The public water supply system covers the whole country and it is based on several types of ground waters. There are the shallow, unconfined aquifers, the deeper, confined aquifers (used for drinking water till 400 meters in depth) and the karstic aquifers. The so called riverbank filtered aquifers are along the Danube and some other rivers; they are gravel and sandy beds. There are about 1600 well fields. 94.1 % of the amount of water supplied originate from groundwater (confined ground water 41.1%, unconfined ground water 2.6 %, karstic water 11.2%, riverbank filtrate 39.4%) and 5.9% from surface water.

It is unusual to find *Cryptosporidium* and *Giardia* parasites in boreholes or deep well water unless fissures are present, thereby allowing surface water into the source. However, they are present in almost all surface waters. If no data are available on the presence of *Cryptosporidium* and *Giardia* in the watersheds, the average concentration can be only estimated very roughly from information on the level of faecal pollution of the watershed. This can be assessed by a sanitary survey and available data on faecal indicator bacteria. In case of pristine, where no direct input of wastes, although minimal human activity, agriculture and wildlife are present, the *E. coli* concentrations are below 10/100 mL, supposedly *Cryptosporidium* is infrequently present. In case of polluted surface water, agriculture is present, wastewater is treated before discharged into the watershed the *E. coli* concentrations are 10-100 or in extreme cases <1000/100 mL, the *Cryptosporidium* concentration estimated is 1-10 oocysts/L. When untreated wastewater or manure is

discharged into the watershed, *E. coli* concentrations are >1000/100 mL, the *Cryptosporidium* concentration can be >100 oocysts/L (Medema et al. 2006).

In this study, we investigated the public drinking water systems in order to gain information on the occurrence and distribution of *Cryptosporidium* and *Giardia* (oo)cysts and estimate the efficacy of applied water treatment techniques in Hungary.

## **3.2. Materials and methods**

### **3.2.1. Sampling sites and sampling design**

From the year 2000 until 2005 suspected contaminated drinking water resources were examined on an irregular basis taking into consideration particular events such as heavy rains or/and dry seasons. The sampling sites are shown in Map 1.

**Springs:** Three springs and three karst wells were investigated. Karsts are a special type of landscape that is formed by dissolution of soluble rocks, including limestone and dolomite. Karst regions contain aquifers that are capable of providing large supplies of water. These aquifers are very productive, but they are more susceptible to contamination, than those in other geological media. In all cases, the water is used without treatment or treatment by chlorination or in the case of one spring, the water is stored in an open pool. There is extensive animal husbandry in the vicinity of the karst wells and in 2 springs. The sample code is 1, 3-5, 12, 17 in Appendix 1 and Map 1.

**Wells:** Two groundwater wells were sampled several times, one of which showed bacteriological problems and was the site of a second giardiasis outbreak in Hungary. During this sampling period, numerous giardiasis cases were identified among local people, mainly among children. This outbreak has been highlighted in the public media but was never reported in a scientific journal. The sample code is 14-15 in Appendix 1 and Map 1.

**Raw and tap water from treatment plants:** Hungary has a total of 16 treatment plants abstracting surface water from lakes and rivers for drinking water consumption. Source

water nears all 16 plants and finished water have been tested for a minimum twice during the investigative period of 2000-2005 in different seasons and/or after rainfalls, when the water level of reservoirs and rivers was raised. Four surface water treatment plants are located around the Lake Balaton, one is located on the River Danube, one on the River Tisza, one on the Eastern Main Canal, two on the River Bódva, and one is located at Brook Nagy of Füzér. In Hungary there are 6 water reservoirs used for drinking water preparation. In general the main steps of the surface water treatment are flocculation by alum and iron salts and/or synthetic organic polymers (alone, or in combination), settling or sedimentation, before sand (or gravel) filtration and chlorination (with chlorine or chlorine dioxide). The maximum level of combined chlorine, chlorite and chlorine ( $\text{Cl}_2$ ) never exceeded 3 mg/L, 0.2 mg/L and 0.5 mg/L in the final water. Chlorine dioxide can be added to a maximum of 0.4 mg/L final concentration.

*Lake Balaton:* The ecological state of Lake Balaton was at its lowest in 1995, when the eutrophication caused problems. It was a result of nutrient pollution such as the release of sewage effluent and run-off fertilizers into the water. Eutrophication promotes excessive plant growth and decay, favours certain weedy species over others, and causes severe reductions in water quality. Enhanced growth of choking aquatic vegetation and phytoplankton (algal bloom) disrupts normal functioning of the ecosystem, causing a variety of problems such as a lack of oxygen in the water. Human society is impacted as well: eutrophication decreases the resource value of the lake such that recreation, fishing and aesthetic enjoyment are hindered. Health-related problems occurred at Lake Balaton too, where eutrophic conditions interfered with drinking water treatment. Nowadays, due to environmental investments, sewage treatment and waste management reforms (at the minimum biological treatment) Lake Balaton is in a good state. Its catchment area is 5,800 km<sup>2</sup> with the River Zala providing the largest source of water and the canalized Sió being the only outflow. The possibility of *Cryptosporidium* and *Giardia* (oo)cysts contamination arises from small inflows, ducts, treated sewage, and from birds. The drinking water treatment includes additional activated carbon filtration. Sample code is 6, 7, 8, 9 in Appendix 1 and Map 1.

*River Danube:* This is the second largest river in Europe and the only major European river to flow from west to east. It receives water from the Black Forest Mountains of Germany and empties after 2,850 km into the Black Sea on the Romanian coast. Along its long route

the Danube flows through 9 countries and drains an area slightly larger, than 817,000 km<sup>2</sup>. It has 300 tributaries, the principal one being the River Tisza in Hungary. A small water treatment plant services the River Danube to produce water for only 8,000 people. Authorized sewage outflows are located at the 1576, 1577, 1577.2, 1580.2 and 1584 km points and water treatment is located at the 1576 km point from the issue, which means that the sewage discharge is before the water treatment. The drinking water treatment includes additional activated carbon filtration. Sample code is 10 in Appendix 1 and Map 1.

*River Tisza:* This river is the second largest river in Hungary and the longest Danube tributary. It is approximately 966 km long with approximately 160 km laying in the Ukraine and Romania and about 800 km in the Great Hungarian Plain. It has a catchment area of 157,000 km<sup>2</sup> with 29.4% being in Hungary. Like the River Danube, it receives many authorized and sometimes illegal sewage outflows, although none of them are located near the water treatment plant. The drinking water treatment includes additional activated carbon filtration and ozonization. Sample code is 2 in Appendix 1 and Map 1.

*Eastern Main Canal:* The main function of this canal is agricultural, and supplies water to fish ponds, year round irrigation-water to the plain and receives the inland water. It does not receive sewage, and the possibility of *Cryptosporidium* and *Giardia* contamination exists from animal husbandry. The drinking water treatment contains additional activated carbon filtration and ozonization. Sample code is 25 in Appendix 1 and Map 1.

*River Bódva:* Its catchment area is 1,730 km<sup>2</sup>. The tributaries are polluted with sewage effluents and at 10.7 km from the issue there is an authorized sewage outflow. Water treatments are located at the 0.1 and 5.7 river km points, which means that the sewage discharge is before the water treatment. If the raw water turbidity is high, the water treatment includes additional activated carbon filtration. If the raw water turbidity is low the water is pumped through sand pools into the groundwater prior to chlorination and delivered into the distribution system. Sample code is 18, 19 in Appendix 1 and Map 1.

*Brook Nagy of Füzér:* Its catchment area is 4 km<sup>2</sup>. This brook flows through forests, where contamination may occur from wild animals. There is also a small water treatment plant at the 0.1 km point of the brook that produces water for around 1000 people. The drinking water treatment does not include flocculation step and includes activated carbon filtration. The sample code is 16 in Appendix 1 and Map 1.

*Water reservoirs:* Reservoir Hasznos is established on the Brook Kövicses (from Spring Kövicses) (catchment area is 36 km<sup>2</sup>), Komravölgy on the River Ipoly (catchment area is 5.4 km<sup>2</sup>), Köszörűvölgy on the Brook Köszörű (from Spring Köszörű) (catchment area is 6.4 km<sup>2</sup>), Csórrét on the Brook Nagy (from Spring Aranybánya and Nyírjes) (catchment area is 8.38 km<sup>2</sup>), Lázbérc on the Brook Bán and Csernely (from Spring Bán and Uppony) (catchment area is 218 km<sup>2</sup>) and Mátrafüred on the Brook Csatorna (catchment area is 25 km<sup>2</sup>, this brook receives water from 9 springs, which are 50-500 m from the brook). Brook Bán, Kövicses, Ipoly, and Nagy (Csórrét) receive authorized sewage. Only in the case of Brook Bán, where water treatment is at river km 10.3, and sewage outflow is at river km 26.3, the sewage discharge is before the water treatment. The reservoirs are surrounded by forests where the possibility of contamination exists by wild animals. Hasznos and Komravölgy have additional activated carbon filtration. Mátrafüred does not include flocculation in water treatment. Sample codes are indicated as 13, 20- 24 in Appendix 1 and Map 1.

**Riverbank filtered water:** The water supply of the city of Budapest and its suburbs originates from riverbank filtration, and approximately 2 million people consume water after riverbank filtration. In total, 700 riverbank filtration wells are located 30-100 m from River Danube, mainly on the Island Szentendrei and on the Island Csepel. On the Island Szentendrei, water flow through sand and gravel layers and is directed to the drinking water distribution system after chlorination. On the Island Csepel water is filtered through gravel layers and after treatment (ozonization, sand and activated carbon filtration, removal of iron and manganese, and chlorination) is directed to the water distribution system. During 2004-2005, the River Danube in Budapest was examined once per week at the 1656 river km point along with its riverbank filtered water. For sampling post riverbank filtration, we selected four sampling points at the drinking water distribution system.



**Map 1:** The map of Hungary, the location and the codes of sampling sites of *Cryptosporidium* and *Giardia* microscopic analysis. The numbers are sample codes as indicated in the Appendix 1.

### 3.2.2. Sample collection, parasite concentration and examination

Sample collection and parasite concentration was performed using Filta-Max foam filters, membrane filtration and chemical flocculation. Oocysts were separated from debris by IMS and were examined after staining by epifluorescent microscope as described in chapters 2.2., 2.4., 2.5.

### 3.3. Results and discussion

236 water samples within Hungary (31 raw water, 44 drinking water, 87 river water, and 71 post RBF samples) were collected and investigated for the presence of *Cryptosporidium* oocysts and *Giardia* cysts.

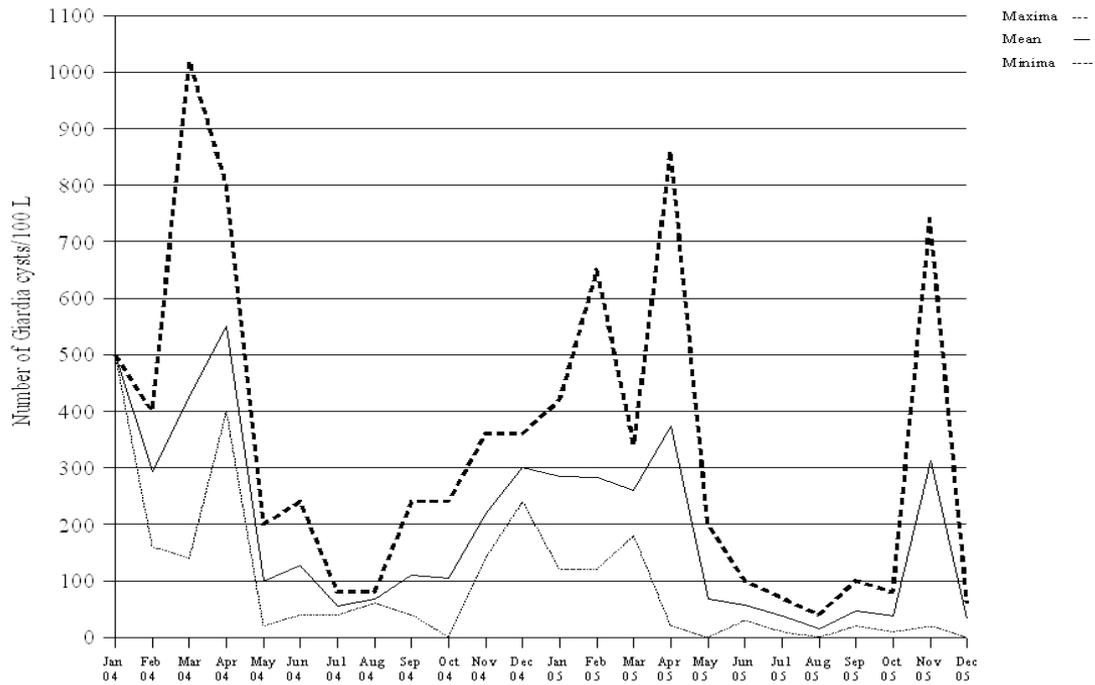
*Spring water:* In one spring water, 2 *Giardia* cysts/100 L were found (sample code 1, Appendix 1 and Map 1) while in another, 4 *Cryptosporidium* oocysts and 3.5 *Giardia* cysts/100 L were detected once. The drinking water in the last case was stored for a long time in an opened pool. The sample code is 17 in Appendix 1 and Map 1.

*Ground water:* No protozoa were detected in any sampled groundwater.

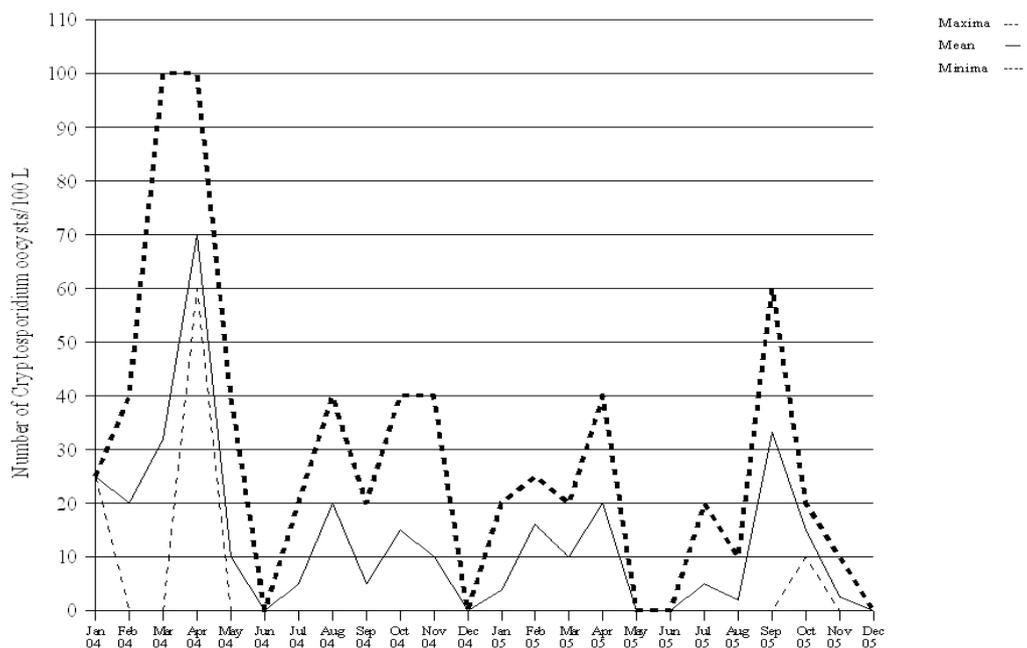
*Raw water:* The raw water of 10 treatment plants was contaminated with both protozoa, ranging from 5 to 50 *Cryptosporidium* oocysts per 100 L and 0.3-1030 *Giardia* cysts/100 L (samples 2, 8-10, 13, 16, 18-20, 22, 24-25, Appendix 1 and Map 1).

*Final (drinking) water:* The final water of 8 water treatment plants was contaminated with *Cryptosporidium* and *Giardia* (oo)cysts varying between 0.1-3 *Cryptosporidium* oocysts and 0.2-63.6 *Giardia* cysts/100 L (samples 2, 8-10, 13, 16, 18-20, 22, 24-25, see also Appendix 1 and Map 1).

*Danube surface river water and riverbank filtered water:* *Cryptosporidium* oocysts (0/100 L) were not detected in ~60% of surface river water samples. In those samples found to be contaminated oocysts values varied between 0-50 /100 L. *Giardia* spp. cysts were detected more frequently, with cyst numbers ranging between 0-500/100 L in ~90% of river water samples, with 1% of the samples containing more than 1000 cysts/100 L. The highest *Giardia* cysts levels were found in January to April in both 2004 and 2005 with a second peak in November 2005. During these times a record high of 1020 *Giardia* cysts/100 L was found with mean numbers ranging between 260-550 cysts/100 L. The lowest *Giardia* cysts levels were found to be in July and August each year with mean numbers ranging between 16-67 cysts/100 L. Similar to *Giardia*, *Cryptosporidium* oocysts concentrations peaked in March and April 2004, with the highest recorded number being 100 oocysts/100 L; the mean varied between 32-70 oocysts/100 L. The minimum and maximum counts for the detection of *Cryptosporidium* and *Giardia* in River Danube samples are shown in Figures 12 and 13 and are presented for each month in the investigated period of 2004 and 2005. Interestingly, no *Cryptosporidium* oocysts or *Giardia* cysts could be detected in the distribution system of the drinking water from riverbank-filtered sources.



**Figure 12:** *Giardia* cysts counts in River Danube at 1656 river km during the years 2004-2005.



**Figure 13:** *Cryptosporidium* oocyst counts in River Danube at 1656 river km during the years 2004 -2005.

**Monitoring design:** Monitoring of *Cryptosporidium* and *Giardia* needed to understand the occurrence of *Cryptosporidium* and *Giardia* in the source waters. Most monitoring programs are using sampling schemes with regular intervals. Such sampling schemes may miss important peak events and it is better to guide monitoring with information about hazardous events that may occur in the catchment and lead to peak contaminations of source waters. Several authors have found relationship between heavy rainfall and high concentrations of *Cryptosporidium* (Semenza and Nichols 2007, Curriero et al. 2001). Also sewer overflows and snowmelt lead to peaks of *Cryptosporidium* concentration in source waters (Gibson et al. 1998). Extreme weather conditions may be a driver for peak events, both in surface and ground water. Other causes also occur, most of the man-made, such as farming practices, accidental spills and water quantity management practices. These events are catchment specific and catchment survey can identify the specific events that could lead to peak in specific source water.

In our investigations, samples were taken from water treatment plants posing a high risk of protozoan contamination; 6 surface water treatment plants were in contact with effluents from sewage treatment plants located between 1 to 40 km away, 3 surface water treatment plants took water from a forested area containing abundant wild animals, and 2 surface water treatment plants' source water was near agricultural activities (livestock rearing). We took into consideration particular events such as heavy rains or/and dry seasons. In all cases the raw water was contaminated, however, except for two water treatment plants parasite removal seemed effective during the investigative period.

**Similar investigations in Hungary and in neighbouring countries:** In Eastern European countries, investigations into contamination of water supplies with *Giardia* and *Cryptosporidium* are limited. In 1987-1988, small Hungarian waterworks (water originating from springs and streams) were examined for the occurrence of *Giardia* cysts using membrane filtration of 70-380 L for sample concentration of the drinking water in order to examine the final pellet by direct microscopy. *Giardia* was detected regularly in one of the springs with the authors emphasizing that the hazard from *Giardia* contaminations in Hungary exists, especially where spring water originates from a forest environment (Andrik and Kőműves 1989). Raw water sources in the Czech Republic were found to contain 0 to 7400 *Cryptosporidium* oocysts per 100 litres and 0 to 485 *Giardia*

cysts per 100 litres (Dolejs et al. 2000) and high levels of contamination have been reported for both protozoa in water supplies in Russia and Bulgaria (Karanis et al. 2006).

**Riverbank filtration is an effective water treatment technology:** Raw water from the River Danube was found to be contaminated with both *Giardia* cysts and *Cryptosporidium* oocysts. Changes in water level and introduction of differently treated sewage led to high variability in the numbers of these protozoa with counts varying over two or three orders of magnitude and peaks found in winter/spring months. One interesting finding pertained to the lack of protozoa after RBF demonstrating the high potential of an RBF system for the reduction or elimination of protozoan. Another group (Weiss et al. 2005) previously recommended RBF for a substantial reduction in microorganisms such as *Giardia*, *Cryptosporidium*, viruses, and potential surrogate parameters.

**Previous serological survey confirm our present findings:** A Hungarian study on serological responses to the 15/17-kDa and 27-kDa cryptosporidial antigens in women using groundwater or surface water for drinking (Frost et al. 2005) has been reported. Serological responses were significantly lower in women who drank water from a confined aquifer or surface water following RBF compared to those drinking water from karst wells or non-RBF treated surface water. Strikingly, among women using bank-filtered water, the intensity of response was less than one-third of that observed for women using conventionally filtered and disinfected surface water. This leads to the hypothesis that RBF has high potential for the reduction or elimination of protozoa.

The serological responses to the used markers are specific for *Cryptosporidium* infection. Infection usually elicits a serological response to the antigen groups that peaks 4-6 weeks after infection. The 15/17-kDa marker declines to baseline levels observed prior to the infection in 4-6 months after infection, while the 27-kDa marker remains elevated for 6-12 months. If people are regularly exposed to drinking water with low concentrations of oocysts, the risk of symptomatic illness or the severity of illness from the infection may be reduced because of the protective immunity. It is also possible that the absence of the clinical disease is because people are exposed to oocysts which are not viable or infectious to human. Since *Cryptosporidium* infection elicits a serological response in most infected humans, surveys for the presence of this response have been used to estimate the

prevalence of prior *Cryptosporidium* infection in populations (Frost et al. 2003). All these information together with our present study confirm that *Cryptosporidium* is present in low concentrations in Hungarian surface waters used by the treatment plants for drinking water preparation.

**Limitations of the detection method:** The current protozoan detection methods likely underestimate the number of organisms and, therefore, when protozoa are detected they should be treated seriously. Infectivity of these (oo)cysts depends on several factors including the species present and whether they are capable of producing human infection, clumping of the (oo)cysts, water temperature, age (Li et al. 2004) and other biological properties of the parasites. We have taken into consideration useful information such as internal structure and DAPI staining for the accurate identification of *Cryptosporidium* and *Giardia* oocysts using microscopy (Ho et al. 1995, Thiriat et al. 1998, Smith et al. 2002, USEPA 2001). PCR identification of (oo)cysts may be more sensitive than microscopic examination, although morphological characteristics cannot be distinguished by PCR (Jiang et al. 2005a) and the presence of empty (oo)cysts also are not detected, since these objects do not contain DNA which targeted by the PCR.

Genotyping isolates found in either source or treated water can give further information on the likely sources of contamination and whether strains may be infectious for humans or not (Ryan et al. 2005a). Classification of *Giardia* and *Cryptosporidium* with molecular typing techniques is still being developed but is promising to distinguish isolates in the environment able to infect humans as well as their transmission patterns by molecular tools (Appelbee et al. 2005, Alves et al. 2006, Xiao et al. 2006a). Different genotypes have been linked to different symptomatology in sporadic human giardiasis and cryptosporidiosis but more information is required regarding the association between different risk factors and different genotypes, particularly for human adapted *Giardia* and *Cryptosporidium* genotypes and for zoonotic genotypes.

**Drinking water treatment efficacy:** Currently, drinking water plants are not prepared for unexpected events or worst-case scenarios in Hungary. Our results make imperative to examine water treatment technologies concerning *Giardia* and *Cryptosporidium* inactivation and removal. Additional treatments are necessary particular at the 14 surface

water treatment plants where the water treatment is not effective against the protozoa (Appendix 1) as well as the introduction of watershed control. Whereas the combination of filtration and chlorine disinfection is considered fundamental for treatment of surface water originated drinking water, unusual raw water conditions or inattentive operation often led to the contamination of purified water and the distribution system (see e.g. number 16, Appendix 1 and Map 1). Moreover, it has been demonstrated in the past that disinfectant levels adequate for *Giardia* treatment are only marginally effective against *Cryptosporidium* (Medema et al. 2006). At typical levels of drinking water chlorination over prolonged periods *Giardia* is killed whereas *Cryptosporidium* can only be rendered harmless via exposure to UV light or special chemical treatment (Medema et al. 2006, Lee et al. 2008). Ozonation is an effective measure in killing both these pathogens; however the most successful method of removing (oo)cysts from a water supply is through filtration (membrane filtration, diatomaceous earth filtration) (Bukhari et al. 2000, Betancourt and Rose 2004). Indeed our study demonstrated that 14 out of 16 surface water treatment plants do not apply ozonation, the most effective treatment for killing this protozoa and neither of them use the most effective removal technology, the membrane filtration.

**Risk level:** *Giardia* can cause disease at a level as low as 3-5 cysts/100 litres in treated drinking water (Wallis et al. 1996) while *Cryptosporidium* requires as few as 10-30 oocysts/100 L to pose a risk of outbreak (Haas and Rose 1995). The highest protozoan level found in Hungarian drinking waters was 63.6 *Giardia* cysts/100 litres and *Cryptosporidium* 3 oocysts/100 litres. During the investigation period, two water treatment plants in Hungary were found to harbour levels of protozoans above this threshold for disease outbreak suggesting the need of re-evaluation of the current water treatment purification systems.

In the United Kingdom, direct monitoring of drinking water is embodied in drinking water regulation. Water supply systems with a risk of *Cryptosporidium* contamination are obligated to sample their treated water at least daily in order to demonstrate average concentrations of *Cryptosporidium* below 10 oocyst/100 L of treated water (DWI 1999). USEPA Surface Water Treatment Rule requires water systems using surface water or ground water in direct contact with surface water to disinfect and/or filter their water in order to render at least 99.9% of *Giardia* cysts harmless or physically removed (USEPA

1989). This level of removal/inactivation is believed to reduce the risk of waterborne giardiasis to less than one person of 10,000 people per year. In addition, it has been determined that raw water should not contain more than 7 *Giardia* cysts/100 litres (USEPA 1989). Raw water from 9 surface water treatment plants in Hungary was found to contain *Giardia* cysts above this level during our study (Appendix 1) making imperative the need of protection measures. One component of the Interim Enhanced Surface Water Treatment Rule regulates *Cryptosporidium* in drinking water by requiring filtered surface water systems serving at least 10,000 people to physically remove at least 99% of *Cryptosporidium*; for systems without filtration a watershed control program must be adapted in order to protect the source water from *Cryptosporidium* contamination (USEPA 1998). In our investigations, the sampling site number 24 has only sand filtration; therefore, a watershed control would be necessary including a catchment survey of the contamination sources and keeping away these sources from the watershed.

In Hungary the statutory orders 201/200 (X.25.) and 47/2005 (III.11.) regulate the handling and testing of drinking water and state that *Cryptosporidium* must be determined for water intended for human consumption if *Clostridium perfringens* is detected. *Clostridium* detection is required at water systems that use surface or ground water in direct contact with surface water. During our studies the bacteriological quality and parameters have been tested in parallel to the *Giardia* and *Cryptosporidium* examinations by either the waterworks and/or the Department of Bacteriology of our Institute according to the Hungarian standard MSZ EN 26461-2:1994. *Clostridium* could not be detected in any drinking water samples.

**Animal reservoirs and water contamination:** It is known that the major reservoir for *C. parvum* is domestic livestock, predominantly cattle, and direct contact with infected cattle is a major transmission pathway of human infection along with indirect transmission through drinking water (Hunter and Thompson 2005). It should be emphasised here, that in Hungary in 2003, the cow and calf (dairy cow, beef cow and heifers in calves) stock totalled 714,000, and the lamb (ewes and shear lings, lambs) stock was estimated to be 1.3 million (Anonymous 2004). In England, the cow and calf and lamb stocks are eight times higher per square kilometre, than in Hungary (Defra Statistics 2004). Based on Hungarian report focused on enteric diseases, *Cryptosporidium* is the third most frequent pathogen of

calves (detected in 70% of the herds) while 22.6% of lambs and 37.5% of goat kids with diarrhoea were found to carry *Cryptosporidium* (Nagy 1995). 562 out of 44,978 stool samples of human patients with gastro-enteritis in Hungary were tested positive for *Giardia* and only 6 for *Cryptosporidium* in 2003 (Anonymous 2003). Accordingly, *Giardia* infections are more frequent than *Cryptosporidium* but more studies need to be undertaken. Currently, there are no data monitoring the presence of these protozoa in wild animals in Hungary. According to data from the Czech Republic deer can be a potential reservoir of *C. parvum* (Hajdusek et al. 2004). A Polish study emphasized that small rodents should be considered as an important reservoir of different *Cryptosporidium* genotypes (Bajer et al. 2003). Review of world-wide reports showed *C. parvum* to be found in 11 wild mammals, mainly rodents, but also insectivores (e. g. common shrew), lagomorphs (e. g. brown hare) and ungulates (e. g. deer) (Sturdee et al. 1999). *G. duodenalis* genotypes A and B are widespread, found in pets, farm and wild animals and the impact of the wildlife cycle cannot be underestimated (Appelbee et al. 2005) nor the risk posed by cats, dogs and other animals (Karanis et al. 1996a, b, Karanis and Ey 1998, van Keulen et al. 2002).

In conclusion, on the basis of the present findings in Hungarian water supplies, protection of the surface sources including effective pollution control and efficient water treatment by optimising conventional water treatment techniques is necessary. Water pollution is one of the most urgent health problems currently facing several European countries and emerging pathogens leading to waterborne disease is a pan-European problem. Safe drinking water is a general concern to all European countries yet it has been recognised that sophisticated surveillance against many diseases and especially those associated with water are not available. There is a need for wider dissemination of information on waterborne and emerging diseases amongst member states of the European Union and also in Hungary.

## 4. DETECTION AND CHARACTERIZATION OF *CRYPTOSPORIDIUM* AND *GIARDIA* IN HUNGARIAN RAW, SURFACE AND SEWAGE WATER SAMPLES BY IFT, PCR AND SEQUENCE ANALYSIS OF THE SSUrRNA AND GDH GENES

### 4.1. Introduction

In both outbreak and non-outbreak settings, the most commonly found genotypes and species in surface and waste waters include the two major *Cryptosporidium* human pathogens (*C. parvum* and *C. hominis*), in addition to *C. andersoni*, which is a common parasite of cattle (Smith et al. 2006). However, other species are also present in water, e.g. mixed populations were found in storm water samples and sequence analysis indicated that most oocysts originated from wildlife (Xiao et al. 2000b). Less is known about the *Giardia* species or *G. duodenalis* Assemblages that are present in water environment. In all wastewater samples examined, only *G. duodenalis* cysts were identified and more importantly, only *Giardia* pathogenic strains (Assemblage A and B) were found (Smith et al. 2006). The prevalence of *G. duodenalis* Assemblages A and B varies from country to country (Karanis and Ey 1998; Smith et al. 2006).

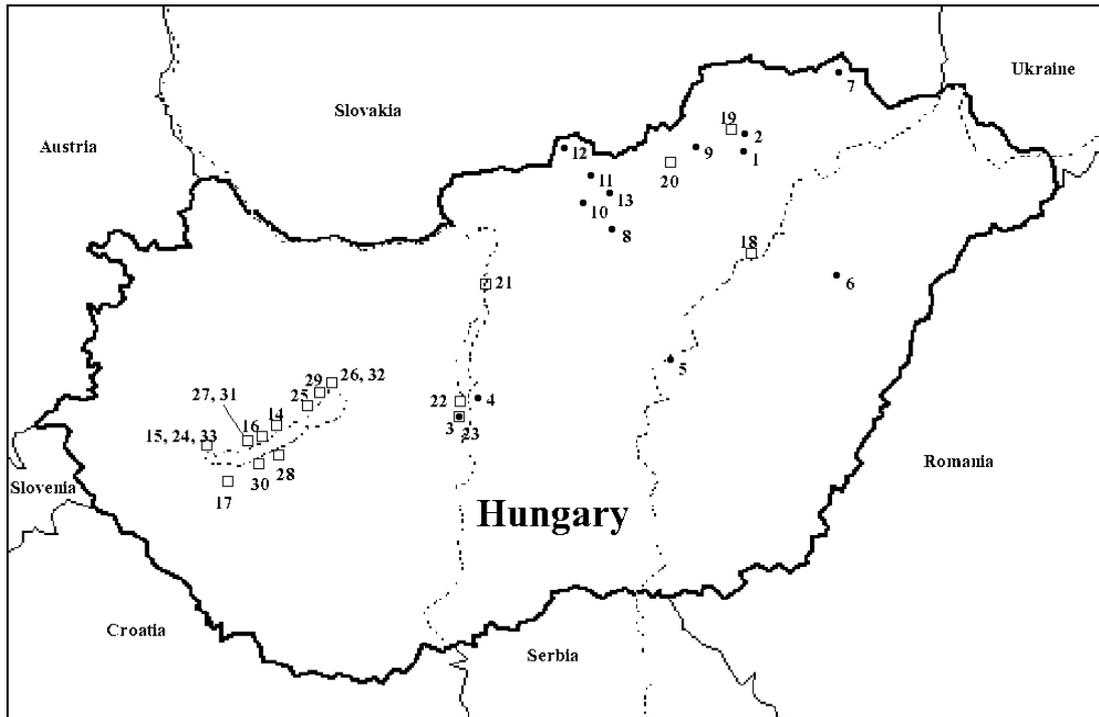
Routine microscopic detection methods do not discriminate among species, genotypes and subtypes leaving water authorities with data indicating *Cryptosporidium* and *Giardia* presence but no additional information of the health significance of the findings. Different genotypes have been linked to different symptomatology in sporadic human giardiasis and cryptosporidiosis cases, but more information is required regarding the association between possible risk factors and different genotypes, particularly for human-adapted *Giardia* and *Cryptosporidium*. Genotyping may identify sources of contamination and it provides information on the presence of human and animal pathogenic strains.

In the present work we aimed the detection and the molecular characterization of *Giardia* spp. and *Cryptosporidium* spp. in water samples to gain information on species composition and genotypes of these waterborne protozoa in Hungary.

## 4.2. Materials and methods

### 4.2.1. Sampling sites and sampling design

36 surface and sewage water samples were collected in the period between 2004-2007. The lakes and rivers chosen for this study are all drinking water sources, namely the River Danube, River Tisza, Eastern Main Canal, River Bódva, Brook Nagy and six water reservoirs: Reservoir Hasznos, Komravölgy, Köszörűvölgy, Csórrét, Lázbérc and Mátrafüred. River Danube, River Tisza, River Bódva and Reservoir Lázbérc (from Brook Bán) receive authorized sewage. 16 untreated surface water (raw water) at the intake of the water treatment plant (sample codes are 1-13 in Map 2 and Appendix 2) and 6 untreated sewage samples were collected from sewage treatment plants which effluent (biologically treated sewage) affect the raw water of drinking water treatment plants. Tiszadorogma sewage treatment effluent is >40 km away from the River Tisza drinking water treatment plant. Szilvászvárad discharge the treated sewage into Brook Bán 16 km away from Reservoir Lázbérc water treatment plant. Edelény sewage treatment plant effluent is 5 and 10 km away from the drinking water treatment plants on River Bódva. Dunaújváros sewage treatment plant effluent is 1 km away from the drinking water treatment plant on River Danube. The sewage of Rácalmás and Budapest also affect the water quality of the River Danube (sample codes are 18-23 in Map 2 and Appendix 2). 14 samples were collected at Lake Balaton, where the survey was expanded to sewage and brook, duct inflows and also to recreational sites of lake. 4 samples from sewage treatment plants' effluent (namely Zánka, Keszthely, Révfülöp, Balatonújlak applying third, lime or iron-salt treatment for phosphorus removing) (sample codes are 14-17 in Map 2 and Appendix 2), 7 samples from brooks/ducts flowing into the lake (Büdös- árok, Brook Kéki, Brook Séd of Vörösberény, Brook Séd of Balatonfüzfő, Brook Burnót, Forró-árok, Keleti-Bozót) (sample codes are 24-30 in Map 2 and Appendix 2) and 3 samples from beaches (Ábrahámhegy, Balatonfüzfő, Keszthely) close to the inflows (sample codes are 31-33 in Map 2 and Appendix 2) were collected.



**Map 2:** The map indicates the sampling sites of raw water of the water treatment plants abstracting surface water (marked by •) and of the sewage treatment plants, ducts, brooks, affecting the quality of the raw water (marked by □). The samples were used for molecular analysis. The numbers are the sample codes as indicated in the Appendix 2.

#### 4.2.2. Sample collection, parasite concentration and examination

Sample collection and parasite concentration was performed using Filta-Max foam filters, membrane filtration and chemical flocculation as described in chapter 2.2. (Oo)cysts were separated from debris by IMS and half of the concentrated pellet was examined after staining by epifluorescent microscope as described above in chapter 2.4. and 2.5. The other half of the final pellet has been used for DNA analysis. The DNA extraction, SSU rRNA and GDH PCR assays, Real-time PCR, RFLP analysis of GDH PCR products, the on chip DNA analysis after RFLP, cloning, sequencing and sequence analysis have been described in chapter 2.7.

#### 4.3. Results and discussion

**Detection of (oo)cysts by IFT:** *Cryptosporidium* oocysts were detected in 4 raw, 3 sewage, 5 surface water and in 3 beach water samples, altogether 15 samples were positive. *Giardia*

cysts were detected in 10 raw water, 10 sewage water, 4 surface water and 1 beach water samples, altogether 25 samples were found to be positive (Appendix 2).

**Detection of *Cryptosporidium* and *Giardia* species by PCR:** 5 raw water, 7 sewage water samples and 1 surface water sample were *Giardia* positive and 2 raw and 8 sewage water were *Cryptosporidium* positive by PCR (Appendix 2).

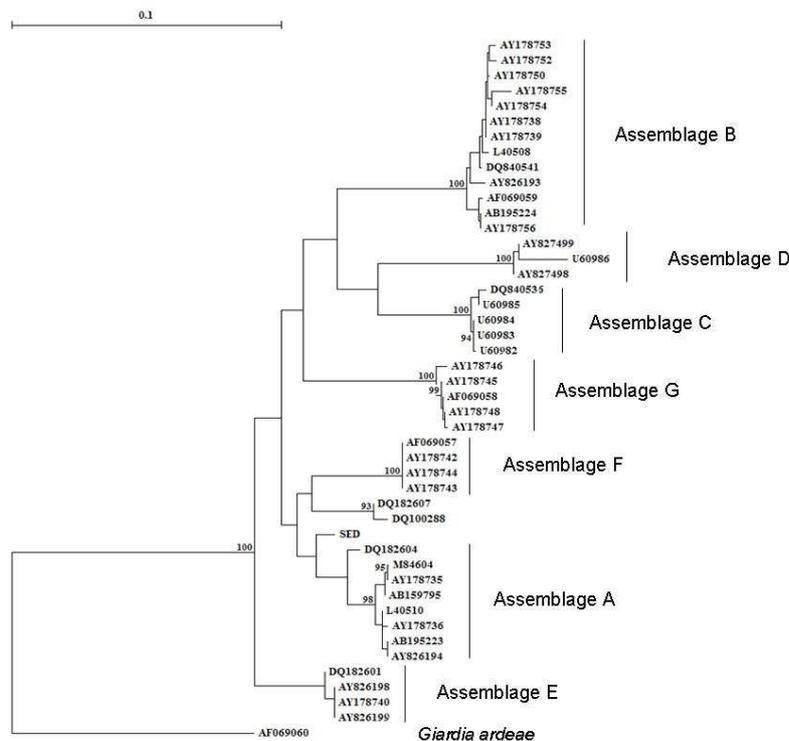
***Cryptosporidium* genotyping:** In one sewage sample *C. parvum* and in another sewage sample *C. meleagridis* have been detected. In 8 other samples (6 sewage and 2 raw water) *Cryptosporidium* PCR product gave very weak band and it could not be sequenced.

***Giardia* genotyping:** 11 out of 12 *Giardia* SSU rRNA PCR products and 9 out of 9 GDH PCR products were successfully sequenced. According to the SSU rRNA PCR sequence analysis in 8 samples *G. duodenalis* Assemblage A, in 2 samples *G. duodenalis* Assemblage B (2 surface water) and in 1 sample *G. duodenalis* Assemblage A plus B (1 sewage water) were detected. According to the GDH sequence data in 7 samples (1 raw water and 6 sewage samples) *G. duodenalis* Assemblage A group II, in 2 samples (1 raw water and 1 surface water) Assemblage A group I and in 2 samples (2 sewage water) *G. duodenalis* Assemblage B group III, in 2 samples (1 raw water and 1 sewage) *G. duodenalis* Assemblage B group IV were found (Appendix 2).

The presented data on the occurrence and species differentiation of *Giardia* and *Cryptosporidium* species in water supplies in Hungary arises several questions on the circulation of (oo)cysts in Hungarian water supplies and it may provide a substantial contribution to the protection of public health in the investigated areas. Most *Giardia* isolates in sewage treatment plants affecting the raw water of drinking water treatment plants were Assemblage A-II although B-III and IV isolates also were detected. We found substantial heterogeneity in sewage samples. Thus, combination of Assemblages A-II and B suggest a human contamination origin.

In one sewage sample *C. parvum* and in other sewage sample *C. meleagridis* were also detected. These *Cryptosporidium* oocysts may have originated from the neighbouring horse and chicken farms directing their wash water to the sewage treatment plant.

Regarding the raw water samples in River Danube, we could track the effect of the inflowing sewages by detecting *G. duodenalis* Assemblage A-II. For the River Tisza, this situation is not clear because the sewage treatment plant's effluent is far away (> 40 km) from the raw water sampling point. In the River Tisza *G. duodenalis* Assemblage A-I animal derived subtype and Assemblage B-IV was detected. In the raw water of Füzér Assemblage A and Assemblage B were detected once. Since sewage inflow is not present in this location, the origin of the contamination remains unknown. In one sample from surface water (Séd) flowing into the Lake Balaton *G. duodenalis* unique sequence was detected, which is available in the GeneBank database under the accession number EU 350516. GDH nucleotide sequence obtained from this surface water sample was aligned with published human and animal GDH sequences by using ClustalW (van der Giessen et al. 2006). Tree was constructed using the neighbour joining algorithm based on evolutionary distances calculated by the Kimura two-parameter model with 1,000 bootstrap sampling: [www.clustalw.ddbj.nig.ac.jp/top-e.html](http://www.clustalw.ddbj.nig.ac.jp/top-e.html). According to this GDH phylogenetic tree the sequence represent a new subtype inside of the *G. duodenalis*-complex, clustered close to the Assemblage A group (Figure 14). According to the SSU rRNA PCR product, the found organism in this water was identical to *G. duodenalis* -Assemblage A.



**Figure 14:** A tree on phylogenetic relationship of the new *Giardia* isolate from Brook Séd examined in this study (marked as SED) to multiple *Giardia duodenalis* Assemblages.

*Giardia*, the aetiological agent of the diarrhoeal disease giardiasis, represents the most common gastro-intestinal protozoan parasites detected in humans, with a prevalence of individuals testing positive for cysts in stool samples of 2-5% in the industrialized world and up to 20-30% in developing countries (Almeida et al. 2006). Infectivity studies utilising *Giardia* isolated from both humans and animals have shown that zoonotic transmission is possible (Thompson 2004). In many situations, giardiasis is clearly transmitted between humans by the faecal-oral route, either directly or by contamination of water by human sewage. However, some epidemics in North America have been linked circumstantially to contamination of water with cysts excreted by animals such as beavers and muskrats, which have reported carriage rates of 7-16% and > 95% respectively (Erlandsen et al. 1990b, Karanis et al. 1996a). Other animal species, including agricultural livestock, are potential contaminators of surface waters. Because the host origin of cysts in water cannot usually be identified with certainty by either field or laboratory investigation and because the zoonotic potential of giardiasis remains an unresolved issue, health authorities are often forced to adopt policies and water management practices that consider any *Giardia* cysts as potentially infectious for humans. Significant health and economic benefits might follow if it could be shown that certain *Giardia* are host-specific or if potentially zoonotic organisms could be identified by the use of specific genetic markers. However, the epidemiological situation of giardiasis in Hungary needs further investigations.

*C. parvum* and *C. meleagridis* were detected in the present study in sewage samples. *C. meleagridis*, a protozoon first observed in turkeys, has been linked by several investigators to cryptosporidiosis in humans. It is the only known *Cryptosporidium* species that infects both avian and mammalian species (Akiyoshi et al. 2003). *C. meleagridis* was readily transmitted from one animal host to another including chickens, mice, piglets, and calves, maintaining genetic homogeneity and stability (Akiyoshi et al. 2003). Sequence data for the SSU rRNA gene of *C. meleagridis* isolated from turkeys in Hungary were found to be identical to the sequence of a *C. meleagridis* isolate from North Carolina (Sréter et al. 2000).

In our another study we investigated the species, genotypes of *Cryptosporidium* in cattle in Hungary and *C. parvum* has been mostly found, which indicates that cattle can be a source

of cryptosporidial infections of human and animals. Currently, there are no data on the presence of *Cryptosporidium* species in wild animals in Hungary.

Identification of different strains of *Giardia* and *Cryptosporidium* species in water still remains problematic. Understanding of the strength and weakness of each technique is needed when using molecular diagnostic tools and it has already been demonstrated, that there are considerable differences between the specificity of the methods (Jiang and Xiao 2003). Moreover, no standardized PCR protocols are available for that purpose, due to the numerous DNA targets analyzed so far by different laboratories (Sato et al. 2005, Smith et al. 2006, Leetz et al. 2007) giving variable results. PCR is strongly inhibited by various water substances usually present in water samples, which is a major disadvantage particularly for the molecular identification of low parasite numbers in raw and highly polluted surface waters (Jiang et al. 2005a). In our study, (oo)cyst microscopical identification was based on immunofluorescence microscopy, phase contrast microscopy and DIC. DIC enhances the visualization of the internal structure of *Giardia* and *Cryptosporidium* (oo)cysts. Empty and amorphous cysts have also been observed, which do not contain DNA targeted by the PCR. The morphology of (oo)cysts depends on various factors and recent studies revealed that long-term storage of oocysts at low temperature has little effect on oocysts morphology (Inoue et al. 2006). IFT microscopy has been previously evaluated for *Giardia* cysts and directly compared to phase contrast microscopy and the results suggested that immunofluorescence microscopy was superior for the detection of *Giardia* cysts in animal faeces (Karanis et al. 1996b).

Information about whether purified (oo)cysts are viable and/or infective is important to determine but methods of (oo)cysts detection by water analysis still has several limitations (Weintraub 2006) and needs further improvements.

According to the presented results we performed genotyping on 69% of the sewage water IFT positive samples, 36% of the raw water, and 8% of the surface water IFT positive samples. The successful molecular detection and genotyping of *Cryptosporidium* and *Giardia* species in water concentrates from non-outbreak sources vary between 6%-100% from wastewater and 8%-93% in surface water (Smith et al. 2006). Semi-nested PCR and direct sequencing was applied for *Cryptosporidium* species detection isolated from sewage and river water in Japan by Hirata and Hashimoto (2006). Their procedure could genotype 54% of FITC stained single oocysts from sewage and 32% from river water. According to

the one year prospective study of two recreational lakes and three river sites located near Paris, *Cryptosporidium* PCR and RFLP have been performed on the water samples and it was found that 40% of the IMS-IFT positive samples were positive by PCR (Coupe et al. 2006).

Extraction of high quality of DNA is a key step in PCR detection and according to the study on the effectiveness of 6 DNA extraction methods (Jiang et al. 2005a) the results showed, that PCR inhibitors were present in all DNA extracts, however the effect of PCR inhibitors could be relieved significantly by the addition of BSA, as it has been confirmed in our experiments. When the levels of performance were compared by using storm and sewage water samples known to have high occurrence of *Cryptosporidium* oocysts, the best results (82% positive of the storm water samples and 53% positive of the sewage samples) were obtained by IMS-QIAmp DNA Mini Kit extraction method (Jiang et al. 2005a). We used the same extraction method in our study as by Jiang et al (2005a) and the results are comparable.

The reproducibility of Method 1623 and PCR in detecting *Cryptosporidium* oocysts in field water samples if low number of (oo)cysts present is inadequate (Xiao et al. 2006b).

Throughout the European Union, several laboratories are using different water analysis methods for the presence of *Giardia* and *Cryptosporidium*, although not on the routine basis. In recent years, molecular techniques for species and genotype identification such as PCR and DNA sequencing have been developed, evaluated and advanced. The methods IFT, IMS and PCR have to be applied in combination to improve the sensitive detection and species or genotype determination. Water industries should be able to interpret the significance of the presence of *Cryptosporidium* and *Giardia* (oo)cysts in their water, to estimate the real removal of *Giardia* and *Cryptosporidium* oocysts in practical water treatment and to define the contamination sources of drinking water supplies.

## 5. GENOTYPE AND SUBTYPE ANALYSIS OF *CRYPTOSPORIDIUM* ISOLATES FROM CATTLE IN HUNGARY

### 5.1. Introduction

Over the past 20 years, cattle have been identified as being a reservoir host for taxa transmitted from animals to humans, however, a remarkable range of species affects cattle, including both cattle-specific, in addition to a zoonotic species. *Cryptosporidium* infection in cattle is commonly caused by *C. parvum*, *C. bovis*, *C. andersoni* and by the recently described *Cryptosporidium* deer-like genotype. *C. parvum* infects the small intestine causing diarrhoeal disease, especially in neonatal calves, *C. andersoni* infects the abomasum of juvenile and mature cattle and this infection has been identified as a cause of reduced milk production (Lindsay et al. 2000, Olson et al. 2004). *C. bovis* and *Cryptosporidium* deer-like genotype are not associated with signs of disease (Santin et al. 2004, Slapeta 2006). The occurrence of these *Cryptosporidium* spp. in cattle shows a host age related susceptibility: *C. parvum* predominates in pre-weaned calves, *C. bovis* and *Cryptosporidium* deer-like genotype in post-weaned calves and *C. andersoni* in older calves and adult cattle (Santin et al. 2004, Robinson et al. 2006). Although a recent study showed that *C. bovis* and *Cryptosporidium* deer-like genotype has also been observed in older and pre-weaned calves (Fayer et al. 2006a, Fayer et al. 2007, Feng et al. 2007b, Thompson et al. 2007). The findings clearly demonstrate that neonatal calves are an important source of zoonotic cryptosporidiosis in humans, although little is known about its transmission dynamics. In the last years, researchers have used highly discriminatory sub-typing techniques (sequence analysis of the gp-60 gene), useful for tracking infection sources and examining the transmission dynamics of *C. parvum* (Strong et al. 2000). The next advance in our understanding of the epidemiology of cryptosporidiosis is likely to come from more detailed characterization of *Cryptosporidium* strains within the same species and genotype (Hunter and Thompson 2005). Identification of the isolates at the subgenotype level will be useful for the understanding of the population structure of *C. parvum* genotypes and for the control of the cryptosporidial infections. The aim of the present work was to use sequences of gp-60 gene to identify subgenotypes of *C. parvum* from cattle farms in Hungary.

## 5. 2. Materials and methods

Seventy nine cattle faecal samples (50 mL) were collected from rectum of pre-weaned calves with diarrhoea from 52 farms in nine counties in Hungary (Map 3) in the period between May-June 2006. The faecal samples were preserved in 2.5 % potassium dichromate and kept on 4 °C until use. After diethyl-ether/PBS 2:1 v/v biphasic concentration and discontinuous sucrose gradient purification microscopic examinations were performed on all samples as described in chapters 2.3., 2.4., 2.5. One fifth (200 µL) of the concentrated sample suspension was processed for genotype analysis. The DNA extraction, SSUrRNA PCR, GP60 PCR, RFLP analysis of SSUrRNA products, sequencing and sequence analysis were performed as described in chapter 2.7.

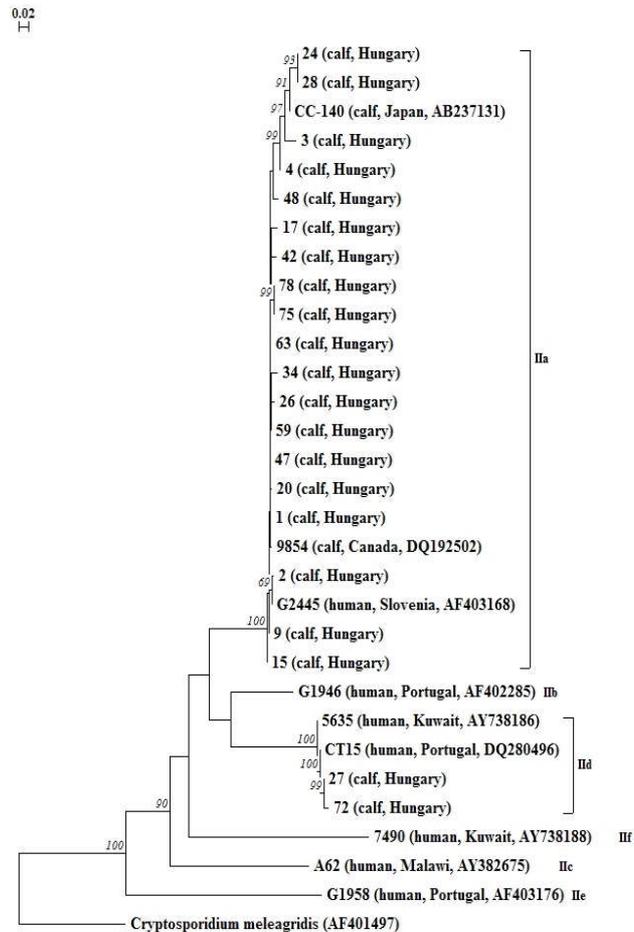


**Map 3:** The map of Hungary indicates the nine counties (grey coloured), where cattle faecal samples were collected (all together 79 samples from 52 farms).

### 5.3. Results and discussion

According to the IFT results, 39 samples (~50%) were *Cryptosporidium* positive, 17 of which contained only one or two oocysts while 22 samples contained more than 100 oocysts (estimated from 10 µl out of the 1.2 mL concentrated sample). Of the 22 samples containing more than 100 oocysts all were found to be PCR positive and subsequent RFLP and gp-60 sequencing results identified *C. parvum* in 21 samples and *Cryptosporidium* deer-like genotype in one. In terms of geographical distribution we were able to detect *C. parvum* in all counties except county Somogy. The county Szolnok was positive for the *Cryptosporidium* deer-like genotype (Map 3). Our results support the earlier epidemiological findings, that *Cryptosporidium* is a frequent pathogen of calves with diarrhoea in Hungary (detected in 70% of the herds) (Nagy 1995).

Alignment of gp-60 sequences obtained with reference sequences downloaded from the GenBank indicated, positives obtained during this study belonged to the *C. parvum* subtype group IIa and IId (Figure 15). Within the IIa group all sequences were identical in the non-repeat region (i.e. had one copy of sequence ACATCA immediately after the trinucleotide repeats) while the trinucleotide repeat region all contained one copy of the TCG repeat and 16, 17 or 18 copies of TCA. In the case of the IId group, the sequences had one copy of the TCG repeat and 19 or 22 copies of the TCA repeat. Altogether, three *C. parvum* IIa subtypes and two *C. parvum* IId subtypes were found. The *Cryptosporidium* subgenotype IIaA16G1R1 being the most common, detected in 15 (71.4%) calves out of 21. The subgenotype IIaA17G1R1 was found in three cases and we found a novel *C. parvum* subgenotype (IIa A18G1R1) inside of the *C. parvum*-complex. Within the IId subtype group the IIdA22G1 and the IIdA19G1 subgenotypes were detected in two samples.



**Figure 15:** Phylogenetic tree of gp-60 sequences of *Cryptosporidium parvum* isolated from cattle. The numbers on the branches are bootstrap values greater, than 50%. The name of the isolates, the hosts, the localities and the accession numbers in the Gene Bank (in case of the retrieved sequences) are shown in parentheses.

Until now the IIaA16G1R1 subtype was described in the United States and Canada and it seems that this subtype is not frequent in North America. It has been found also in cattle in Serbia, Montenegro, The Netherlands and Germany, but it has not yet been detected in human patients (Peng et al. 2003, Mistic and Abe 2006, Trotz-Williams et al. 2006, Xiao et al. 2006a, Wielinga et al. 2007, Broglia et al. 2008).

The *C. parvum* subtype IIaA17G1R1 played an important role in the three *Cryptosporidium* outbreaks in the United Kingdom as it was detected in nine human patients and also in water samples. This subgenotype has been found in calves and humans in Slovenia and in The Netherlands; in cattle in Ireland and is known as the Moredun

isolate found in cattle in the United States (Peng et al. 2001, Chalmers et al. 2005, Xiao et al. 2006a, Thompson et al. 2007, Wielinga et al. 2007).

The IIA18G1R1 new subtype was recently described in cattle from Serbia, Montenegro and The Netherlands (Misic and Abe 2006, Wielinga et al. 2007).

The subtypes IIA22G1 and IIA19G1, which were detected in Hungarian calves in this report, were detected in human patients in Portugal (Alves et al. 2006). The IIA22G1 has been found additionally in cattle in Belgium and Germany and in human in Switzerland (Geurden et al. 2007, O'Brien et al. 2008, Broglia et al. 2008).

The IIA16G1R1 subtype was detected in Hungary in counties Baranya, Csongrád, Jász-Nagykun-Szolnok, Borsod-Abaúj-Zemplén, Győr-Moson-Sopron, Veszprém and Fejér. The IIA17G1R1 subtype was detected in counties Borsod-Abaúj-Zemplén, Szabolcs-Szatmár-Bereg and Csongrád, the IIA18G1R1 subtype in county Fejér. The IIA22G1 subtype was found in county Szolnok and the IIA19G1 subtype in county Csongrád.

County Csongrád in Hungary is bordering Serbia. The subgenotype IIA16G1R1 has been detected in both places, however, it is difficult to follow the animal transport ways.

From another point of view, the subtypes IIA16G1R1 and IIA18G1R1 found in our neighbouring country Serbia and the IIA17G1R1 found in another neighbouring country Slovenia, have also been detected in Hungary.

The potential of human infection of subtype IIA16G1R1 (found in 71.4% of the Hungarian samples) and of the new subtype IIA18G1R1 (found in one Hungarian sample) is yet to be determined. Since the IIA22G1, the IIA19G1 and the IIA17G1R1 subgenotypes have already been detected in human patients, a clear public health risk in terms for the potential of zoonotic transmission exists in Hungary (Alves et al. 2003, 2006, Chalmers et al. 2005).

*Cryptosporidium* deer-like genotype has been recently isolated in various age groups of cattle from United States, China (Santin et al. 2004, Fayer et al. 2006a, Feng et al. 2007b), Malaysia (Halim et al. 2007), Denmark (Langkjaer et al. 2007), Northern Ireland (Thompson et al. 2007) and Kenya (Szonyi et al. 2008). The zoonotic significance of this little known genotype is yet to be determined.

As indicated above in chapter 1.1.3., several species of *Cryptosporidium* are known to be transmissible between humans and animals with *C. parvum* being the most common zoonotic species identified in domestic ruminants (Alves et al. 2003, 2006, Nagy 1995).

The ecotourism scenario such as farm stay or petting farm is a new attraction nowadays also in Hungary. Visitors are allowed to stay in the farmhouse to experience farm life while petting farms usually incorporate educational element which mainly attracts children. Possibility of ingesting viable oocyst increases the risk of disease transmission. Environmental pollution with human and domestic-animal faecal material is recognized as a potential pathogenic pathway for wildlife infections with the zooanthropomorphic protozoan parasite. Our previous studies confirmed *Cryptosporidium* oocyst contamination in various types of Hungarian surface water. In order to prevent these pathways potential control measures and the role of veterinary and medical professionals in the prevention of cryptosporidiosis need to be defined. A benefit of this subgenotyping approach is its ability to differentiate various effects such as geographic variation and relationships to demographic and epidemiological data (Peng et al. 2001), thereby giving the possibility to determine infection sources accurately.

## 6. EPIDEMIOLOGICAL STUDY ON *GIARDIA* IN TWO HUNGARIAN VILLAGES AND GENOTYPE ANALYSIS OF THE *GIARDIA* ISOLATES DETECTED IN DRINKING WATER AND HUMANS

### 6.1. Introduction

As described before (chapter 1.2.4.) the well known spectrum of clinical manifestations seen in human giardiasis are short lasting, mild, transient intestinal complaints, that resolve completely and a rather characteristic complex of symptoms consisting of an acute onset of diarrhoea, abdominal cramps, bloating and flatulence often accompanied by nausea and weight loss lasting for up to 7 weeks. In undernourished hosts and in children, the infection can become chronic with profound diarrhoea, weight loss, disturbance of absorption and growth (Farthing 1996, Adam 2001, Lebwohl et al. 2003). Furthermore, people may be infected without any relevant symptoms, and it has even been suggested that some people benefit from their carrier state, e.g. healthy day care children with asymptomatic *G. duodenalis* infection showed no disadvantage and perhaps even an advantage in nutritional status and freedom from other illnesses (Ish-Horowicz et al. 1989). It is not fully understood why some individuals develop clinical giardiasis while others remain asymptomatic, however, host factors such as immune status, nutritional status, age, concurrent enteritic infections and environmental factors as well as differences in virulence and pathogenicity of *G. duodenalis* strains are recognized as important determinants for the severity of infection (Thompson 2004).

In the previous studies we examined the occurrence and removal of *Giardia* and *Cryptosporidium* (oo)cysts in Hungarian water treatment plants (chapter 3. and 4.). Water treatment plants of Füzér and Mátrafüred were found to be inadequate for their *Giardia* removal. In the raw water of Mátrafüred 840-850 cysts/100L, in the drinking water of Mátrafüred 27-63/100L, in the raw water of Füzér 40 csysts/100L and in the drinking water of Füzér 12 cysts/100L were detected (chapter 3. and 4.). Cysts with distinct nuclei and amorphous, empty cysts have also been recorded. This concentration of cysts in the drinking water according to Wallis et al. (1996) potentially able to induce human infections. Also in the previous study (chapter 3.) the riverbank filtration was found to be effective regarding the protozoa removing.

The aim of the present study was to describe epidemiological investigations focused on the prevalence of *G. duodenalis* infections in asymptomatic individuals in Füzér and Mátrafüred in Hungary, where *Giardia* cysts have been detected in their drinking water sources and to compare with the situation in a control city (Budapest) with consistent negative *Giardia* cysts water findings. Furthermore, the genotypes of *G. duodenalis* found in human faeces and in the local waters have been determined by sequencing the fragment of SSU rRNA and GDH genes and the sequences gained from water have been compared with sequences gained from faeces.

## **6.2. Materials and methods**

### **6.2.1. Information about the sampling sites**

The sampling sites for this study were selected according to the type of their drinking water: the inhabitants of Füzér and Mátrafüred consume drinking water abstracted from surface water (water reservoir and brook) and the inhabitants of Budapest consume Danube riverbank filtrated water. The *Giardia* infections of 100 randomly selected humans from each settlements consuming different water sources were compared and in the same time of this epidemiological study the drinking water from each settlements were examined for the presence of *Giardia* cysts.

**Geography and population at sampling areas:** Füzér is located on the north-eastern part of Hungary at the Hungarian–Slovakian border. Mátrafüred is located on the northern part of Hungary between the Mátra Hills. Füzér has altogether 550 inhabitants, Mátrafüred has 700. Budapest is the capital of Hungary and it has about two million inhabitants (Map 4).

**Drinking water preparation to supply the settlement of Füzér:** The Brook Nagy flows through the village of Füzér. A small water treatment plant located at the 0.1 km point from the brook's origin (before the brook enters the village area) produces the drinking water. The treatment of water includes sand filtration, activated carbon filtration and chlorination. Sheeps are mainly kept within the catchment area and the main crops are maize, rape, cereals. This water treatment plant surrounded by a forest.

**Drinking water preparation to supply the settlement of Mátrafüred:** Mátrafüred water reservoir is on the Brook Csatorna, which receives water from 9 springs, which are located

in 50-500 m from the brook. The water treatment includes only sedimentation, sand filtration and chlorination. This water treatment plant is surrounded by a forest, agricultural activity is not done.

**Budapest, control city:** The water supply of the city of Budapest and its suburbs originates from riverbank filtration, and approximately 2 million people consume riverbank filtrated water. In total 700 riverbank filtration wells are located 30-100 m from River Danube, mainly on the Island Szentendrei and on the Island Csepel. On the Island Szentendrei, water flows thorough sand and gravel lawyers and is directed to the drinking water system after chlorination. On the Island Csepel water is filtered through gravel layers and after treatment (ozonization, sand and activated carbon filtration, removal of iron and manganase, and chlorination) is directed to the water distribution system.

The bacteriological parameters of the drinking water of the investigated waterworks are regularly monitored. The CFU (Colony Forming Unit) counts (Colony count/22 °C, Colony count/37° C, *Escherichia coli*, Coliform, Enterococci, *Pseudomonas*, *Clostridium*) in Füzér and Budapest have always met the drinking water requirements. Mátrafüred occasionally, rarely showed problems with the Coliform (4 CFU) and Enterococci (2 CFU) counts.



**Map 4:** The location of Füzér, Mátrafüred and Budapest.

### **6.2.2. Collection and processing of the faecal samples for the detection of *Giardia* cysts**

Fresh human faecal samples were randomly obtained from a total of 300 (3×100) volunteer persons in Füzér, Mátrafüred and Budapest city. Individuals participated voluntarily after a clear explanation of the research objectives. The sample collection was performed with the permit of the Council of Health Science and volunteers signed a written informed consent. If the subjects were children informed consent was obtained from their guardians. The study was organized with the help of the local general practitioners and the steril, wide-mouth screw-capped sample storage containers were supplied. The fresh faecal samples were collected in Füzér on 7 June 2007, in Mátrafüred on 14 June 2007 and in Budapest on 20 September 2007. The age gap of the examined persons was between 9-88 years, females and males with different occupation, and all of them have consumed the local distributed drinking water for a minimum of 5 years.

The IMS of *Giardia* from faecal samples, the protein analysis (*Giardia* microplate assay), the microscopic examination, the DNA extraction, the SSU rRNA and GDH PCR, the RFLP analysis on GDH PCR products, the on chip analysis of the RFLP products, the sequencing and sequence analysis was performed as described above in chapters 2.3.-2.7.

### **6.2.3. Structured epidemiological interview**

A comprehensive, pre-coded, validated, written questionnaire (Appendix 3.) and personal interview were used for collecting data. Persons at increased risk for *Giardia* infection include: travellers (endemic areas), children in child care settings, close contacts of infected persons, persons who ingest contaminated drinking water, persons who swallow contaminated recreational water, persons taking part in outdoor activities who consume unfiltered, untreated water or who fail to practice good hygienic behaviours and persons who have contact with infected animals. The questionnaire covered demographic data, family life, education, travel history according to Stuart et al. (2003). Individuals were interviewed directly or in the case of young children the parents were interviewed. The interviewers had been trained previously.

#### **6.2.4. Water sample collection, parasite concentration and examination for the epidemiological investigations in Füzér and Mátrafüred**

Before the faecal sample collection, taking into consideration that the prepatent period of *Giardia* infection is 5-21 days (Thompson et al. 2007), 10 L of raw water, 50 L of drinking water and 50 L of filter backwash water were collected into cans from the waterworks of Füzér and Mátrafüred and the samples were transferred to the laboratory for further analysis. The detailed information of the collected samples is shown in Table 8.

After membrane filtration of the collected samples, cysts were separated from debris by IMS and half of the sample concentrate was examined after staining by epifluorescent microscope as described in chapter 2.2., 2.4.-2.5. The other half was used for DNA extraction and molecular studies. The DNA extraction, SSU rRNA, GDH PCR assays, sequencing and sequence analysis were performed as described in chapter 2.7.

### **6.3. Results and discussion**

#### **Detection of *Giardia* by microplate assay and IFT:**

**Stool samples examined by microplate assay and by IFT:** In total 300 stool samples have been examined, 100 from each settlement. 4 (4%) stool samples from Füzér, 1 (1%) sample from Mátrafüred and 1 (1%) sample from Budapest city have been found positive by both the microplate assay and by IFT (Table 9).

**Water samples (IFT):** In the raw water of Füzér 118 cysts/100 L, in the drinking water and in the filter backwash water of Füzér 4 cysts/100L were detected. In the raw water of Mátrafüred we could not detect *Giardia* cysts, in the filter backwash water of Mátrafüred 4 cysts/100L and in the drinking water 5 cysts/100L were detected (Table 8).

#### **Detection of *Giardia* by PCR and sequence analysis:**

**Stool samples (PCR):** One stool sample from Mátrafüred, 4 samples from Füzér and 1 sample from Budapest have been found positive by the SSU rRNA PCR and two samples (one from Füzér and one from Budapest) by the GDH PCR. According to the sequence analysis of the SSU rRNA gene in two positive (sample codes: 1172 and 1811) samples of Füzér *G. duodenalis* Assemblage A and B and in one sample (sample code: 1061) *G.*

*duodenalis* Assemblage A, in one sample (sample code: 1841) Assemblage B was detected. The GDH sequence analysis confirmed the presence of Assemblage A in the sample '1061'. Comparing this sequence with reference sequences downloaded from the GenBank, it was clustered to group A-II showing 100% similarity with the sequence L40510 of human origin. According to the sequence analysis of the SSU rRNA gene in the positive sample of Mátrafüred (sample code: 2211) *G. duodenalis* Assemblage A was detected. In the control city, Budapest, one stool sample (sample code: 3115) contained *G. duodenalis* Assemblage A by the SSU rRNA PCR product and the GDH sequence analysis confirmed the presence of Assemblage A-II showing similarity with the sequence L40510 of human origin and one single nucleotide polymorphism was detected (SNP) (Table 9).

**Water samples (PCR):** In Füzér, the raw water sample was found to be positive by SSU rRNA PCR assay and the sequence analysis showed the presence of *G. duodenalis* Assemblage B. Subgenotyping was not possible, because we could not get GDH PCR product from water samples. In samples collected from Mátrafüred, PCR did not yield any electrophoresis product (Table 8).

Settlement Sampling date	Sample type	<i>Giardia</i> detected by IFT/ 100 L	PCR results	Results of the sequence analysis
<b>Füzér</b> 07.06.2007.	Raw water	118	+	<i>G. duodenalis</i> Assemblage B
	Backwash water	4	-	-
	Drinking water	4	-	-
<b>Mátrafüred</b> 07.06.2007.	Raw water	0	-	-
	Backwash water	4	-	-
	Drinking water	5	-	-

**Table 8:** IFT detection and PCR-sequencing results of *Giardia* species from raw, bachwash and drinking water of Füzér and Mátrafüred.

Patient Code	<i>Giardia</i> microplate assay	Microscopy	SSU rRNA PCR	GDH PCR	Sequence analysis of SSU rRNA PCR products	Sequence analysis of GDH PCR products
1061	+	+	+	+	<i>G. duodenalis</i> Assemblage A	<i>G. duodenalis</i> Assemblage AII
1172	+	+	+	-	<i>G. duodenalis</i> Assemblage B+A	-
1811	+	+	+	-	<i>G. duodenalis</i> Assemblage A+B	-
1841	+	+	+	-	<i>G. duodenalis</i> Assemblage B	-
2211	+	+	+	-	<i>G. duodenalis</i> Assemblage A	-
3115	+	+	+	+	<i>G. duodenalis</i> Assemblage A	<i>G. duodenalis</i> Assemblage AII

**Table 9:** IFT, microplate assay detection and PCR-sequencing results of *Giardia* species from human faecal samples collected in Füzér and Mátrafüred.

**Information about *Giardia* positive patients:** The aim of the study was to find correlations between the contaminated drinking water consumption and human infections with *Giardia duodenalis* in the investigated areas in Hungary. The female patient from Budapest city (sample code: 3115) had travelled to South Asia and reported gastroenteritis there, therefore it was excluded that she had an endemic infection. The 4 patients from Füzér (sample codes: 1061, 1172, 1811, 1841) and the 1 patient from Mátrafüred (sample code: 2211) had no history of travelling abroad. Four of them (3 persons from Füzér, 1 from Mátrafüred) reported that they may have swallowed recreational water, all of them had outdoor activities using unfiltered water, 4 of them (3 persons from Füzér, 1 from Mátrafüred) had contact with animals, 2 persons from Füzér had contact with children under 5 years, 2 of the persons' relatives (one from Füzér and one from Mátrafüred) and a person from Füzér had diarrhoea in the last 4 months. The details of the infected people are summarized in Table 10.

<b>Patient Code</b>	<b>1061</b>	<b>1172</b>	<b>1811</b>	<b>1841</b>	<b>2211</b>	<b>3115</b>
<b>Age (Yr)</b>	44	71	37	31	64	26
<b>Occupation of patients</b>	Ambulance man	Pensioner	Heating fitter	House wife	Pensioner	Assistant
<b>Animal contact</b>	-	+	+	+	+	+
<b>Contact with person having diarrhoea</b>	-	-	-	+	+	-
<b>Child contact &lt;5 yr</b>	-	-	+	+	-	+
<b>The possibility of consuming contaminated food</b>	+	+	+	+	+	+
<b>Outdoor activities and consuming unfiltered water</b>	+	+	+	+	+	+
<b>Recreation</b>	+	-	+	+	+	+
<b>The patient reported diarrhoea 4 months before</b>	-	-	+	-	-	-

**Table 10:** Information about the *Girardia* positive patients in Mátrafüred, Füzér and Budapest according to the questionnaire. At Füzér, the patient code starts with 1, at Mátrafüred with 2 and at Budapest with 3.

**Information derived from the questionnaire in general:** In the two villages (Füzér and Mátrafüred) and in the control city (Budapest) 56, 55% and 80% of the people reported, that they may have swallowed recreational water, 65, 56% and 70% took part in outdoor activities and consumed unfiltered, untreated water, 88, 71% and 63% had contact with animals, 44, 33% and 40% had contact with children under 5 years, 25, 30% and 24% of the persons' relatives and 17%, 16% and 7% of the persons had diarrhoea in the last 4 months. Questionnaire summary is shown in Table 11.

Settlement	Füzér	Mátrafüred	Budapest
Age (Yr)	9-88	13-88	18-69
Animal contact (%)	88	71	63
Contact with person having diarrhoea (%)	25	30	24
Child contact <5 yr (%)	44	33	40
The possibility of consuming contaminated food (%)	80	84	93
Outdoor activities and consuming unfiltered water (%)	65	56	70
Recreation (%)	56	55	80
The patient reported diarrhoea 4 months before (%)	17	16	7

**Table 11:** Information derived from the questionnaire during the epidemiological investigations in Füzér, Mátrafüred and Budapest.

**Efficacy of the detection methods:** In our study we used effective concentration and sensitive detection methods for the determination of *Giardia* infections in faecal material and in water samples from selected areas in Hungary to find out the epidemiological relevance of *G. duodenalis* occurrence and transmission between humans and the environment. The microplate assay is a protein based test, which can detect the *Giardia* infections in stool specimens without microscopical indication of cysts or trophozoites. The IMS assay selectively concentrates only *Giardia* cysts in the investigation material. The IFT and DIC microscopy enhances the visualization of the *Giardia* cysts themselves and the internal morphology. Empty or amorphous cysts have also been recorded during the water sample microscopy, which can not provide appropriate quality of DNA for the following PCR analysis. The PCR analysis enhances the possibility the detection of the parasites present in the samples and the analysis of the amplified sequence give information about genotypes or Assemblages. As we discussed in chapter 4 the PCR detection could also be problematic, because of various inhibitors usually present in faecal and/or in water samples. However, the combination of the microscopy and molecular analysis lead to effective outcomes.

Wielinga and Thompson (2007) sorted and aligned in total 405 *G. duodenalis* sequences to examine the substitutions within and between the Assemblages. It was found that all of the genes could reproducibly group isolates into their Assemblages and that than A-I and A-II sub-Assemblage groups were robust and identifiable at all loci. However, the Assemblage B subgroups were not reproducible at half of the loci (SSU rRNA and beta-giardin) because there was insufficient sequence data of reference isolates available for comparison. In our study we used the GDH sequence analysis. The GDH gene display a high degree of polymorphism in *G. duodenalis*, which allows differentiation at intra-genotype level and sufficient number of GDH sequence are available in the GenBank for comparison.

***Giardia* genetic diversity:** On basis of the sequence analysis of the SSU rRNA PCR product (a 292 bp sequence), isolates can be classified into genetic Assemblages A or B. This region has previously been shown to be reliable in differentiating between genotypes in Australia (Hopkins et al. 1997). The PCR-sequencing was performed on all samples extracted by Mini Kit after IMS and extracted directly by Stool Kit. In two cases (samples 1172 and 1811) the results were conflicting, since in sample 1172 Assemblage A was determined extracted by Stool Kit and Assemblage B in sample extracted by Mini Kit after IMS. In case of sample 1811 Assemblage B was determined in sample extracted by Stool kit and Assemblage A in sample extracted by Mini Kit after IMS. Therefore it is possible that these samples contained mixed genotypes and depending on the PCR extraction method is not excluded that not all of the genotypes are included in the final detection.

It has been reported that *Giardia* isolates recovered from humans and other mammalian species fall into one of the two major genetic groupings or Assemblages, each containing a number of genetic subgroups. In Assemblage A, the isolates can be grouped into two distinct subgroups: A-I consists of a mixture of closely related animal and human isolates, whereas A-II appears to be restricted mainly to humans, although it recently has been found in cattle and horses (Traub et al. 2005, Mendonca et al. 2007). Assemblage B comprises a genetically diverse group of genotypes isolated principally from humans and some other mammalian species (Thompson 2000).

***Giardia duodenalis* transmission ways:** There are a lot of indications that animals and humans serve as contaminants of water supplies, but the frequency of zoonotic,

anthroponotic, anthrozoonotic, or zooantroponotic transfer of *Giardia* infections and the question who is infecting who remain an unclear issue. Identifying the environmental sources of contamination and routes of infection, particularly for human giardiasis, requires an 'in depth' understanding of which genotypes are host adapted (and do not infect humans) and which are probably transmissible to humans. According to the molecular investigations we can not definitely pronounce that the infection source of the people is the drinking water, since we could not perform the subgenotyping on water samples and on all human samples. This would be possible if we could confirm that the same strains in water are able to infect people who consumed this water.

In order to confirm significant associations between risk factors and *Giardia* infection in humans statistical analysis would be necessary (multiple logistic regression model), but in our case the number of positive samples are low, therefore the statistical analysis was not possible. Most of the people have contact with pets and livestock (dog, cat or cattle, sheep) and other people, take part in outdoor activities, recreation. We can not absolutely rule out, that the infection sources are the ones mentioned above. However, according to the questionnaire data people have almost the same lifestyle and habits in the capital and in the country. The main difference between the capital and the villages was that the inhabitants consumed different drinking water, and in Füzér and Mátrafüred twice as many people reported, that they had had diarrhoea in the last 4 months, than in Budapest. Among the *Giardia* positive patients only one person from Füzér reported diarrhoea in the last 4 months, and in this particular case a mixed infection was observed. This information support the hypothesis, that the *Giardia* infections present in Füzér are most probably originated from water.

The zoonotic potential of *G. duodenalis* has been discussed by various authors (van Keulen et al. 2002, Traub et al. 2004, Lalle et al. 2005, Savioli et al. 2006) but its real clinical significance is not clear. The major zoonotic risk should be from those genotypes of *Giardia* in Assemblage A and to a lesser extent, genotypes in Assemblage B (Thompson 2000). Humans, dogs, cats, domestic livestock (cattle, sheep, pig, horse) and certain species of wildlife (fallow deer, white tailed deer) were described as hosts of *G. duodenalis* Assemblage A (Trout et al. 2003, Traub et al. 2005, Uehlinger et al. 2006, Lalle et al. 2007, Langkjaer et al. 2007, Leonhard et al. 2007, Mendonca et al. 2007, Souza et al. 2007, Geurden et al. 2008). Humans, domestic livestock (cattle, sheep) and some species of

wildlife (beaver, wild monkey, coyote) were described as hosts of *G. duodenalis* Assemblage B (Itagaki et al. 2005, Aloisio et al. 2006, Fayer et al. 2006b, Trout et al. 2006, Castro-Hermida et al. 2007, Coklin et al. 2007, Mendonca et al. 2007). The contamination of the drinking water of Füzér and Mátrafüred with those genotypes may also have originated from the wildlife, since the water sources are surrounded by forests. In Füzér, it may have additionally originated from domestic livestock (sheep). There are not sewage contamination sources of human origin in these areas. In Hungary there are limited data about the prevalence of *Giardia* infection in domestic livestock, pets and wildlife. In 2006, 79 faecal samples were collected from pre-weaned calves in Hungary and the presence of both *Giardia* cysts and *Cryptosporidium* oocysts was examined. Only 3 out of 79 samples were *Giardia* positive and according to the SSU rRNA sequence data, it was *G. duodenalis* Assemblage E (Unpublished). Preliminary investigations were performed on the prevalence of *Giardia* infection in kennel dogs from Hungary by microscopic examinations and using a *G. duodenalis* specific coproantigen test. In order to investigate the genotypes of *Giardia*, a nested PCR specific for *Giardia* 18S rRNA was used. All sequenced samples displayed the sequences described for Assemblage D and C dog specific *G. duodenalis* strains. These results indicate, however, that dog giardiasis is highly prevalent in the studied geographical areas, but it does not present severe zoonotic potential. In the course of the study, the higher sensitivity of the copro-antigen test compared to microscopy, and the significant decline in the infection rate with the increasing age of the dogs sampled was clearly pointed out (Szénási et al. 2007).

The largest waterborne giardiasis outbreak described to date occurred in Norway, 2004, affecting around 1500 people (Robertson et al. 2006). The outbreak was caused by *G. duodenalis* Assemblage B, but genotyping of patient samples was complex and gave conflicting results. Genotyping of *Giardia* cysts found in contaminated water was not possible (Robertson et al. 2006).

**Correlation between the clinical manifestations and the *G. duodenalis* Assemblage:** By examination of stool samples of 353 children under 5 years of age, Read et al. (2002) found that Assemblage B were more prevalent in asymptomatic children, than Assemblage A. Almeida et al. (2006) studied 190 asymptomatic persons (13 adults, 177 children) in a Portuguese parochial Centre dedicated to social solidarity and they reported *G. duodenalis*

Assemblage B in 5 children and Assemblage A in 2 other children. It should be noted that these children were all asymptomatic with no obvious indications of diarrhoea but several children had high parasite loads. All of these information are in agreement with the fact that *Giardia* infections have higher incidence in children less than 5 years of age as their immune system is not fully developed (Adam 2001). Homan and Mank (2001) studied a group of symptomatic patients (8-60 years of age) and observed correlation between the genotype and the degree of severity of the symptoms. They found strong correlation between Assemblage A and intermittent diarrhoea and Assemblage B and persistent diarrhoea, respectively. In our study all the *Giardia* infected subjects (31-71 years of age) were asymptomatic. The 37- and the 71-year-old patients had mixed infection and the 44, 64 and 26 years of age patients were infected by *G. duodenalis* Assemblage A, the 31 year old patient was infected by Assemblage B. In case of these patients probably host factors may affect the presence of clinical manifestations more than the biological and genetic properties of the detected *Giardia* Assemblages. This finding is in agreement with a recent study where there was no statistically significant relationship between Assemblage and symptomatic or asymptomatic *Giardia* infections of patients if more than five years of age only were considered (Shagun et al. 2008).

**Giardiasis in Europe:** The prevalence of giardiasis in humans has been reported to be 2-7% in Europe, e.g. 5.4% in patients with gastroenteritis and 3.3% in asymptomatic persons in The Netherlands, 4% in asymptomatic patients in Portugal (Almeida et al. 2006, de Wit et al. 2001) and 1.2-2.1% in patients with gastroenteritis in Hungary (Anonymous 2003). All in all 6 (2%) out of 300 Hungarian asymptomatic human stool samples were found to be *Giardia* positive indicating similar European average reported. The ratio was higher (4%) in Füzér, where *Giardia* cysts were detected in drinking water sources by microscopic and also molecular methods and it was lower (1%) in Mátrafüred, where *Giardia* cysts were detected only by microscopy in the water. DNA amplification in those samples was not possible probably for the reasons discussed above. The fact that *Giardia* cysts could be identified by microscopy is a clear evidence for a contamination in the investigated samples. The prevalence of giardiasis was lower (1%) in Budapest, where *Giardia* cysts have never been detected in the drinking water sources.

**Regulative aspects and conclusions:** The results make necessary appropriate intake regulation with respect to high pathogen loads, as the risk increases with the time of exposure to pathogen contaminants as it has been similarly suggested by others (Aström et al. 2007). A recent study confirmed that source and treatment system causative events of outbreaks often occurred concomitantly, the distribution system causative events occurred less frequently. Livestock and rainfall in the catchment with no/inadequate filtration of water sources also contributed to outbreaks. 90% of protozoan outbreaks were due to filtration deficiencies. By contrast, for bacterial, viral gastroenteritis and mixed pathogen outbreaks, 75% of treatment events were disinfection deficiencies (Risebro et al. 2007). Similar study in Malaysia highlights the need to look into the possibility of other risks of *Giardia* infections such as water and food transmission routes (Mohammed Mahdy et al. 2008).

Even in Füzér *Giardia duodenalis* Assemblage B was detected in the water source and in 3 patients too, it remains unproved that *Giardia* cysts detected in drinking water play a role in the presence of higher number of asymptomatic giardiasis in this settlement. The presented data give a message to the public health authorities about the significance of the investigations and about the importance of protecting the public health in the investigated areas. We also believe that this study has a worldwide significance due to the similar situation in other countries. However, more studies are necessary to clarify the situation of *Giardia* infections via drinking water and in relation to the symptomatic or asymptomatic individuals and the isolation of *Giardia* strains from livestock, pets and wildlife is also necessary that maybe infective to human.

## 7. GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The emergence and spread of infectious diseases such as giardiasis and cryptosporidiosis in human populations is an increasing problem around the world. These waterborne pathogens can pose threats to drinking water supplies, recreational waters, source waters for agriculture and aquaculture, as well as to aquatic ecosystems and have tremendous adverse impacts in developing countries. While developed countries have been more successful in controlling waterborne pathogens, water quality problems are still prevalent. Contamination of water is known to occur from a range of sources including municipal wastewater effluents, agricultural wastes, and wildlife.

According to our investigations in Hungary, *Giardia* cysts and/or *Cryptosporidium* oocysts were detected by microscopy in 55 % of the raw water samples and 34 % of the drinking water samples. Two water treatments were found to be inadequate from the point of protozoa removal: neither of them includes the flocculation step in their treatment. *Cryptosporidium* and *Giardia* were detected regularly in the River Danube, but never in riverbank filtered water suggesting the effectiveness of RBF as a purification method. According to our investigations the waterworks having risk to protozoa contamination had been informed and they already started control measures regularly.

PCR analysis confirmed that 36 % of the investigated water samples were positive for *Giardia* and 28 % positive for *Cryptosporidium*. Furthermore, *G. duodenalis* Assemblage A and Assemblage B human pathogenic Assemblages were identified. Regarding *Cryptosporidium*, the species *C. parvum*, the most reported human pathogen has been found in addition to *C. meleagridis* which is pathogenic for humans and birds. Sequence analysis revealed a new subtype of *G. duodenalis* - complex, clustered close to the Assemblage A group. According to the molecular investigations the contamination sources, specifically the affect of sewage inflows could be tracked. In some cases the contamination sources are unknown.

This present study provides the first report on simultaneous detection and genotyping of *G. duodenalis* and *Cryptosporidium* species from water supplies in Hungary. The described

detection techniques for *Giardia* and *Cryptosporidium* species in the environmental water, human and animal reservoirs in Hungary now routinely applied, will contribute to the protection measures and public health information. Our work also demonstrates the difficulties of dealing with low (oo)cyst concentrations in water and faecal samples. The presented assays are applicable to clinical (human and non-human hosts), environmental samples and food, suggesting their attractiveness for further investigation of clinical, water and food samples. The use of this molecular tools will be helpful in the assessment of the zoonotic potential of various *Cryptosporidium* and *Giardia* spp. and the sources of human infections. They also play a significant role in characterization transmission dynamics in endemic and epidemic areas.

Safe drinking water supplies are critical for protecting public health and livelihood of the Hungarians depends on the availability of safe drinking water. In some portions of the nation drinking water is a scarce resource and there is no possibility to choose between the different water sources, while in other areas abundant water supplies are available. Because of the geographical location and geological conditions of Hungary the water supplies originate mainly from groundwater and less from riverbank filtration and surface water. The status of *Giardia* and *Cryptosporidium* threats to drinking water and aquatic ecosystems was not known in Hungary and these investigations throw a light into the current situation of the contamination of the water supplies in the country. Surveillance and scientific research were (and are) required to better understanding the nature of these pathogen threats.

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# APPENDIX

**Appendix 1:** *Cryptosporidium* and *Giardia* (oo)cysts detected by microscopy in Hungarian drinking water resources and in drinking water samples.

Sample codes, dates of sampling, the characteristics of the investigated water sources and the number of *Cryptosporidium* and *Giardia* (oo)cysts in 100 L water are shown. Complementary description in chapter 3.

<b>Code</b>	<b>r=raw d=drinking water</b>	<b>Date of sampling</b>	<b><i>Cryptospori- dium</i> oocysts /100 L</b>	<b><i>Giardia</i> cysts /100 L</b>	<b>Water source/Water treatment characteristics</b>
1	d	09/10/2000	0	2	Karst spring (Jósvafő)/chlorination
	d	13/11/2000	0	0	
2	r	22/03/2001	0	0.3	Surface water (River Tisza)/ flocculation, filtration, ozonation, activated carbon, chlorination
	d	22/03/2001	0	0	
	r	10/09/2001	0	6	
	d	10/09/2001	0	1.2	
	r	25/03/2002	5	60	
	d	25/03/2002	2.6	0	
	r	12/08/2002	0	0	
	d	12/08/2002	0	0.2	
3	d	09/04/2001	0	0	Karst spring (Veszprémfajsz)/chlorination
4	d	09/04/2001	0	0	Karst spring (Nemesvámos)/chlorination
5	d	09/04/2001	0	0	Karst spring (Balatocsicsó)/chlorination
6	r	08/04/2002	0	0	Surface water (Siófok- Balatonszéplak, Lake Balaton)/flocculation, filtration, activated carbon and chlorination or chlorine dioxide
	d	08/04/2002	0	0	
	r	18/11/2002	0	0	
	d	18/11/2002	0	0	
7	r	07/05/2002	0	0	Surface water (Fonyód, Lake Balaton)/flocculation, filtration, activated carbon and chlorination or chlorine dioxide
	d	07/05/2002	0	0	
	r	09/09/2002	0	0	
	d	09/09/2002	0	0	
8	r	21/05/2002	0	0	Surface water (Balatonalmádi, Lake Balaton) /flocculation, filtration, activated carbon and chlorination or chlorine dioxide
	d	21/05/2002	0	0	
	r	04/11/2002	0	5	

	d	04/11/2002	0.1	0	
9	r	21/05/2002	0	0	Surface water (Balatonfüred, Lake Balaton) /flocculation, filtration, activated carbon and chlorination or chlorine dioxide
	d	21/05/2002	0	0	
	r	04/11/2002	0	0	
	d	04/11/2002	0	0.1	
10	r	10/06/2002	135	5	Surface water (Dunaújváros, River Danube)/ flocculation, filtration, activated carbon and chlorination
	d	10/06/2002	0.4	0	
	r	13/09/2005	300	40	
	d	13/09/2005	0	0	
12	d	31/03/2003	0	0	Karst spring (Szalonna)/chlorination
13	r	31/03/2003	0	0	Surface water (Lázbérc Reservoir)/flocculation, filtration, chlorination
	d	31/03/2003	0	0	
	r	21/06/2005	90	20	
	d	21/06/2005	0	0	
	r	06/09/2005	0	0	
	d	06/09/2005	0	0	
14	d	08/03/2004	0	0	Ground water (Szentes)/chlorination
	d	08/03/2004	0	0	
15	d	28/06/2005	0	0	Ground water (Kéked)/chlorination
16	d	28/06/2005	0	3	Surface water (Füzér, Brook Nagy) /sand filtration, activated carbon and chlorination
	r	16/08/2005	40	0	
	d	16/08/2005	12.8	0	
17	d	28/06/2005	4	0	Spring (Pusztafalu)/chlorination
	d	16/08/2005	3.5	0	
18	d	05/07/2005	0	0	Surface water (Borsodszirák, River Bódva) /flocculation, filtration, activated carbon and chlorination
	r	06/09/2005	20	0	
	d	06/09/2005	0	0	
19	r	05/07/2005	1030	30	Surface water (Sajóecseg, River Bódva)/flocculation, filtration, chlorination
	d	05/07/2005	0	0	
	r	06/09/2005	60	20	
	d	06/09/2005	0	0	

20	r	12/07/2005	0	0	Surface water (Komravölgy Reservoir) /flocculation, filtration, activated carbon and chlorine dioxide
	d	12/07/2005	0.7	1.4	
	r	23/08/2005	0	0	
	d	23/08/2005	0	0	
21	r	26/07/2005	0	0	Surface water (Hasznos Reservoir) /flocculation, filtration, activated carbon and chlorine dioxide
	d	26/07/2005	0	0	
22	r	26/07/2005	10	0	Surface water (Csórrét Reservoir)/flocculation, filtration, chlorination
	d	26/07/2005	1	1	
	r	23/08/2005	10	0	
	d	23/08/2005	0	0	
23	r	26/07/2005	0	0	Surface water (Köszörűvölgy Reservoir)/flocculation, filtration, chlorination
	d	26/07/2005	0	0	
24	r	09/08/2005	840	0	Surface water (Mátrafüred Reservoir)/sedimentation, sand filtration, chlorination
	d	09/08/2005	27.1	0	
	r	14/09/2005	850	0	
	d	14/9/2005	63.6	0	
25	r	10/09/2001	8	0	Canal from River Tisza (Eastern Main Canal)/flocculation, filtration, ozonation, activated carbon, chlorination
	d	10/09/2001	0	0	
	r	13/09/2005	0	50	
	d	13/09/2005	0	0	

All together 75 samples, 31 raw water, 44 drinking water.

**Appendix 2:** *Cryptosporidium* and *Giardia* species detected by IFT, PCR and characterized by sequence analysis in Hungarian drinking water resources, surface and sewage water. The samples collected and examined, sample codes and sampling dates are shown. The numbers of *Cryptosporidium* and *Giardia* detected by IFT have been expressed in (oo)cysts per 10 L water. Beside the PCR results the target gene for *Giardia* and *Cryptosporidium* and the sequence data are indicated. Complementary description in chapter 4.

Sample code	Origin of the sample and sampling date	Number of the <i>Cryptosporidium</i> oocysts detected by IFT /10 L	Number of the <i>Giardia</i> cysts detected by IFT /10 L	<i>Cryptosporidium</i> SSUrRNA PCR and sequence data	<i>Giardia</i> PCR and sequence data	
					SSU rRNA	GDH
<b>Raw waters</b>						
1	River Bódva Sajóecseg 06.09.2005.	2	5	-	-	-
2	River Bódva Borsodszirák 06.09.2005.	0	2	-	-	-
3	River Danube Dunaújváros 13.09.2005.	4	30	-	-	-
4	River Danube Tass 28.08.2007.	1.5	25.5	+ could not be sequenced	+ <i>G. duodenalis</i> Assemblage A	+ <i>G. duodenalis</i> Assemblage AII

5	River Tisza Szolnok 12.09.2007.	0	1.5	-	+ <i>G. duodenalis</i> Assemblage B	+ <i>G. duodenalis</i> Assemblage AI and BIV
6	Eastern Main Canal Balmazújváros 13.09.2005.	5	0	-	-	-
7a	Brook Nagy, Füzér 16.08.2005.	0	4	-	+ <i>G. duodenalis</i> Assemblage A	-
7b	Brook Nagy, Füzér 04.06.2007.	0	12	-	+ <i>G. duodenalis</i> Assemblage B	-
8a	Mátrafüred reservoir 14. 09. 2005.	0	85	-	-	-
8b	Mátrafüred reservoir 09.08.2005.	0	84	-	-	-
9a	Lázbérc reservoir 06.09.2005.	0	0	-	-	-
9b	Lázbérc reservoir 24.10.2007.	0	0	+ could not be sequenced	+ could not be sequenced	-

10	Hasznos reservoir 26.07.2005.	0	0	-	-	-
11	Köszörűvölgy reservoir 26.07.2005.	0	0	-	-	-
12	Komravölgy reservoir 12.07.2005.	0	0	-	-	-
13	Csórrét reservoir 26.07.2005.	0	1	-	-	-

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**Sewage treatment plants**

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14	Zánka 21.09.2004. effluent	0	240	-	-	-
15	Keszthely 13.07.2004. effluent	0	6.6	+ could not be sequenced	-	-
16	Révfülöp 02.08.2004. effluent	6	125	+ could not be sequenced	+ <i>G. duodenalis</i> Assemblage A	-
17	Balatonújlak 13.09.2004. effluent	4.4	375.5	+ could not be sequenced	-	-

18	Tiszadorogma 16.08.2007. raw	0	8000	+ <i>C. meleagridis</i>	+ <i>G. duodenalis</i> Assemblage A	+ <i>G. duodenalis</i> Assemblage AII
19	Edelény 16.08.2007. raw	0	4560	+ could not be sequenced	+ <i>G. duodenalis</i> Assemblage A and B	+ <i>G. duodenalis</i> Assemblage AII and BIII
20	Szilvásvárad 16.08.2007. raw	0	57600	+ <i>C. parvum</i>	+ <i>G. duodenalis</i> Assemblage A	+ <i>G. duodenalis</i> Assemblage AII and BIV
21	Budapest 16.10.2007. raw	0	3200	+ could not be sequenced	+ <i>G. duodenalis</i> Assemblage A	+ <i>G. duodenalis</i> Assemblage AII
22	Rácalmás 28.08.2007. raw	0	14640	-	+ <i>G. duodenalis</i> Assemblage A	+ <i>G. duodenalis</i> Assemblage AII and BIII
23	Dunaújváros 16.10.2007. raw	260	14400	+ could not be sequenced	+ <i>G. duodenalis</i> Assemblage A	+ <i>G. duodenalis</i> Assemblage AII

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**Brooks, ducts**

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24	Büdös-árok Keszthely 13.07.2004.	1.2	173.7	-	-	-
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25	Brook Kéki Balatonfüred 26.07.2004.	2	0	-	-	-
26	Brook Séd Balatonfüzfő 02.08.2004.	6	0	-	-	-
27	Brook Burnót Ábrahámhegy 02.08.2004.	7	3	-	-	-
28	Forró-árok Balatonlelle 13.09.2004.	0	0	-	-	-
29	Brook Séd Vörösberény 21.09.2004.	1	1	-	+ <i>G. duodenalis</i> Assemblage A	+ <i>G. duodenalis</i> Assemblage AI
30	Keleti-Bozót Fonyód 21.09.2004.	0	2.2	-	-	-

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**Recreation sites, close to the inflows (beaches)**

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31	Ábrahámhegy 05.08.2004.	23	0	-	-	-
32	Balatonfüzfő 05.08.2004.	2.5	0	-	-	-
33	Keszthely 05.08.2004.	3.2	8	-	-	-

**Appendix 3:**

Structured epidemiological interview

The questionnaire

Identity code:

Sampling date:

1. Age:
2. Gender: male/female
3. Marital status: married/single
4. How long have you lived in Mátrafüred? Year/month
5. How many people live together in your family?
6. How many children are in your family under 5 years old?
7. How many children are in your family under 16 years old?
8. What is your occupation?
9. Besides Mátrafüred, please name one more settlement, where you spend most of your time!
10. Highest qualification: Less, than primary school/ primary school/vocational school /grammar school/university
11. From where does your drinking water originate? Local distributed drinking water/own well/own well near to lake or river/tap water further purified with other water purification system/other...../I do not know
12. Do you regularly drink unboiled tap water or prepare soft drink from unboiled tap water?  
Yes/no How many glasses a day?
13. Do you regularly drink mineral water? Yes/no How many glasses a day?
14. Do you use tap water for washing your food? Yes/No
15. Do you wash the raw fruits and vegetables before consuming?  
Always/sometimes/rarely/never/ I do not know/I do not eat fruits and vegetables
16. How often do you eat salads? Always/sometimes/rarely/never/ I do not know/I do not eat fruits and vegetables
17. Do you eat the fruits or vegetables unpeeled if their skin is eatable?  
Always/sometimes/rarely/never/ I do not know
18. Do you eat the outer part of the lettuce or cabbage? Yes/No/ I do not know/I do not eat this kind of vegetables

19. Is there in your family a child less than 5 years old? Yes/no
20. How many children are in your family less than 1 year old?
21. How many children are in your family 1-2 year old?
22. How many children are in your family 3-5 year old?
23. In the last 12 months have you moved to another place? Yes/no
24. In the last 12 months have you touched pets of yours or of other people's? Yes/no
25. In the last 12 months have you touched less, than 1 year old animals? Yes/no
26. In the last 12 months have you touched livestock or animals in the zoo? Yes/no
27. In the last 12 months have you been on farms or zoo petting sites? Yes/no
28. In the last 12 months have you drunk of lakes, rivers or untreated well water? Yes/no
29. In the last 12 months have you swum or had a bath in
  - a. outdoor pool? Yes/no
  - b. covered pool? Yes/no
  - c. lakes, rivers, brooks? Yes/no
  - d. bathing tube or shower? Yes/no
  - e. aqua parks? Yes/no
30. In the last 12 months has been a baby in your house? Yes/no
31. In the last 12 months have you touched a baby living another family? Yes/no
32. In the last 12 months have you touched a baby's nappy? Yes/no
33. In the last 12 months have you taken care of a family member having diarrhoea? Yes/no
34. In the last 12 months have you had pipe repair works in your house? Yes/no
35. In the last 12 months have you had outdoor activities? Yes/no
36. In the last 12 months have you travelled abroad? Yes/no Where?
37. Have you had diarrhoea in the last 4 months taking more than 4 days? Yes/no/I do not remember
38. Has had somebody in your family diarrhoea in the last 4 months taking more than 4 days? Yes/no/I do not remember
39. Have you ever been told by your general practitioner that you have *Giardia/Cryptosporidium* infection? Yes/no/I do not remember What time?
40. Has somebody in your family ever been told by your general practitioner that his/her has *Giardia/Cryptosporidium* infection? Yes/no/I do not remember What time?