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Cryptosporidium and *Giardia*: detection in environmental and faecal samples

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Academic Dissertation

To be publicly discussed with the permission of
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ISBN 952-91-9808-6 (paperback)
ISBN 952-10-2867-X (PDF)
Yliopistopaino 2006

To my boys, great and small

Ei hän väistä vaaroja,
ei säiky jalopeuraa,
ei hän käänny takaisin,
ken tähteänsä seuraa.

V.A. Koskenniemi

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1 Acknowledgements

This study was performed at the Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, University of Helsinki during the period of 1998-2005. These years have been the most productive in my life; not only have I become a mother of two lively sons and renovated an old house, but also managed to carry on and finish this challenging project. The project was supported by the Academy of Finland, the National Technology Agency, the Ministry of Agriculture and Forestry, the Ministry of Defence, the Finnish Defence Forces, Intervet Oy, the Department of Food and Environmental Hygiene, the Foundation of Finnish Veterinary Medical Association, the Walter Ehrström Foundation, the Faculty of Veterinary Medicine, the Helsinki University Fund, the NOVA mobility grant and the Helsinki University Grant for Finishing Doctoral Dissertation. I thank professor Hannu Korkeala, head of the department, for providing the working facilities and for all support during these years. I wish to thank professor Lars-Axel Lindberg for organizing my salary from the Academy of Finland and for the interest he has shown to my work.

I want to express my gratitude to my supervisor, professor Marja-Liisa Hänninen for her braveness to take up this totally new protozoan project. Together we have built up cryptosporidial and giardial laboratory diagnostics from the scratch. You have always encouraged me to grow as an independent researcher for which I am truly grateful. I also want to thank you for the understanding you have shown for my decision to combine research and family life.

I owe my gratitude to the reviewers of this thesis, Joke van der Giessen and Lucy Robertson, for their precious thoughts and comments on this work.

I wish to thank all my fellow researchers and co-authors of the original publications for successful co-operation. Many warm thanks to Heidi Enemark for sharing her valuable time and vast knowledge on protozoan research, and the warm-hearted hospitality during my stays at the Danish Veterinary Institute. I thank Ari Hörman for pleasant co-operation with water-related items and for all practical help considering the thesis process. Jenni Kolehmainen and Pialiisa Toropainen are acknowledged for their enthusiastic help in sampling and treating the dog samples. The personnel at the department are acknowledged for the pleasant working atmosphere. My warmest thanks go to Urszula Hirvi for guiding me to the world of PCR and for being a constant sunshine of the laboratory.

My colleagues and friends Päivikki Perko-Mäkelä, Anu Näreaho and Mari Nevas are acknowledged for the countless refreshing and inspiring cola break discussions and for their cheerful company in leisure activities. I also want to address my sincere thanks to all my valuable friends for keeping contact during these busy years. Sharing all my thoughts with my life-long friends Tiina and Joanna has been meaningful throughout the years.

This work would not have been finished without the concrete help of my family and relatives. I owe my devoted thanks to my parents Pirjo and Esko and to my sister Karkki for the endless baby-, dog- and horse-sitting, help in renovating and for all support during my life. I also want to thank my gutsy grand mother Ester for loading me with lots of self-confidence by showing that hard work can get you anywhere!

I want to extend my gratitude to my parents-in-law Gitta and Ralf, my godparents Riitta and Eku and my cousin Anne for keeping an eye on my boys while I have been working. My tender thanks to lapphund Naava for patiently forgiving me the hurries and finnhorse Alinda for challenging me physically by taking me down to earth whenever she thought it was necessary.

My husband Patrik deserves all my love and gratitude for his never ending support and guiding in scientific matters. You have taken good care of our family while I have been away with the work either mentally or physically. I love you a lot! I also want to express my love to my little ones, Eedi and Ebbe, who have given me the joy and pride of motherhood and who with their existence have shown me the true meaning of life. You mean everything to me!

Espoo, December 20, 2005

2 Abbreviations

ANOVA, Analysis of variance
API, Analytical Profile Index
ASTM, American Society of Testing and Materials
COWP, *Cryptosporidium* oocyst wall protein
DNA, deoxyribose nucleic acid
dNTP, deoxynucleotide triphosphate
EDTA, ethylene diamine tetra-acetic acid
ELISA, enzyme-linked immunosorbent assay
FITC, fluorescein isothiocyanate
GES, guanidine thiocyanate
GDH, glutamate dehydrogenase
GSA, *Giardia* specific antigen
IC-PCR, immunocapture-PCR
ICR, Information Collection Rule
IF, immunofluorescence
IFA, immunofluorescence assay
IMS, immunomagnetic separation
ISO, International Organization for Standardization
mAb, monoclonal antibody
MPN, most probable number
NCFA, Nordic Committee on Food Analysis
NIDR, National Infectious Disease Registry
NTU, nephelometric turbidity unit
OD, optical density
pAb, polyclonal antibody
PBS, phosphate-buffered saline
PBS-Tween 20, PBS containing 0.05% Tween 20
PCR, polymerase chain reaction
RE, recovery efficiency
RFLP, restriction fragment length polymorphism
rRNA, ribosomal ribose nucleic acid
TE, buffer containing 0.1 M Tris-HCl and 0.2 M EDTA
TSC agar, tryptose sulphite cycloserine agar
USEPA, United States Environmental Protection Agency
UV, ultraviolet
WTP, wastewater treatment plant

3 ABSTRACT

Cryptosporidium and *Giardia* are highly infectious protozoan parasites capable of causing gastrointestinal illness in both animals and humans. The waterborne transmission of these parasites has been well documented worldwide. The significance of *Cryptosporidium* and *Giardia* as waterborne pathogens is based on their generalized presence in the environment and robustness against environmental conditions. Low numbers of (oo)cysts are sufficient for infection. In addition, infected individuals can excrete massive amounts of infective (oo)cysts. Furthermore, since low densities of (oo)cysts are detected in environmental samples, sensitive detection methods are needed.

In the present thesis, molecular methods for detection of *Cryptosporidium* and *Giardia* were developed and applied in studies in which the occurrence of *Cryptosporidium* and *Giardia* in sewage sludge and surface water was determined. Furthermore, the usefulness of indicator parameters (occurrence of *Clostridium perfringens*, *Escherichia coli*, enterococci and water turbidity) to predict the occurrence of *Cryptosporidium* and *Giardia* in environmental samples was evaluated. *Cryptosporidium* and *Giardia* could be found both in untreated and treated sewage sludge and in surface water. However, no correlation between indicator parameters and the occurrence of *Cryptosporidium* and *Giardia* was detected.

The occurrence and zoonotic character of *Cryptosporidium* and *Giardia* in possible reservoirs for human infections was determined by examining faeces of asymptomatic dogs. For this purpose, the present detection techniques were evaluated. The *Giardia* ELISA technique studied performed well and was capable of detecting animal-specific genotypes. The accuracy of the *Cryptosporidium* ELISA studied was low and due to high rates of false-positive results, cannot be recommended for detection of *Cryptosporidium* in canine faeces. *Cryptosporidium* and *Giardia* were frequent findings in asymptomatic dogs, however, since all species and genotypes found were considered animal-specific, the zoonotic transmission risk from these dogs appears restricted.

The findings indicate that *Cryptosporidium* and *Giardia* are present and cycle in Finland´s environment. Furthermore, direct analysis is the best way to confirm the presence of these protozoans in environmental samples.

4 List of original publications

The thesis is based on the following original articles:

I Rimhanen-Finne R, Ronkainen P, Hänninen ML. (2001) Simultaneous detection of *Cryptosporidium parvum* and *Giardia* in sewage sludge by IC-PCR. *Journal of Applied Microbiology* 91:1030-5.

II Rimhanen-Finne R, Hörman A, Ronkainen P, Hänninen ML. (2002) An IC-PCR method for detection of *Cryptosporidium* and *Giardia* in natural surface waters in Finland. *Journal of Microbiological Methods* 50:299-303.

III Hörman A, Rimhanen-Finne R, Maunula L, Bonsdorff von CH, Torvela N, Heikinheimo A, Hänninen ML. (2004) *Campylobacter* spp., *Giardia* spp., *Cryptosporidium* spp., Noroviruses and indicator organisms in surface water in southwestern Finland, 2000-2001. *Applied and Environmental Microbiology* 70:87-95.

IV Rimhanen-Finne R, Vuorinen A, Marmo S, Malmberg S, Hänninen ML. (2004) Comparative analysis of *Cryptosporidium*, *Giardia* and indicator bacteria during sewage sludge hygienization in various composting processes. *Letters in Applied Microbiology* 38:301-5.

V Rimhanen-Finne R, Enemark HL, Kolehmainen J, Toropainen P, Hänninen ML. Evaluation of immunofluorescence microscopy and enzyme-linked immunosorbent assay in detection of *Cryptosporidium* and *Giardia* infections in asymptomatic dogs. Submitted in *Journal of Clinical Microbiology*.

The original articles are referred to by the Roman numbers I–V in the text. The articles have been reprinted with permission from Blackwell Publishing (I and IV), Elsevier Science (II) and American Society for Microbiology (III).

5 Introduction

Methods for detecting protozoan parasites were developed following the first reported waterborne outbreaks of giardiasis in Aspen, CO, USA in 1965 and cryptosporidiosis in Braun Station, TX, USA in 1984. In the early 1990s, the American Society of Testing and Materials (ASTM) proposed a method for detecting *Cryptosporidium* oocysts and *Giardia* cysts in low-turbidity water. Similarly, the UK Standing Committee of Analysis developed a preliminary method for detecting waterborne *Cryptosporidium* and *Giardia*. The largest reported waterborne outbreak in Milwaukee, WI, USA in 1993, caused by *Cryptosporidium parvum*, affected over 400 000 individuals and raised the level of public awareness of protozoan organisms, leading to increased levels of detection in laboratories. As a consequence of this enhanced awareness, the United States Environmental Protection Agency (USEPA) approved in 1995 an Information Collection Rule (ICR) method for detecting (oo)cysts in water. These early methods were based on cartridge filtration, flotation to separate (oo)cysts from debris and immunofluorescence (IF) microscopy to identify and enumerate the (oo)cysts. The methods, however, were criticized for being costly and difficult to perform as well as presenting low recovery efficiencies, leading to efforts to develop new, innovative technologies for protozoan analysis.

In 1999, USEPA approved method 1622 for *Cryptosporidium* detection, and soon after method 1623 for simultaneous detection of *Cryptosporidium* and *Giardia* in water by capsule filtration, immunomagnetic separation (IMS) and IF microscopy. While most researchers agree that method 1623 performs better than the ICR method, it has still been criticized for being too labour-intensive and time-consuming as well as demonstrating variable recoveries. As with previous methods, experience is required to perform and interpret results of methods 1622 and 1623. The methods are probably also too expensive to adopt in many countries. Furthermore, since identification in methods 1622 and 1623 relies on IF microscopy, which differentiates *Cryptosporidium* and *Giardia* only on genus level, improvements in detection techniques has been proposed.

To increase the quality of Finnish research in environmental health, the Finnish Research Programme on Environmental Health (1998-2001) was established by the Academy of Finland and the National Technology Agency in Finland. One of the aims of this program was to develop methods for detection of *Cryptosporidium* and *Giardia* from environmental samples. No previous information concerning the occurrence of *Cryptosporidium* and *Giardia* in Finland´s environment was available. Similarly, methods for detecting these parasites were lacking. This thesis is partly based on studies conducted under the Finnish Research Programme on Environmental Health.

6 Review of the literature

6.1 *Cryptosporidium* and cryptosporidiosis

6.1.1 Classification and taxonomy

Protozoan parasites of the genus *Cryptosporidium* belong to the phylum Apicomplexa and are classified as Coccidea together with other enteric parasites such as *Eimeria*, *Isospora* and *Toxoplasma* (Levine, 1980; Levine *et al.*, 1980). The taxonomy of the genus *Cryptosporidium* is incomplete and ongoing. A unified species concept and criteria for species definition, characterization and identification appear to be lacking. Traditionally, species were named after the host in which they were found (Levine *et al.*, 1980; Levine, 1984). Morphological features have been used to designate genus and species, although they cannot be considered as a reliable tool to distinguish between *Cryptosporidium* species (Fall *et al.*, 2003). Revision of the taxonomy of *Cryptosporidium* species was proposed according to the new molecular data on isolates from various host animals (Morgan *et al.*, 1999; Xiao *et al.*, 2000a). Genetic analyses have indicated that two distinct *Cryptosporidium* species are responsible for most human infections. *Cryptosporidium parvum* (formerly *C. parvum* "bovine" or "cattle" genotype; genotype 2 or C) is considered as a zoonotic, and *Cryptosporidium hominis* (formerly *C. parvum* "human" genotype; genotype 1 or H) as an anthroponotic species (Peng *et al.*, 1997; Spano *et al.*, 1998; Sulaiman *et al.*, 1998; Morgan-Ryan *et al.*, 2002). In this review of the literature, the names *C. parvum* and *C. hominis* will be used. Infections caused by *C. meleagridis*, *C. felis* and *C. canis* (also known as *C.* dog type or *C.* dt) in immunocompetent persons have been reported (Pedraza-Diaz *et al.*, 2001a; Xiao *et al.*, 2001). In cases of immunodeficiency, patients are also known to be infected by other *Cryptosporidium* parasites, including *C. meleagridis*, *C. felis*, *C. canis*, *C. suis* (also known as *C.* pig genotype), *C. baileyi* and *C. muris* (Ditrich *et al.*, 1991; Xiao *et al.*, 2000b; Pedraza-Diaz *et al.*, 2001b; Caccio *et al.*, 2002; Palmer *et al.*, 2003). So far, eight valid *Cryptosporidium* species have been reported to be capable of infecting humans (Table 1). Additionally, new *C. parvum* genotypes such as *Cryptosporidium* cervine geno-

Table 1. *Cryptosporidium* species capable of causing infections in humans.

Species	Original host[1]	Reference
C. hominis[2]	*Homo sapiens* (human)	Morgan-Ryan *et al.*, 2002
C. parvum[3]	*Mus musculus* (house mouse)	Tyzzer, 1910
C. baileyi	*Gallus gallus* (chicken)	Ditrich *et al.*, 1991
C. meleagridis	*Meleagris gallopavo* (turkey)	Pedraza-Diaz *et al.*, 2001a, Xiao *et al.*, 2001
C. felis	*Felis catis* (cat)	Pedraza-Diaz *et al.*, 2001b, Xiao *et al.*, 2001, Caccio *et al.*, 2002
C. canis[4]	*Canis familiaris* (dog)	Pedraza-Diaz *et al.*, 2001b, Xiao *et al.*, 2001
C. suis[5]	*Sus scrofa domesticus* (pig)	Xiao *et al.*, 2002
C. muris	*Mus musculus* (house mouse)	Palmer *et al.*, 2003

[1]from Fayer *et al.*, 1997
[2]also known as *C. parvum* human genotype; genotype 1 or H, or type I
[3]also known as *C. parvum* bovine genotype; genotype 2 or C, or type II
[4]also known as *C.* dt or *C.* dog type
[5]also known as *C.* pig genotype

Table 2. Morphometric characteristics of oocysts.

Species	Measure (µm)	Reference
C. hominis	4.9 × 5.2 (4.4–5.4 × 4.4–5.9)	Morgan-Ryan *et al.*, 2002
C. parvum	5.0 × 4.5 (4.5–5.4 × 4.2–5.0)	Upton & Current, 1985
C. baileyi	6.1 ± 0.4 × 4.8 ± 0.2	Ditrich *et al.*, 1991
C. meleagridis	4.0 × 4.5	Fayer *et al.*, 1997
C. felis	4.0 × 4.6 (3.0–4.0 × 3.2–5.1)	Sargent *et al.*, 1998
C. canis	4.7 × 5.0 (3.7–5.9 × 3.7–5.9)	Fayer *et al.*, 2001
	4.2 × 4.8 (3.8–4.6 × 4.6–5.1)	Fall *et al.*, 2003
C. suis	4.2 × 4.6 (4.0–4.3 × 4.4–4.9)	Ryan *et al.*, 2004
C. muris	5.6 × 7.4 (5.3–6.5 × 6.6–7.9)	Upton & Current, 1985
	6.1 × 8.1 (5.6–6.4 × 8.0–9.0)	Palmer *et al.*, 2003

type have been identified in human stools (Ong *et al.*, 2002). It appears likely that also other new species or genotypes may cause occasional zoonotic infections in humans in the future.

6.1.2 Morphology of the oocyst

The small size of *Cryptosporidium* oocysts makes them indistinguishable from each other based on morphology by light microscopy (Fall *et al.*, 2003; Table 2). The oocysts are spherical or ovoid in appearance, and contain four parallel sporozoites surrounded by a smooth oocyst wall (Fig. 1). In the wall, a faint suture can be seen through which the sporozoites exit during excystation (Upton *et al.*, 1985; Morgan-Ryan *et al.*, 2002).

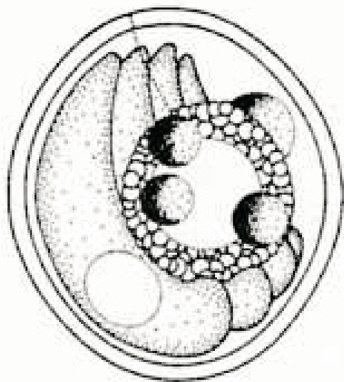

Figure 1. *Cryptosporidium* oocyst (Upton and Current, 1985).

6.1.3 Life cycle

The first detailed description of the *Cryptosporidium* life cycle was published in 1910 (Tyzzer, 1910). The use of differential interference microscopy and electron microscopy in a more recent study confirmed many of the observations made by Tyzzer and revealed additional features of the endogenous development of the parasite (Current *et al.*, 1986). The only exogenous stage of the *Cryptosporidium* life cycle is the sporulated, thick-walled oocyst, which is excreted in the faeces of an infected host (Fig. 2). The endogenous part of the life cycle begins when the infectious oocyst is ingested. In the gastrointestinal lumen, the oocyst releases sporozoites, which parasitize the villous enterocytes. The sporozoites differentiate intracellularly into trophozoites (uninucleate meronts) that undergo asexual multiplication by nuclear division leaving behind type I and type II meronts. The merozoite stages leave from inside the type I meront to infect other host cells and develop into uninucleate meronts, producing more merozoites. Merozoites from type II meronts initiate sexual multiplication as they differentiate into either male microgamonts or female macrogamonts. Fertil-

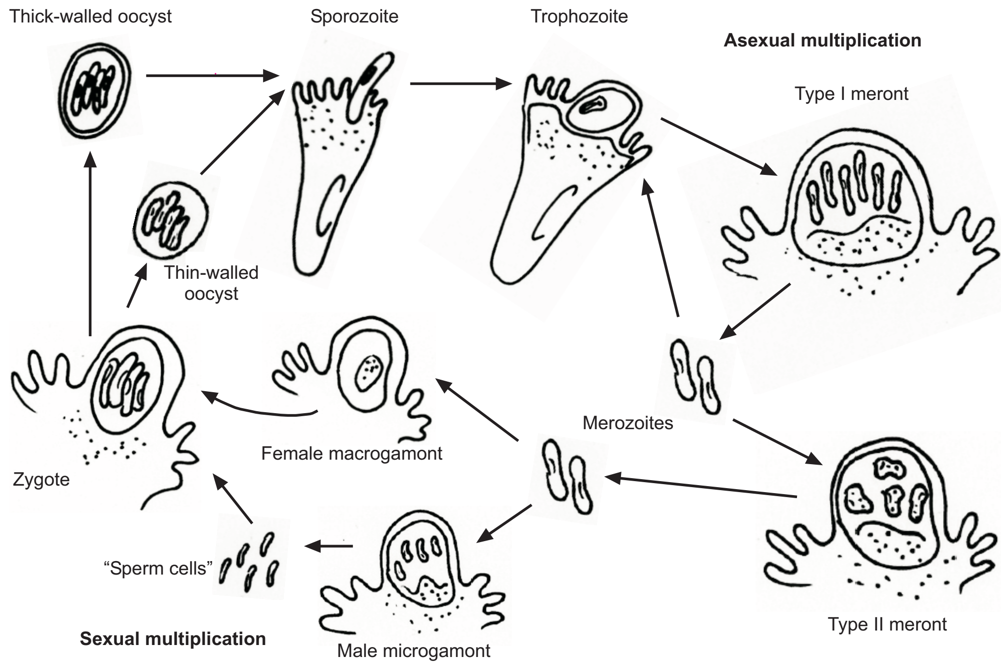

Figure 2. Life cycle of *Cryptosporidium*.

ized macrogamonts develop into thick-walled oocysts, which are excreted from the body, or into thin-walled oocysts responsible for repeated asexual and sexual multiplication (autoinfection) within the same host.

6.1.4 Human cryptosporidiosis

Cryptosporidial infections can be transmitted to a person directly from another person (Baxby *et al.*, 1983; Koch *et al.*, 1985; McNabb *et al.*, 1985; Addiss *et al.*, 1991b), an animal (Anderson *et al.*, 1982a; Current *et al.*, 1983; Tzipori *et al.*, 1983; Rahaman *et al.*, 1984; Pohjola *et al.*, 1986a; Pohjola *et al.*, 1986b) or through the environment via contaminated water (D'Antonio *et al.*, 1985; Mac Kenzie *et al.*, 1994; Atherton *et al.*, 1995; Fig. 3). The infection results from oral ingestion of as low a dose as approximately 30 infectious oocysts (DuPont *et al.*, 1995) invading the microvillous border of epithelial cells in the intestines (Current *et al.*, 1983; Current *et al.*, 1986). The infectivity, however, is affected by the type of infecting isolate and the immune status of the host (Teunis *et al.*, 2002b; Teunis *et al.*, 2002a). The type I merozoite cycle and capability for autoinfecting host cells probably connive in the low infectious dose as the infective forms multiply inside the host. The reported incubation periods range from 3 to 22 days, for an average of 1 week (Jokipii *et al.*, 1983; Jokipii *et al.*, 1985a; DuPont *et al.*, 1995). Cryptosporidiosis more often affects children < 3 years of age and is a cause of death in immunocompetent infants < 2 years of age in the developing countries (Molbak *et al.*, 1993; Xiao *et al.*, 2001). However, in immunocompetent individuals the infection usually results in a self-limited gastrointestinal illness, with diarrhoea as the main symptom. Other major symptoms include abdominal cramps and nausea. Fever, vomiting, headache and

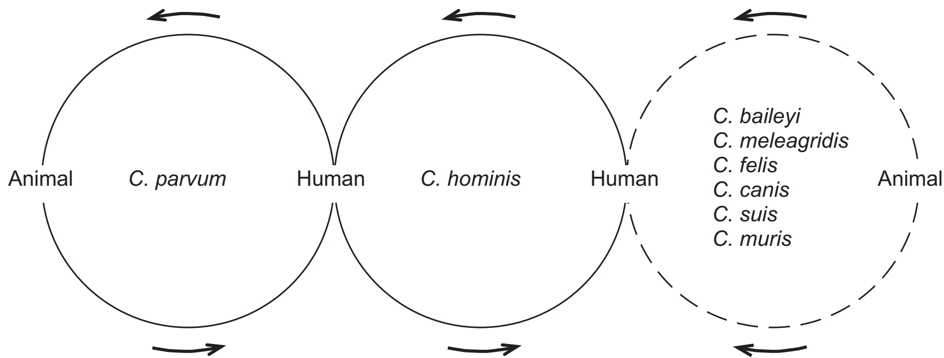

Figure 3. Transmission cycles of human *Cryptosporidium* infections. Dotted cycle demonstrates rare transmission.

growth retardation as well as asymptomatic infections have also been reported (Current *et al.*, 1983; Jokipii *et al.*, 1983; Jokipii *et al.*, 1985a; DuPont *et al.*, 1995; Checkley *et al.*, 1998). The duration of gastrointestinal illness ranges from 1 to 10 days (Current *et al.*, 1983; Jokipii *et al.*, 1983). Infections caused by *C. hominis* result in longer duration of oocyst shedding (mean 13.9 days vs. 6.4 days) and higher excreted oocyst quantities compared with infections caused by *C. parvum* (McLauchlin *et al.*, 1999; Xiao *et al.*, 2001). An infected person can excrete $>10^5$ oocysts/g of faeces (McLauchlin *et al.*, 1999). However, no differences in the clinical pictures between *C. hominis* and zoonotic infections were detected (Xiao *et al.*, 2001). In immunocompromised patients, cryptosporidiosis causes chronic, fulminant, transient or relapsing diarrhoea, which has been even associated with mortality (Koch *et al.*, 1985; Manabe *et al.*, 1998). The infection may spread throughout the alimentary canal and other mucosal surfaces, including the stomach and biliary, urinary or respiratory tracts (Casemore *et al.*, 1985; Harari *et al.*, 1986; Ditrich *et al.*, 1991; Clemente *et al.*, 2000; Megremis *et al.*, 2004). A broad-spectrum antiparasitic salicylamide derivative of nitrothiazole, nitazoxanide, reduces the duration of diarrhoea and oocyst shedding in both immunocompetent and immunocompromised patients (Rossignol *et al.*, 1998; Rossignol *et al.*, 2001; Amadi *et al.*, 2002). However, higher doses and longer duration of therapy may be needed for HIV-positive malnourished children to benefit from the drug (Amadi *et al.*, 2002). Before approval of nitazoxanide in November 2002, no drug for treatment of cryptosporidiosis had been approved. Antiretroviral therapy that improves the patient's immune response induces self-cure of cryptosporidiosis or, in some cases, protects against it (Manabe *et al.*, 1998; Megremis *et al.*, 2004).

6.2 *Giardia* and giardiasis

6.2.1 Classification and taxonomy

The genus *Giardia* belongs to the phylum Sarcomastigophora and is classified as Zoomastigophorea in the order Diplomonadida (Levine *et al.*, 1980). Several species of *Giardia* have been described, based on host occurrence. In the 1950s, as doubts over validity of the host specificity arose, Filice proposed criteria based mainly on trophozoite morphology to re-evaluate *Giardia* species (Filice, 1952). *Giardia duodenalis* (also referred to as *G. intestinalis* or *G. lamblia*), which is hosted by mammals is to date the only species found in humans. The use of multiple names reflects the current confusion in the taxonomy. In this review

Table 3. Assemblages and subgroups of *Giardia duodenalis*.

Assemblage	Subgroup	Host range[1]
A[2]	AI	Human, cat, dog
	AII	Human
	AIII	Cat
	AIV	Cat, guinea pig, alpaca
B[3]	BI	Monkey, dog
	BII	Monkey
	BIII	Human
	BIV	Human, cat, dog
C		Dog, cat
D		Dog, cat
E		Cattle, sheep, pig, cat
F		Cat
G		Rat

[1]according to Monis *et al.*, 2003 and Read *et al.*, 2004
[2]also known as Polish type or groups ½
[3]also known as Belgian type or group 3

of the literature, the name *G. duodenalis* as proposed by Filice and Thompson *et al.* will be used (Filice, 1952; Thompson *et al.*, 2000). Molecular analysis has made genetic characterization of *G. duodenalis* isolates possible, showing *G. duodenalis* as a complex of morphologically indistinguishable genotypes (assemblages) rather than a uniform species (Table 3). To date, the assemblages A and B (also called Polish and Belgian types or genetic groups 1/2 and 3) appear to be responsible for all human infections (Homan *et al.*, 1998; Monis *et al.*, 2003a; Read *et al.*, 2004) and can be further grouped into distinct clusters, AI-AIV and BI-BIV, by allozyme analysis (Monis *et al.*, 2003a). The information available suggests that clusters AI, AII, BIII and BIV may be zoonotic in character, whereas clusters AIII, AIV, BI and BII have been recovered from various animals so far (Monis *et al.*, 2003a; Read *et al.*, 2004; Traub *et al.*, 2004; Traub *et al.*, 2005).

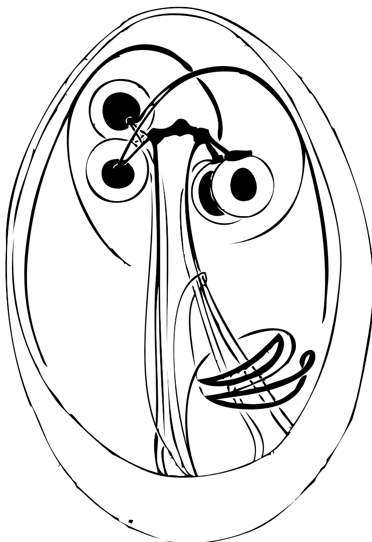

Figure 4. *Giardia* cyst (Filice, 1952).

6.2.2 Morphology of the cyst

In 1952, F.P. Filice wrote: "The descriptions of *Giardia* in the literature are so varied that one would almost suspect that every author discussed a different species." The most common stage of cysts seen in the faeces consists of four nuclei at the narrow end of the cyst and the thick portions of sucking-disc fibrils connected with the flagella in the posterior end. The cyst is surrounded by a rigid cyst wall (Filice, 1952; Fig. 4). The cysts have an oval appearance and size criteria varying from 5 to 20 µm (Jokipii, 1971; Petersen, 1972; Rose *et al.*, 1989; Jakubowski *et al.*, 1991; LeChevallier *et al.*, 1991; Payment *et al.*, 1993; Ortega *et al.*, 1997). Interestingly, in many studies based on microscopy, the morphologic criteria of *Giardia* were not described.

6.2.3 Life cycle

The life cycle of *Giardia* is noninvasive and simpler than that of *Cryptosporidium*. It includes developmental processes of excystation and encystation (Fig. 5). Exposure to gastric acids leads to excystation of the ingested cyst. The excyzoite form divides rapidly, forming four trophozoites (Bernander *et al.*, 2001) that infect the small intestine (Filice, 1952). During the vegetative stage, the trophozoite genome cycles between tetraploidy (4N) and octaploidy (8N) as a result of division of the cell and nucleus

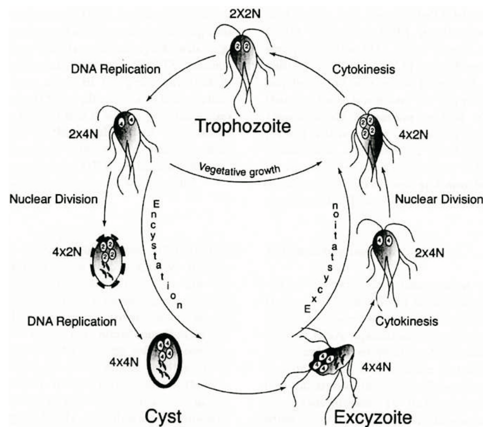

Figure 5. Life cycle of *Giardia* (Svärd *et al.*, 2003). Permission obtained from Elsevier.

(Bernander *et al.*, 2001). On their way through the intestine, some of the trophozoites encyst. Encysting trophozoites become stiff, rounded and unable to attach to the intestinal epithelium (Filice, 1952; Svärd *et al.*, 2003). A repeated DNA replication without intervening cell division (endoreplication) occurs resulting in a cyst with four nuclei covered by the cyst wall. During episodes of diarrhoea, the trophozoites may be excreted with the intestinal contents, but the actual exogenous stage is the cyst that is excreted in the faeces.

6.2.4 Human giardiasis

Person-to-person transmission of *Giardia* has been well documented, especially among children and staff members in day care centres (Sterner *et al.*, 1971; White *et al.*, 1989; Addiss *et al.*, 1991a; Tjernström *et al.*, 1992; Hoque *et al.*, 2001; Linnane *et al.*, 2001). *Giardia* can be transmitted to a person through the environment, mainly via contaminated water (Moore *et al.*, 1969; Ljungström *et al.*, 1992; Fraser *et al.*, 2000; Linnane *et al.*, 2001). Although *Giardia* has been considered as a zoonotic parasite for over 20 years, direct evidence of its zoonotic potential is lacking (Thompson, 2004). However, there is some recent evidence of both human, canine, feline and equine infections with *G. duodenalis* assemblages A and B, clusters AI, AII, BIII and BIV (Monis *et al.*, 2003a; Read *et al.*, 2004; Traub *et al.*, 2004; Traub *et al.*, 2005) that may support the zoonotic character of *G. duodenalis*. *Giardia* infection may result from ingestion of 10-100 cysts (Rendtorff, 1954). One reason for the low infectious dose may be the division of one cyst into four trophozoites capable of colonizing the intestinal wall. Incubation periods (the time from infection to symptoms) of 3-20 days (average 1 week) have been reported (Jokipii *et al.*, 1974; Jokipii *et al.*, 1977; Jokipii *et al.*, 1985a; Nash *et al.*, 1987). The prepatent period (the time from ingesting the cyst to cyst excretion) ranges from 1 to 3 weeks (Rendtorff, 1954; Jokipii *et al.*, 1977; Nash *et al.*, 1987). An infected individual can excrete nearly 10^7cysts/g of faeces and approximately 10^{10}cysts/day (Porter, 1916; Rendtorff, 1954; Danciger *et al.*, 1975). The excretion of cysts can be intermittent (Rendtorff, 1954; Danciger *et al.*, 1975). The clinical picture of giardiasis varies, ranging from asymptomatic infection or acute self-limited diarrhoea to severe, chronic diarrhoea associated with abdominal pain, nausea, flatulence, headache, fever, foul-smelling stools, malabsorption or loss in weight (Porter, 1916; Rendtorff, 1954; Moore *et al.*, 1969; Jokipii, 1971; Sterner *et al.*, 1971; Jokipii *et al.*, 1974; Danciger *et al.*, 1975; Nash *et al.*, 1987; Fraser *et al.*, 2000; Homan *et al.*, 2001; Read *et al.*, 2002). *Giardia* infection is common in children younger than 5 years of age living in an environment of compromised hygiene (Meloni *et al.*, 1993). In such children, growth retardation and impaired cognitive function associated with giardiasis were reported (Fraser *et al.*, 2000; Berkman *et al.*, 2002). The duration of relapsing gastrointestinal illness can be as long as 10 months and asymptomatic carriers can excrete cysts for years (Jokipii *et al.*, 1974). There is some evidence of a difference in clinical symptoms between in-

fections caused by *G. duodenalis* assemblage A and B even though contrasting findings have been reported. In a study undertaken in the Netherlands, assemblage A was associated with mild, intermittent diarrhoea, whereas assemblage B caused profound diarrhoea with weight loss and fatigue (Homan *et al.*, 2001). A study from Australia showed a correlation between asymptomatic cases and assemblage B infection. Children infected with assemblage A were more likely to have diarrhoea than those infected with assemblage B (Read *et al.*, 2002). Future studies with defined identification of species and assemblages will hopefully reveal if differences in symptoms between assemblages exist. Metronidazole, the traditional treatment for giardiasis, has been reported to be ineffective in about one-third of the cases (Sterner *et al.*, 1971; Petersen, 1972). Nitazoxanide, however, is reserved for patients who fail metronidazole treatment or for small children who do not adhere to metronidazole due to the length of the therapy or the bad taste of the medicine (Bailey *et al.*, 2004).

6.3 Significance of *Cryptosporidium* and *Giardia* as environmental pathogens

Cryptosporidium oocysts and *Giardia* cysts are excreted into the environment in the faeces of infe-cted individuals. Since the number of excreted (oo)cysts is high and the time of excretion long, the incidence of cryptosporidiosis and giardiasis in human and animal populations is an important factor contributing to transmission through the environment (Fig. 6). Sewage effluent from wastewater treatment plants (WTPs), pasture runoff or spreading of contaminated sludge or manure on agricultural land are the main sources of water system pollution that may enable the (oo)cysts to circulate in the ecosystem, leading to threats to human and animal health.

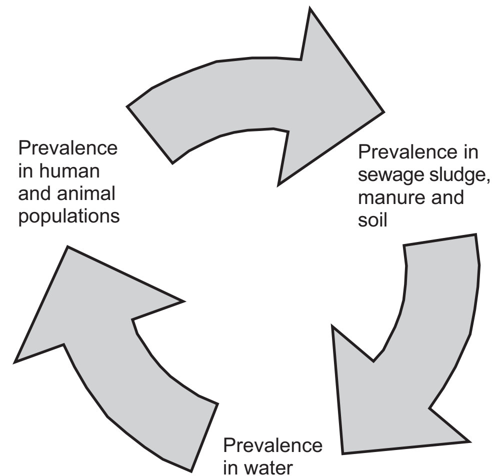

Figure 6. Environmental cycle of *Cryptosporidium* and *Giardia*.

6.3.1 Prevalence of cryptosporidiosis and giardiasis in human populations

Cryptosporidium and *Giardia* share worldwide distribution as human enteric pathogens and are among the most commonly identified human pathogenic parasites (Kyrönseppä *et al.*, 1976; Meloni *et al.*, 1993; O'Donoghue, 1995; Casemore *et al.*, 1997; de Wit *et al.*, 2001a; de Wit *et al.*, 2001b; Hoque *et al.*, 2001; Monis *et al.*, 2003b). In a study conducted in the Netherlands however, *Giardia* was detected in a similar percentage (5%) in gastroenteritis patients and controls (de Wit *et al.*, 2001c). In studies on faecal occurrence of (oo)cysts the prevalences vary, reflecting the study design (e.g. asymptomatic vs. symptomatic cases) and the socioeconomic conditions in the societies where surveys have been conducted. Prevalence is also highly dependent on the diagnostic methods used as well as the proficiency of the personnel performing the laboratory analyses. These factors complicate comparison of the available data, however, O'Donoghue (1995) collected data on human *Cryptosporidium* prevalence based on coprological surveys. The results indicate that *Cryptosporidium* infection is less

prevalent in the industrialized countries (prevalence 0.1-27.1% in diarrhoeic patients; 0-2% in asymptomatic individuals) compared with the developing countries (0.1-31.5%; 0-9.8%). Using meta-analysis, the prevalence of *Cryptosporidium* in asymptomatic individuals was estimated to be 1% and in symptomatic patients 3% in the Nordic countries (Hörman *et al.*, 2004), while *Giardia* prevalence was estimated to be 3% in asymptomatic and 6% in symptomatic persons. In the industrialized countries, *Giardia* prevalence varies from 2% to 5% and has been reported to range from 20% to 30% in the developing countries (Sterner *et al.*, 1971; Petersen, 1972; Marshall *et al.*, 1997; Ortega *et al.*, 1997). The higher prevalence in the developing countries is probably associated with the vicious cycle of poor sanitation and contaminated water supplies.

The first study of *Giardia* incidence in Finland was carried out during 1925-1928 (Svensson, 1928). Using iodine-eosin and iron-alum-haematoxylin staining, *Giardia* was found in 4.6% (n = 2014) hospital patients in Finland and Sweden. Forty years later, *Giardia* was detected in 19% of 124 patients with diarrhoea and in 11% of asymptomatic controls (n = 72), as well as in 8.6% of 139 asymptomatic persons when formalin-ether concentration and iodine staining was used (Jokipii, 1971; Jokipii *et al.*, 1974). Furthermore, *Giardia* was the most common pathogenic parasite detected among asymptomatic persons in an institution for the mentally retarded, hospital patients, military servicemen and prisoners (Kyrönseppä *et al.*, 1976). During the 1980s, the prevalence of *Giardia* in hospital patients without gastrointestinal symptoms and healthy persons was about 1% (Kyrönseppä, 1993). In 4545 patients with gastrointestinal symptoms, the prevalence of *Cryptosporidium* was 2.6% and *Giardia* 2.7% (Jokipii *et al.*, 1985a). However, in small intestinal biopsy *Giardia* trophozoites could not be found from 100 patients with gastrointestinal symptoms (Björknäs *et al.*, 1995). Although cryptosporidiosis is known to be a disease of young children (Molbak *et al.*, 1993; Checkley *et al.*, 1998; de Wit *et al.*, 2001c), no oocysts were detected when 423 healthy children < 8 years of age were studied (Vuorio *et al.*, 1991), even if most of the children in this study (322/423) were < 3 years of age. Two asymptomatic cryptosporidiosis cases were reported in children < 1 year of age in Finland (Pohjola *et al.*, 1986a; Lähdevirta *et al.*, 1987). In asymptomatic children < 15 years of age, *Giardia* was detected at prevalences varying around 1-2% (Kyrönseppä *et al.*, 1976; Kyrönseppä, 1993). The prevalence studies showed that giardiasis was considered as an endemic disease in Finland, whereas cryptosporidiosis was regarded mainly as an agent of travellers' diarrhoea (Jokipii, 1971; Jokipii *et al.*, 1974; Jokipii *et al.*, 1983; Jokipii *et al.*, 1985a; Jokipii *et al.*, 1985b).

6.3.2 Prevalence of cryptosporidiosis and giardiasis in calves

In the 1970s cryptosporidiosis was considered merely as the cause of neonatal calf diarrhoea (Tzipori *et al.*, 1980; Anderson, 1981; Anderson *et al.*, 1982b), but since the 1980s calves have been regarded as a reservoir for zoonotic *Cryptosporidium* (Anderson *et al.*, 1982a; Current *et al.*, 1983; Rahaman *et al.*, 1984; Pohjola *et al.*, 1986a; Pohjola *et al.*, 1986b). In studies conducted in the Netherlands, Canada and Sweden, *Cryptosporidium* spp. or *C. parvum* and *G. duodenalis* were frequently found in both asymptomatic (*Cryptosporidium* prevalence 5%, *Giardia* 23-34%) and diarrhoetic (*Cryptosporidium* 11-38%, *Giardia* 7-29%) veal (Appelbee *et al.*, 2003) or dairy (Huetink *et al.*, 2001; Björkman *et al.*, 2003) calves younger than 5 months of age. In preweaned dairy calves 1-7 weeks of age, the prevalence of *G. duodenalis* ranged 9-93% (mean 40%) on 14 farms studied in the USA (Trout

et al., 2004). In Australia, *C. parvum* was detected in 48% and *Giardia* in 89% of calves < 3 months of age studied on two dairy farms (Becher *et al.*, 2004). Livestock-specific assemblage E of *G. duodenalis* appears to be the most frequent genotype in calves (Appelbee *et al.*, 2003; Monis *et al.*, 2003a; Becher *et al.*, 2004; Berrilli *et al.*, 2004; Read *et al.*, 2004), but the potentially zoonotic assemblage A has also been found (O'Handley *et al.*, 2000; van Keulen *et al.*, 2002; Trout *et al.*, 2004).

6.3.3 Prevalence of cryptosporidiosis and giardiasis in dogs

Pets live in close association with humans, especially small children who come easily in contact with them or their droppings in the environment. The first cryptosporidial infections in dogs were reported in the early 1980s (Tzipori *et al.*, 1981; Wilson *et al.*, 1983) and already in the late 1980s questions about the possible zoonotic nature of canine cryptosporidiosis as well as giardiasis arose (Simpson *et al.*, 1988; Sykes *et al.*, 1989; Winsland *et al.*, 1989; Rahman, 1990). However, to date there is still insufficient information to determine the role of pets in zoonotic transmission (Monis *et al.*, 2003b). As is the case in human prevalence studies, the variety of study designs and detection techniques makes the comparison of *Cryptosporidium* and *Giardia* prevalence in dogs troublesome. Marked variation in occurrence of (oo)cysts may occur in canine faeces. Thus, *Cryptosporidium* has been detected with a prevalence range of up to 45% in dogs (Jokipii *et al.*, 1985a; Simpson *et al.*, 1988; Bugg *et al.*, 1999; Fayer *et al.*, 2001; Abe *et al.*, 2002; Hackett *et al.*, 2003). In three studies, no oocysts were found in 579 dogs from Finland, Scotland and Australia (Jokipii *et al.*, 1985a; Simpson *et al.*, 1988; Bugg *et al.*, 1999). The prevalence of *Giardia* in dogs varies with a range of up to 51% (Hahn *et al.*, 1988; Haralabidis *et al.*, 1988; Simpson *et al.*, 1988; Sykes *et al.*, 1989; Winsland *et al.*, 1989; Castor *et al.*, 1990; Rahman, 1990; Meloni *et al.*, 1993; Nolan *et al.*, 1995; Diaz *et al.*, 1996; Bugg *et al.*, 1999; Jacobs *et al.*, 2001; Oliveira-Sequeira *et al.*, 2002; Capelli *et al.*, 2003; Hackett *et al.*, 2003; Traub *et al.*, 2004; Ponce-Macotela *et al.*, 2005), and the infection was reported to be more prevalent in dogs < 1 year of age (Hahn *et al.*, 1988; Sykes *et al.*, 1989; Castor *et al.*, 1990; Rahman, 1990; Bugg *et al.*, 1999; Jacobs *et al.*, 2001). No difference in prevalence between age-groups < 3 years and > 3 years was detected (Winsland *et al.*, 1989). Although most of the prevalence studies were conducted with asymptomatic individuals, no marked difference was seen in the occurrence of (oo)cysts in the faeces between diarrhoetic and healthy dogs (Sykes *et al.*, 1989; Winsland *et al.*, 1989; Jacobs *et al.*, 2001; Hackett *et al.*, 2003). Prevalence studies have shown that *Cryptosporidium* and *Giardia* are common findings in symptom-free dogs, suggesting that canine cryptosporidiosis and giardiasis may be asymptomatic infections rather than frequent causes of enteric disease. However, more detailed case-control studies are needed before any conclusions could be made. Furthermore, since most of the studies on *Cryptosporidium* and *Giardia* in dogs were conducted before the advent of molecular tools, thus relying only on microscopic identification, more information on the species or genotypes of *Cryptosporidium* and *Giardia* detected in dogs would be needed as well. Studies in which species or genotype was identified show that dogs may harbour *C. parvum* and zoonotic assemblages of *G. duodenalis* (assemblages A and B) in addition to their own species (*C. canis*) and assemblages C and D (Fayer *et al.*, 2001; Ponce-Macotela *et al.*, 2002; van Keulen *et al.*, 2002; Abe *et al.*, 2003; Monis *et al.*, 2003a; Berrilli *et al.*, 2004; Hajdusek *et al.*, 2004; Traub *et al.*, 2004; Eligio-Garcia *et al.*, 2005).

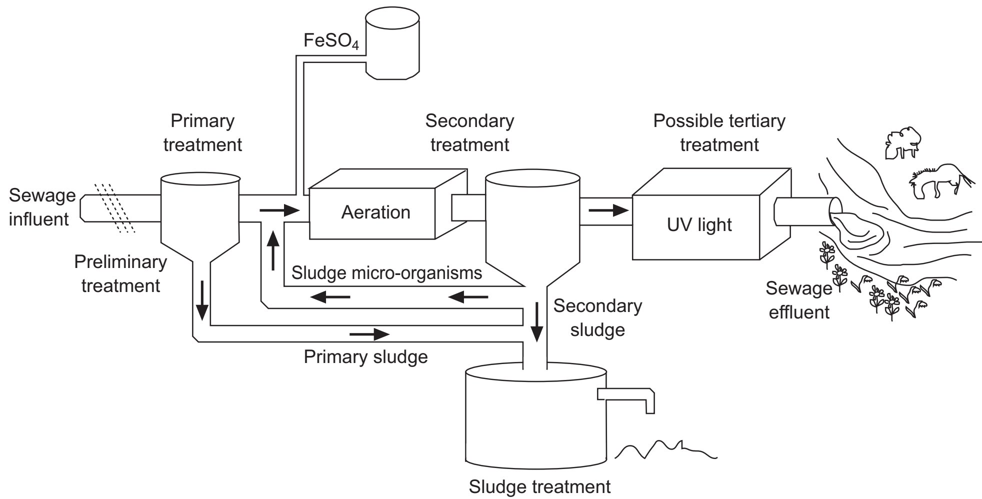

Figure 7. Multistage wastewater treatment process.

6.3.4 Occurrence in the environment and factors affecting survival of (oo)cysts

Wastewater treatment is a multistage process for renovating sewage before it reenters the water system (Fig. 7). Conventional treatment used in the industrialized countries has not been focused on the reduction of pathogens, but on removal of organic substances needing oxygen for decomposition (Carr, 2001). During preliminary treatment, large particles are removed from the incoming wastewater (sewage influent). Primary treatment separates the suspended solids and grease from the influent. The clarified wastewater flows on to the secondary treatment, during which phosphorus is precipitated from the wastewater by adding $FeSO_4$ and dissolved organic matter is removed by micro-organisms. Tertiary treatment would be needed to reduce the level of pathogens in the outgoing wastewater (sewage effluent). Thus, effluent could be treated by filtration, chlorination or by using ultraviolet (UV) light. The tertiary treatment adds to the cost of wastewater treatment process and may therefore be unsuitable for adoption in many countries (Carr, 2001).

Variable numbers of (oo)cysts have been reported both in sewage influent and effluent probably reflecting the variation in detection techniques used (Madore *et al.*, 1987; Jakubowski *et al.*, 1991; Robertson *et al.*, 1995; Bukhari *et al.*, 1997; Chauret *et al.*, 1999; Robertson *et al.*, 2000; Heitman *et al.*, 2002). These studies have shown that *Cryptosporidium* and *Giardia* are frequently detected in sewage. Furthermore, cysts appear to be a more prevalent finding compared with oocysts (Robertson *et al.*, 1995; Bukhari *et al.*, 1997; Chauret *et al.*, 1999; Robertson *et al.*, 2000; Heitman *et al.*, 2002). For example, more cysts (mean range 2809-16 429/l of influent; 7-8386/l of effluent) than oocysts (mean range 0-143/l of influent; 0-343/l of effluent) were detected in WTPs from Scotland (Robertson *et al.*, 2000). Similarly, in England 10-170 oocysts and 10-13 600 cysts/l of influent, and 10-60 oocysts and 10-720 cysts/l of effluent were detected (Bukhari *et al.*, 1997). It is noteworthy that in areas where water supplies are limited, even raw sewage is used in irrigation of food crops (Amahmid *et al.*, 1999).

Sewage sludge is a by-product of wastewater treatment containing a mixture of

organic and inorganic compounds (Fig. 7). The primary sludge settles out during primary treatment, while the secondary sludge consists of microorganisms from the secondary treatment (Carr, 2001). During wastewater treatment, *Cryptosporidium* oocysts and *Giardia* cysts are sedimented into the raw sludge, at concentrations of 10^5-10^7 (oo)cysts/kg (Straub *et al.*, 1993; Thiriat *et al.*, 1997; Gale, 2005). The goal of sludge treatment is to reduce the sludge volume, odour and level of human, animal and plant pathogens. After treatment, the sludge can be spread on fields, returning the organic matter and nutrients to the soil. Land application of sludge may, however, allow the (oo)cysts to cycle in the ecosystem (Straub *et al.*, 1993; Gale, 2005; Fig. 6). Models for risk assessment and risk management have been developed for pathogens in wastewater and sewage sludge and applied for identifying and controlling the risk of *Cryptosporidium* and *Giardia* (Westrell *et al.*, 2004; Gale, 2005). The risk of *Cryptosporidium* and *Giardia* infection to humans from consumption of vegetable crops harvested from agricultural lands on which treated sewage sludge had been applied was determined by Gale (2005). Using a computer simulation, he assessed the risk from vegetable crop consumption to be 50 *Giardia* infections per year and one *Cryptosporidium* infection every second year in the UK (a population of 60 million persons). As Gale stated, "the problem with quoting quantitative predicted risks is that the degree of uncertainty is quickly forgotten" and the experiment may easily turn into "uncertainty in the uncertainty". During extrapolation, any uncertainty in the experimental data is multiplied. In the case of *Cryptosporidium* and *Giardia*, there is still not enough understanding of the source and pathway of infection, which was needed to be taken into account in Gale´s study.

Surface water is contaminated by faecal (oo)cysts mainly through sewage effluents, manure discharge or pasture runoff (Madore *et al.*, 1987; LeChevallier *et al.*, 1991; Richardson *et al.*, 1991; Atherton *et al.*, 1995; Smith *et al.*, 1995; Hansen *et al.*, 1998; Robertson *et al.*, 2001; Fig. 6). Wildlife may also play a role as a source of surface water contamination. *Cryptosporidium* and *Giardia* occur widely in wildlife, but the wild animals harbour host-specific species and genotypes that are not, to date, considered as a significant risk for human health (Appelbee *et al.*, 2005; Polley, 2005).

Cryptosporidium oocysts and *Giardia* cysts are common findings in surface water in the USA as well as in Europe (Ongerth *et al.*, 1987; LeChevallier *et al.*, 1991; Smith *et al.*, 1995; Hansen *et al.*, 1998; Smith *et al.*, 1998; Robertson *et al.*, 2001). In 26 surface water systems from Sweden, *Cryptosporidium* was found in 32% (16/50) and *Giardia* in 26% (13/26) of the samples studied (Hansen *et al.*, 1998). In Norway, *Cryptosporidium* was found in 13.6% (20/147), *Giardia* in 7.5% (11/147) and both in 11% (16/147) of the surface water systems studied (Robertson *et al.*, 2001). In the USA and UK, occurrences range between 4% and 100%, depending on the land-use activities near the system (Smith *et al.*, 1995; Rose *et al.*, 2002). Likewise, association between a large number of livestock animals near the water system and the presence of *Cryptosporidium* and *Giardia* was detected in the Norwegian study (Robertson *et al.*, 2001). Similarly, in agricultural fields to which the cattle had access, *Cryptosporidium* and *Giardia* were detected more often compared with sites at which the cattle were not allowed to pasture (Barwick *et al.*, 2003). At peak shedding, calves are known to excrete 10^9-10^{10} (oo)cysts/kg of faeces (Xiao *et al.*, 1994; Fayer *et al.*, 1998; O'Handley *et al.*, 1999), a load potentially able to contaminate the environment effectively. Although cattle have often been implicated as a source of waterborne cryptosporidiosis outbreaks, recent genotyping results have in many cases demonstrated sewage of human origin being the real source of water pollution (Caccio *et al.*, 2005).

The concentrations of (oo)cysts in surface water are usually low, ranging from 1 to 18 (oo)cysts/10 l (Smith *et al.*, 1995; Hansen *et al.*, 1998; Robertson *et al.*, 2001; Hänninen *et al.*, 2005). In the Netherlands, higher concentrations of (oo)cysts in surface water has been detected. The average concentration, corrected with the average recovery efficiency, was 4.5 and 5.4 oocysts/l and 22 and 95 cysts/l in rivers Rhine and Meuse, respectively (Medema *et al.*, 2001). In the USA, concentrations as high as 5 800 oocysts and 241 cysts/l of surface water have occasionally been reported (Madore *et al.*, 1987; LeChevallier *et al.*, 1991; Smith *et al.*, 1995).

The resistance of *Cryptosporidium* and *Giardia* towards environmental conditions have been well described in several papers (deRegnier *et al.*, 1989; Robertson *et al.*, 1992; Whitmore *et al.*, 1995; Robertson *et al.*, 2004). Thus, *Cryptosporidium* oocysts can survive in cattle faeces at temperature of 4ºC and in river water for nearly 6 months (Robertson *et al.*, 1992), while *Giardia* cysts survived for almost 2 months at 0-2 ºC in river water (deRegnier *et al.*, 1989). When cyst viability was compared with water quality parameters such as turbidity, temperature and pH, only temperatures < 10ºC prolonged the survival of *Giardia* cysts in surface water (deRegnier *et al.*, 1989). Furthermore, a proportion of oocysts was capable of surviving for at least 1 month at -22 ºC, although fast-freezing in liquid nitrogen killed them effectively (Robertson *et al.*, 1992). In sewage sludge treated with mesophilic anaerobic digestion, the oocysts remained viable for at least 18 days, and in sludge-treated soil for over a month (Whitmore *et al.*, 1995). However, under the environmental conditions encountered in Norway, the (oo)cysts were not able to survive over winter in soil (Robertson *et al.*, 2004). Laboratory studies and the lessons learned from waterborne outbreaks have shown that (oo)cysts may survive both physical and chemical water treatment processes (Robertson *et al.*, 1992; Mac Kenzie *et al.*, 1994; Smith *et al.*, 1995; Nygård *et al.*, 2004).

6.3.5 Waterborne outbreaks caused by Cryptosporidium and Giardia

Numerous waterborne outbreaks of cryptosporidiosis and giardiasis have caused public health concern and economic losses during recent years (e.g. (Moore *et al.*, 1969; D'Antonio *et al.*, 1985; Richardson *et al.*, 1991; Ljungström *et al.*, 1992; Mac Kenzie *et al.*, 1994; Atherton *et al.*, 1995; Mac Kenzie *et al.*, 1995; Rose *et al.*, 2002). Faecal contamination of potable water by sewage is a common cause of waterborne outbreaks (Moore *et al.*, 1969; D'Antonio *et al.*, 1985; Neringer *et al.*, 1987; Richardson *et al.*, 1991; Ljungström *et al.*, 1992). Agricultural runoffs, especially after heavy rainfalls, were also suspected of causing outbreaks (Mac Kenzie *et al.*, 1994; Atherton *et al.*, 1995; Goh *et al.*, 2004). In some giardiasis outbreaks, infected beavers were claimed to have been the source of water system contamination (Isaac-Renton *et al.*, 1993; McIntyre *et al.*, 2000). Failures or deficiencies in waterworks management have also played a role in outbreaks (Shaw *et al.*, 1977; Neringer *et al.*, 1987; Hayes *et al.*, 1989; Mac Kenzie *et al.*, 1994; Nygård *et al.*, 2004).

The largest outbreak of cryptosporidiosis occurred in Milwaukee, WI, USA in 1993, affecting over 400 000 people (Mac Kenzie *et al.*, 1994; MacKenzie *et al.*, 1995). Human sewage and agricultural runoff after unusually heavy rains together with failures in waterworks management were suspected of causing the outbreak. The total cost of the Milwaukee outbreak was estimated to have been nearly 100 million dollars (Corso *et al.*, 2003). In Nordic countries, the reported waterborne outbreaks of giardiasis or cryptosporidiosis appear to be rare (Neringer *et al.*, 1987; Ljungström *et al.*, 1992; Nygård *et al.*, 2004). However, to-

gether with campylobacters, *Giardia* has been the most commonly identified agent of waterborne outbreaks in Sweden (Anderssson *et al.*, 2001). In 1982, an outbreak of giardiasis affected a small community of 600 inhabitants in Blekinge, Sweden after contamination of municipal water with sewage (Neringer *et al.*, 1987). A recent outbreak of giardiasis occurred in Bergen, Norway in autumn 2004 (Nygård *et al.*, 2004). Samples taken from the water supply confirmed the presence of cysts in treated water, which together with interviews of infected individuals indicated that the public water supply was the source of the outbreak. An efficient procedure for removing *Giardia* from the source water was apparently lacking at the waterworks.

6.4 Detection of *Cryptosporidium* and *Giardia* in environmental and faecal samples

The detection procedure for *Cryptosporidium* and *Giardia* in environmental samples consists of three stages including concentration of (oo)cysts, separation of (oo)cysts from extraneous material and identification of *Cryptosporidium* and *Giardia*. The most procedures aimed for detection of *Cryptosporidium* and *Giardia* in surface water are based on the USEPA approved method 1623 (United States Environmental Protection Agency, 1999).

6.4.1 Concentration and separation

6.4.1.1 Surface water

In the 1980s and 1990s, large volumes (100-1000 l) of water were concentrated with yarn-wound cartridge filtration to recover *Cryptosporidium* oocysts and *Giardia* cysts (Nieminski *et al.*, 1995; Shepherd *et al.*, 1996). Recovery efficiencies (RE) in protocols using cartridge filtration were modest, ranging 8-9% for *Cryptosporidium* and 12-28% for *Giardia* at various (oo)cyst inoculation levels (Nieminski *et al.*, 1995; Hsu *et al.*, 2001a).

A wide range of recovery rates for protocols using membrane filtration techniques have been reported. However, comparison between the various protocols is difficult due to differences in water quality as well as the techniques and inoculation levels chosen by the authors. For example, recoveries of 30% for *Cryptosporidium* and 47% for *Giardia* at inoculation levels of 750-850 (oo)cysts/10 l were reported after filtration, centrifugation, pellet washes and IF microscopy (Shepherd *et al.*, 1996). At levels of 100-1000 oocysts/10 l, recoveries of 54-68% using pleated membrane and 68-81% using foam disc filtration for concentration and electrochemiluminescence assay for identification were obtained (Lee *et al.*, 2004). The protocols including IMS are based on the USEPA Method 1623 (United States Environmental Protection Agency, 1999). The mean REs for IMS-based protocols including concentration, separation and identification of *Cryptosporidium* and *Giardia* range from < 1% to 85% depending on the techniques and inoculation level used as well as the turbidity of the water sample (Table 4). Thus, Feng *et al.* (2003), using pleated membrane filtration, showed that the highest mean level of *Cryptosporidium* recovery (85%) was obtained at a turbidity of 5 nephelometric turbidity units (NTUs). In another study, the mean recoveries at a turbidity level of 99 NTUs were < 1% for *Giardia* and 36-37% for *Cryptosporidium*, when 100 (oo)cysts/10 l were inoculated (DiGiorgio *et al.*, 2002). The recoveries were ~ 50% for both *Giardia* and *Cryptosporidium* at a turbidity level of 11 NTUs. These data may suggest that a moderate level of turbidity enhances the recovery. Furthermore, the turbidity of the water sample also affects the filtration capacity: at a turbidity of 88 NTUs, pleated membrane devices were sometimes able to filter a mean of only 1.7 l of water, instead of the standard 10 l, without clogging (DiGiorgio *et al.*, 2002).

Table 4. Summary of IMS- and IF microscopy-based protocols for detection of *Cryptosporidium* and *Giardia* in surface water.

Spike/ volume filtered	No. of trials	Turbidity (NTU)	Concen- tration	Recovery efficiency, % (SE)		Reference
				Cryptosporidium	*Giardia*	
Pleated membrane						
100/10 l	3	11	1)	51 (0.02)[ECHV] 43 (0.01)[EC]	53 (0.05)[ECHV] 61 (0.06)[EC]	DiGiorgio *et al.*, 2002
100/10 l	3	99	1)	36 (0.02)[ECHV] 37 (0.05)[EC]	0.47 (0.00)[ECHV] 0.83 (0.01)[EC]	DiGiorgio *et al.*, 2002
100/10 l	8		2)	51 (4)	37 (3.8)	Wohlsen *et al.*, 2004
100/10 l	18	1-15	3)	25 (4.4)	41 (4.5)	Ferguson *et al.*, 2004
100-150/10 l	15	15	4)	15 (3.2)		Simmons *et al.*, 2001
2010/10 l	3	5	5)	85 (5.2)		Feng *et al.*, 2003
	3	20		50 (11.6)		Feng *et al.*, 2003
	3	40		25 (8.4)		Feng *et al.*, 2003
Flatbed membrane						
100/10 l	5		6)	3 (2.7)	8 (3.4)	Wohlsen *et al.*, 2004
100/10 l	18	1-15	7)	36 (6.4)	48 (3.5)	Ferguson *et al.*, 2004
Foam disc filtration						
100/10 l	8		8)	19 (2.2)	24 (3.5)	Wohlsen *et al.*, 2004
100/50 l	8		8)	41	50	McCuin *et al.*, 2003
100/10 l	17	1-15	9)	20 (2.3)	41 (4.7)	Ferguson *et al.*, 2004
Ultrafiltration						
100-150/10 l	15	15	10)	42 (7.2)		Simmons *et al.*, 2001
100/10 l	18	1-15	11)	26 (4.9)	23 (2.1)	Ferguson *et al.*, 2004

Description of concentration:

1) Filter through polyethersulphone (Envirochek standard, EC) or polyester track-etch (Envirochek high volume, ECHV) membrane material capsule, elute with up to 275 ml of Laureth12 buffer, centrifuge 1100 x g for 15 min.

2) Filter through ECHV, backwash for 5 s, elute with 3 x 100 ml PBS-Tween-Antifoam buffer, centrifuge 1100 x g for 15 min.

3) Filter through EC, elute with 2 x 125 ml of PBS-Tween-Antifoam buffer in wrist action shaker 3 x 5 min, centrifuge 3000 x g for 30 min.

4) Filter through EC, elute with up to 275 ml of Laureth12 buffer in wrist action shaker 2 x 5 min or in shaker platform 2 x 15 min, centrifuge 1164 x g for 20 min.

5) Filter through EC, elute with up to 275 ml of Laureth12 buffer, centrifuge 1500 x g for 15 min.

6) Filter through cellulose acetate flatbed membrane filter (pore size 1.2 µm, diameter 142 mm), elute with 3 x 20 ml of PBS-Tween80 and scrape the filter surface, centrifuge 1100 x g for 15 min.

7) Filter through track-etched polycarbonate flatbed membrane filter (pore size 2 µm, diameter 293 mm), 2-4 x elute the filter with tetrasodium pyrophosphate-Tween80 buffer, centrifuge 1620 x g for 10 min.

8) Filter through Filta-Max compressed foam disc filter (pore size 1 µm) and then through polysulphone flatbed membrane filter (pore size 3 µm), elute with 2 x 600 ml of PBS-Tween20 and manually knead the membrane twice together with 2 x 10 ml of PBS-Tween20, centrifuge 1500 x g for 15 min.

9) Filter through Filta-Max, and then through cellulose nitrate flatbed membrane filter (pore size 3 µm), elute with 5 ml of PBS-Tween20, centrifuge 1100 x g for 15 min.

10) Filter through polysulphone Hemoflow F80A ultrafilter designed for hemodialysis, elute with PBS-Laureth12 buffer, centrifuge 1164 x g for 20 min.

11) Filter through Hemoflow F80A, elute with PBS-Laureth12 buffer, centrifuge 3000 x g for 30 min.

To separate (oo)cysts from background debris, density gradient centrifugation has been used conventionally (Nieminski *et al.*, 1995; Hsu *et al.*, 2001a). The method is based on particle size, making it non-specific because extraneous material of the same size will be concentrated simultaneously. Using flatbed membrane filtration and Percoll-Percoll gradient centrifugation, oocyst recovery was 9%, whereas cyst recov-

ery was 49% at an inoculation level of 1000 (oo)cysts/40 l (Nieminski *et al.*, 1995). Recoveries were similar (16% for *Cryptosporidium* and 38% for *Giardia*) when flatbed membrane filtration and Percoll-sucrose flotation was used (Hsu *et al.*, 2001a). With Percoll-sucrose flotation and Percoll-Percoll gradient centrifugation, recoveries of 12% and 63% from river water were reported on as high an inoculation level as 7 x 10^5-10^6 oocysts/ml (Chesnot *et al.*, 2004). In any event, this level of contamination in surface water is hardly realistic.

Immunomagnetic separation (IMS) serves as an (oo)cyst-specific separation method since the paramagnetic beads are coated with antibodies against *Cryptosporidium* and *Giardia*. Using IMS, (oo)cysts have successfully been captured from environmental water samples (Hsu *et al.*, 2001b; McCuin *et al.*, 2001; Feng *et al.*, 2003). The recovery rates of commercial IMS kits vary to a large extent (Bukhari *et al.*, 1998; Rochelle *et al.*, 1999). Recoveries of 62-100% in high-turbidity (50-11 480 NTU) environmental samples have been reported using Dynal Company's (Oslo, Norway) IMS Kit (Rochelle *et al.*, 1999; Hsu *et al.*, 2001b; McCuin *et al.*, 2001).

6.4.1.2 Sewage sludge

Formalin-ethyl acetate concentration, commonly used to concentrate stool samples (Weber *et al.*, 1991), was tested on sewage sludge samples (Chesnot *et al.*, 2004). It, however, resulted in remarkable oocyst loss, and cannot be recommended for use on sewage sludge. To separate (oo)cysts from sewage sludge, Massanet-Nicolau (2003) presented a method based on (oo)cyst sedimentation in phosphate-buffered saline (PBS) and IMS. REs associated with sedimentation (40-60% for both *Cryptosporidium* and *Giardia*) were higher than those associated with sucrose flotation (< 10% for both *Cryptosporidium* and *Giardia*) on a mean inoculation level of 140 oocysts and 107 cysts/g. Purification by flotation or density gradient centrifugation has given recoveries of ~ 30% for *Cryptosporidium* at inoculation levels of 10^5-10^6 oocysts/ml, whereas by filtration mean recoveries of 74% were reported (Chesnot *et al.*, 2004). The flotation and density gradient centrifugation included washing steps lacking from the filtration technique, which could have affected the results. Likewise, Robertson *et al.* (2000) stated that concentration techniques requiring the least manipulation give the highest REs for *Cryptosporidium* and *Giardia* in sewage. To purify (oo)cysts from sewage sludge, IMS has also been used (Massanet-Nicolau, 2003; Iacovski *et al.*, 2004).

6.4.1.3 Faeces

Several techniques for concentrating (oo)cysts from faecal samples have been described, although in many of these the results are varying and cannot ensure recovery quantification. These techniques are known to result in considerable losses of oocysts (Casemore *et al.*, 1985; Weber *et al.*, 1991; Weber *et al.*, 1992). The concentration procedure, however, has improved (oo)cyst detection sensitivity compared with direct smears (Baughn *et al.*, 1971; Garcia *et al.*, 1983). In the study of Baughn and Morales (Baughn *et al.*, 1971) only 31% of the *Giardia*-positive samples were detected when direct smears were studied, compared with concentration by formalin-ether sedimentation, which increased the detection to 72%. Poor oocyst concentration was obtained in formalin-ethyl acetate sedimentation (Weber *et al.*, 1991; Weber *et al.*, 1992), however, no oocysts were detected, using sugar or zinc sulphate flotation (Weber *et al.*, 1992). Recoveries achieved by Weber *et al.* (1991) ranged from 2% to 7% when formalin-ethyl acetate concentration was used, depending on the number of oocysts inoculated into the formed faeces. Similarly, formalin-ether sedimentation was

superior to zinc sulphate flotation or sugar flotation in concentrating oocysts (Mtambo *et al.*, 1992). By contrast, compared with formalin sedimentation, sugar flotation was a more sensitive technique for concentrating *Cryptosporidium* oocysts (Garcia *et al.*, 1983). McNabb *et al.* (1985) found that sugar flotation and formalin-ethyl acetate sedimentation perform equally well for oocyst concentration. More cysts, however, were detected with formalin-ethyl acetate sedimentation. Furthermore, water-ether concentration recovered up to 75% of seeded *Cryptosporidium* oocysts, whereas zinc sulphate flotation and sugar flotation performed more weakly, with recoveries of 22-41% and 24-65%, respectively (Bukhari *et al.*, 1995). To detect *Giardia* cysts, the efficiencies of centrifugation combined with zinc sulphate flotation and formalin-ethyl acetate sedimentation were similar, since 7/143 and 6/143 samples positive for *Giardia* were detected, respectively (Oliveira-Sequeira *et al.*, 2002). Compared with traditional faecal concentration techniques, IMS was a more sensitive method for concentrating and purifying (oo)cysts from human and animal stool as detected by various identification techniques (Webster *et al.*, 1996; Pereira *et al.*, 1999; Power *et al.*, 2003; Souza *et al.*, 2003).

6.4.2 Identification

6.4.2.1 Immunofluorescence (IF) microscopy

Routine identification of environmental (oo)cysts on the genus level is based on morphometry and morphology by microscopy. In IF microscopy, a fluorophore-labelled antibody attaches to cell wall antigens of (oo)cysts. Thus, visualization of the shape and size of (oo)cysts is emphasized (Rose *et al.*, 1989). Both polyclonal (pAb) and monoclonal (mAb) antibodies have been used to identify (oo)cysts in water (Ongerth *et al.*, 1987; Rose *et al.*, 1989). The mAbs are believed to improve the sensitivity of identification technique because they are generated against a certain epitope of target organism (Rosoff *et al.*, 1989). Some anti-*Cryptosporidium* mAbs, however, cross-react with background debris of the sample or algae commonly found in surface waters, interfering with identification due to non-specific fluorescence (Rodgers *et al.*, 1995). Similarly, cross-reactivity of mAbs with faecal yeasts has been reported (Sterling *et al.*, 1986).

Rose *et al.* (1989) proposed criteria based on the degree of fluorescence and the size and shape of the (oo)cysts to identify water-borne cryptosporidia and giardia by IF microscopy (Table 5). The detection limits for IF microscopy in human or bovine faeces range 10–50 000 oocysts/g, depending on the characteristics of the stool specimens and the concentration techniques used (Anusz *et al.*, 1990; Weber *et al.*, 1991; Weber *et al.*, 1992; Webster *et al.*, 1996; Pereira *et al.*, 1999).

Table 5. Criteria for identifying *Cryptosporidium* oocysts and *Giardia* cysts by IF microscopy.[1]

Fluorescence
- The degree of fluorescence at least 50% of that in fresh control organisms
- Distinct fluorescence around the (oo)cyst wall

Morphology
- *Cryptosporidium*: spherical shape and size of 4–6 μm, folding in the oocyst wall
- *Giardia*: oval shape and size of 8–18 by 5–15 μm

[1]according to Rose *et al.*, 1989

6.4.2.2 Enzyme-linked immunosorbent assay (ELISA)

As with IF, the enzyme-linked immunosorbent assay (ELISA) relies on binding of antibodies (mAbs or pAbs) to *Cryptosporidium* or *Giardia* antigens in faeces. Several antigens are known to be associated with *Giardia* infection and it is believed that the pAb-based ELISA reacts with multiple antigens,

whereas the mAb-ELISA would not be able to detect different species of *Giardia* (Rosenblatt *et al.*, 1993). The monoclonal *Giardia*-specific antigen (GSA)-65 was suggested to be the predominant antigen in the stools of giardiasis patients (Rosoff *et al.*, 1989). However, it is not yet precisely known whether the presence of GSA-65 in stool is universal to all *Giardia* species or whether it is restricted to only the human specific *Giardia* species or assemblages (Addiss *et al.*, 1991a). The ELISA results are interpreted through use of a colour shift indicating the presence of soluble antigen. The colour change is assessed spectrophotometrically measuring the optical density (OD) or by visual examination. When IF microscopy has been used as a reference method, sensitivities of 68-99% for *Cryptosporidium* and 89-100% for *Giardia* together with specificities of 99-100% for both *Cryptosporidium* and *Giardia* have been reported using commercial ELISA kits designed for human faecal specimens (Garcia *et al.*, 1997; Aldeen *et al.*, 1998; Johnston *et al.*, 2003).

6.4.2.3 Molecular identification techniques

In molecular identification, highly processed (oo)cysts are needed. Density gradient centrifugation techniques for purifying stool samples have been used (Arrowood *et al.*, 1987; Ortega-Mora *et al.*, 1992; Suresh *et al.*, 1996; Trout *et al.*, 2004). Numerous (oo)cyst disruption techniques, e.g. freezing and thawing, shaking with zirconia beads or incubation with proteinase K, and DNA purification techniques including e.g., phenol-chloroform extraction and binding of DNA to glass milk or activated silica have been described (Stinear *et al.*, 1996; Spano *et al.*, 1998; McLauchlin *et al.*, 1999; Lowery *et al.*, 2000; McLauchlin *et al.*, 2000; Xiao *et al.*, 2001; Read *et al.*, 2004). Furthermore, IMS is a method used to purify oocysts before DNA extraction in environmental and faecal samples (Stinear *et al.*, 1996; Webster *et al.*, 1996; Hallier-Soulier *et al.*, 2000; Lowery *et al.*, 2000; Xiao *et al.*, 2001; Massanet-Nicolau, 2003; Jiang *et al.*, 2005). Currently, commercial kits for purifying DNA directly from faeces are available and widely used in DNA isolation of *Cryptosporidium* and *Giardia* (Caccio *et al.*, 2002; Enemark *et al.*, 2002; Caccio *et al.*, 2003; Read *et al.*, 2004; Lalle *et al.*, 2005a).

The advantage of molecular identification techniques is that they are able to detect genus-, species- or genotype-specific nucleic acid sequences of *Cryptosporidium* and *Giardia*. To date, several molecular techniques for identifying *Cryptosporidium* or *Giardia* are available. According to current knowledge, at least two genomic loci from an isolate should be analyzed at the same time to provide reliable identification. One of these should target a universal coding region (highly or moderately conserved coding regions) and the other should be suitable for species identification and subtyping analyses (Caccio *et al.*, 2005). The coding regions such as 18S ribosomal ribonucleic acid (rRNA), structural and housekeeping genes are analyzed in detection of *Cryptosporidium* and *Giardia* species and genotypes. These include genes encoding *Cryptosporidium* oocyst wall protein (COWP), heat shock protein 70 (Hsp70) and 60 kDa glycoprotein (gp60) of *Cryptosporidium* (Stinear *et al.*, 1996; Spano *et al.*, 1998; McLauchlin *et al.*, 1999; Hallier-Soulier *et al.*, 2000; Lowery *et al.*, 2000; McLauchlin *et al.*, 2000; Xiao *et al.*, 2000a; Xiao *et al.*, 2001; Enemark *et al.*, 2002; Heitman *et al.*, 2002; Chalmers *et al.*, 2005). The highly or moderately conserved gene regions used in the detection of *Giardia* include e.g. 18S rRNA, glutamate dehydrogenase (GDH) and ß-giardin genes (Homan *et al.*, 1998; Read *et al.*, 2002; Caccio *et al.*, 2003; Read *et al.*, 2004; Traub *et al.*, 2004; Lalle *et al.*, 2005b).

Because of similarity within the *Cryptosporidium* 18S rRNA and COWP genes between species, various species can be am-

plified by targeting these genes (Rochelle *et al.*, 1997(Xiao *et al.*, 2000a). By targeting a universal gene region combined with species and genotype identification by PCR-restriction fragment length polymorphism (PCR-RFLP) or sequencing, various species/genotypes can be differentiated (Spano *et al.*, 1998; McLauchlin *et al.*, 1999; McLauchlin *et al.*, 2000; Xiao *et al.*, 2000a; Enemark *et al.*, 2002; Heitman *et al.*, 2002). For instance, distinct COWP nucleotide sequences have been obtained from nine *Cryptosporidium* species and eight different *C. parvum* genotypes (Xiao et al., 2000a). Similarly, by sequencing the ß-giardin amplification product and using the GDH gene targeted PCR-RFLP, various assemblages of *G. duodenalis* have been differentiated (Homan *et al.*, 2001; Read *et al.*, 2004; Lalle *et al.*, 2005b).

Mini- and microsatellites are polymorphic sequence repeats in eukaryote genomes (Caccio *et al.*, 1999; Caccio *et al.*, 2001). Fingerprinting techniques targeting mini- and microsatellite loci are available for *Cryptosporidium* and can be used to identify subgenotypes and clonal lineages e.g. in defining the epidemiology of an infection (Caccio *et al.*, 2005). For instance, by sequencing the amplified mini- or microsatellite product, *C. parvum* and *C. hominis* subtypes can be identified (Caccio *et al.*, 2001; Enemark *et al.*, 2002; Chalmers *et al.*, 2005). For *Giardia*, well-aimed subtyping tools are still required.

The presence of inhibitors and low amount of target DNA in environmental samples are considered as major factors affecting DNA amplification (Stinear *et al.*, 1996; Lowery *et al.*, 2000; McIntyre *et al.*, 2000; Caccio *et al.*, 2003; Jiang *et al.*, 2005). Furthermore, PCR is vulnerable to contamination by non-specific DNA or carryover from previously amplified DNA leading to false-positive amplification (Kwok *et al.*, 1989; Rochelle *et al.*, 1997). Moreover, although PCR amplification would have been successful in one laboratory, it does not always succeed in another one (Rochelle *et al.*, 1997). Despite these facts and with proper optimization of PCR conditions, PCR is considered as a repeatable and sensitive identification technique for faecal and environmental samples (Johnson *et al.*, 1995; Stinear *et al.*, 1996; Kostrzynska *et al.*, 1999; Hallier-Soulier *et al.*, 2000; Lowery *et al.*, 2000). Using PCR, 1-10^5 oocysts have been detected (Johnson *et al.*, 1995; Stinear *et al.*, 1996; Hallier-Soulier *et al.*, 2000; Lowery *et al.*, 2000). Compared with IF microscopy, the PCR has shown to increase detection sensitivity 10-10^4-fold in environmental and faecal samples in some studies (Webster *et al.*, 1996; Lowery *et al.*, 2000).

7 Aims of the study

The aim of the thesis was to develop methods for detecting *Cryptosporidium* and *Giardia* in environmental samples and to determine whether *Cryptosporidium* and *Giardia* are present in the environment of Finland. The specific aims were as follows:

1. To develop IMS-based molecular methods for detecting *Cryptosporidium* and *Giardia* in sewage sludge and surface water (I, II).

2. To determine the presence of *Cryptosporidium* and *Giardia* in untreated and treated sewage sludge and surface water (I-III, IV).

3. To evaluate the usefulness of indicator parameters to predict the occurrence of *Cryptosporidium* and *Giardia* in sewage sludge and surface water (III, IV).

4. To evaluate current techniques for detecting *Cryptosporidium* and *Giardia* in canine faecal samples (V).

5. To determine the occurrence and zoonotic character of *Cryptosporidium* and *Giardia* infections in asymptomatic dogs (V).

8 Materials and methods

8.1 Development of immunocapture (IC)-PCR assays (I, II)

8.1.1 Control organisms

C. parvum (Iowa isolate) oocysts and *G. duodenalis* (H3 isolate) cysts were obtained from Waterborne Inc. (New Orleans, LA, USA) as purified suspensions in antibiotic solution of PBS with penicillin, streptomycin, gentamycin and 0.01% Tween 20 to prevent the growth of contaminating bacteria. The concentration of purified (oo)cysts was enumerated from five or six stock solution aliquots in a haemocytometer and the stock solution was mixed by vortexing prior to inoculation, which was performed within a few days to a month from the enumeration. The (oo)cysts were stored at 4°C.

8.1.2 Detection limits of IC-PCR assays

To evaluate the analytic sensitivity of the IC-PCR assays, sewage sludge and surface water samples not containing *Cryptosporidium* or *Giardia* in PCR were inoculated with dilutions of purified control (oo)cyst stock (Table 6). The homogeneity of the seed stock was ensured by vortexing before each sample was seeded. One unseeded sewage sludge and one surface water sample were used as negative controls in each dilution series.

8.1.3 Concentration and separation of (oo)cysts

Seeded surface water samples (2 l) were concentrated by filtration through a polycarbonate flatbed membrane filter (pore size 1.2 mm, diameter 142 mm; Millipore, Tullagreen, Cork, Ireland). The trapped (oo)cysts were eluted from the filter with 10 ml of PBS containing 0.05% Tween20 (PBS-Tween20) in a 50-ml polycarbonate tube by agitation at full speed in a wrist action shaker for 10 min. The (oo)cysts were captured from the extraneous material using IMS (Dynabeads GC-Combo) as recommended by the manufacturer (Dynal, Oslo, Norway). However, after capture 5 ml of PBS-Tween20 were added to the tube containing the immunobead-parasite complexes and the tubes were rotated for 5 min. This step was repeated once with 5 ml and once with 1 ml of PBS-Tween20. The complexes were then suspended in 50 µl of distilled water.

Seeded sewage sludge (1 ml) was homogenized with 9 ml of distilled water and the (oo)cysts captured from extraneous material using IMS (Dynal). At the end of the capture the immunobead-parasite complexes were suspended in 100 µl of distilled water.

Table 6. Evaluating the sensitivity of IC-PCR assays. Bold font denotes concentrations above the detection limit.

Matrix		Sample size	Concentration of (oo)cysts/sample							N:o of repeats	Study
Sludge	*Cryptosporidium*[1]	1 ml	**12 500**	**1250**	**625**	125	50	10	0	5	I
	Giardia[2]		**12 500**	**1250**	**625**	125	50	10	0	5	I
Water	*Cryptosporidium*[1]	2 l	**1000**	**100**	**50**	10	5	1	0	2	II
	Giardia[2]		**1000**	**100**	**50**	10	5	1	0	2	II

[1]Iowa isolate
[2]H3 isolate

8.1.4 Extraction and purification of DNA

To remove PCR inhibitors, 25 µl and 50 µl of 25% Chelex 100 (Bio-Rad, Hercules, CA, USA) were added to the immunobead-parasite complex suspension of surface water and sewage sludge samples, respectively. DNA from the captured (oo)cysts was released by five cycles of freezing (30 min at -70ºC) and thawing (30 min at +70ºC) followed by denaturation (10 min at 100ºC). The suspensions were centrifuged for 1 min at 13 000 rpm and supernatants containing the extracted DNA were stored at -20ºC.

To amplify the PCR products successfully from the sewage sludge samples, additional purification was included. A quantity (200 µl) of TE-buffer (0.1 M Tris-HCl, 0.2 M EDTA), containing 10 M guanidine thiocyanate (GES) and 50 µl of activated silica (Sigma-Aldrich, St. Louis, Missouri, MO, USA) was added to the supernatant. The suspension was incubated for 10 min at room temperature, centrifuged and the pellet washed with 0.1 M TE containing 10 M GES, ethanol and acetone as described by McLauchlin *et al.* (1999). The purified DNA was eluted in 150 µl of sterile distilled water and stored at -20ºC.

8.1.5 PCR amplification

The primers Cry9/Cry15 amplify a 550-bp fragment specific for *Cryptosporidium* oocyst wall protein (COWP) gene (Spano *et al.*, 1997), and the primers Lax A/LaxB a 194-bp fragment of an unknown genomic region of *C. parvum* (Balatbat *et al.*, 1996). The primers Gdh1/Gdh4 amplify a 768-bp fragment specific for the glutamate dehydrogenase (GDH) gene of *Giardia* spp. (Homan *et al.*, 1998). The primers were synthesized by a commercial laboratory (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany). The amplification reaction mixture contained 2.5 U HotStarTaq DNA Polymerase, 1 x PCR buffer containing 1.5 mmol/l $MgCl_2$ and 200 mol/l of each dNTP (HotStarTaq Master Mix, Qiagen GmbH, Hilden, Germany), 12.6 pmol of the primers Cry9/Cry15, 50 pmol of the primers LaxA/LaxB and Gdh1/Gdh4, and 5 µl of the template in a total volume of 50 µl. Negative and positive controls (approximately 0.2 µg of purified DNA from the control organisms) were run simultaneously. The PCR was performed with optimized PCR cycles (Table 7) in a thermal cycler (DNA Engine PTC-200, MJ Research, Waltham, MA, USA).

8.1.6 Visualization of amplified products

A 14-50-µl quantity of amplified product was visualized after electrophoresis in an ethidium bromide-stained 2-3% agarose gel under UV light. Molecular-weight marker VI (Boehringer Mannheim GmbH, Mannheim, Germany) served as a DNA size-marker. The specificity of the amplified PCR product was confirmed by southern hybridization. The products were transferred to a nylon membrane (MagnaGraph nylon transfer membrane 0.45 Micron, Osmonics Inc., Westborough, MA,USA) and hybridized at 65ºC for 16 h with digoxigenin labelled probes prepared from the amplification products of control organisms according to the manufacturer´s instructions (DIG DNA Labelling and Detection Kit, Boehringer Mannheim). Products amplified by LaxA/LaxB were hybridized at 42ºC for 4 h with 3'- end digoxigenin labelled (DIG Oligonucleotide 3'- End Labelling Kit, Roche Diagnostics GmbH, Mannheim, Germany) internal probe, 5' - TGG CCA ATG ATG AAT TAA CC - '3, selected by Hallier-Soulier and Guillot (Hallier-Soulier *et al.*, 1999). The hybridization products were detected colorimetrically with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salts.

Table 7. PCR primers and temperature conditions.

Target region	Predena-turation	No. of cycles	Dena-turation	Annealing	Extension	Final extension	Expec-ted size (bp)	Study	Reference
Cryptosporidium									
COWP[1]	94°C 15 min	30	94°C 1 min	55°C 1 min	72°C 1 min	72°C 10 min	550	I-III	Spano *et al.*, 1997
Unknown[2]	94°C 15 min	40	94°C 30 s	60°C 45 s	72°C 1 min		194	II	Balatbat *et al.*, 1996
18S rDNA[3]	95°C 10 min	45	94°C 55 s	56°C 27 s	72°C 50 s	72°C 7 min	925	V	Enemark *et al.*, 2002
Giardia									
GDH[4]	94°C 15 min	35	94°C 1 min	55°C 1 min	72°C 1 min	72°C 7 min	768	I-III	Homan *et al.*, 1998
GDH[5]	95°C 10 min	40	94°C 30 s	57°C 30 s	72°C 30 s	72°C 7 min		V	Read *et al.*, 2004
	95°C 10 min	45	94°C 30 s	56°C 25 s	72°C 30 s	72°C 7 min	432		
18S rDNA[6]	95°C 10 min	40	94°C 30 s	57°C 30 s	72°C 30 s	72°C 7 min		V	Hopkins *et al.*, 1997;
	95°C 10 min	45	94°C 30 s	56°C 25 s	72°C 30 s	72°C 7 min	174		Read *et al.*, 2002

[1]=5´GTA GAT AAT GGA AGA GAT TGT G3'; 5´GGA CTG AAA TAC AGG CAT TAT CTT G3'
[2]=5'GCG AAG ATG ACC TTT TGA TTT G3'; 5'CCT TGG AGT CTT CTT CTT AGG A3'
[3]=5'AAC AGT TAT AGT TTA CTT GAT AAT C3'; 5'TGA AGG AGT AAG GAA CAA CC3'
[4]=5'ATC TTC GAG AGG ATG CTT GAG3'; 5'AGT ACG CGA CGC TGG GAT ACT3'
[5]=5'GTT RTC CTT GCA CAT CTC3'; 5'TCA ACG TYA AYC GYG GYT TCC GT3'
5'GTT RTC CTT GCA CAT CTC3'; 5'CAG TAC AAC TCY GCT CTC GG3'
[6]=5'CAT CCG GTC GAT CCT GCC3'; 5'GTC GAA CCC TGA TTC TCC GC3'
5'GAC GCT CTC CCC AAG GAC3'; 5'CTG CGT CAC GCT GCT CG3'

Table 8. Sampling procedures and detection techniques for environmental and faecal samples.

Matrix	Sampling time	Sampling site	N:o of samples	Sample size studied	Detection techniques	Study
Untreated sludge	April to June 2000	WTP	44	1 ml	IC-PCR	I
Treated sludge	March to October 2001	WTP	38	450 mg	IF microscopy	IV
Surface water-grab	September 2000 to October 2001	Lake, river	154	10 l	IC-PCR, IF microscopy	II-III
Surface water-capsule	November 2000 to January 2001	River	25	6-50 l	IC-PCR	II
Dog faeces	February 2003 to December 2004	Ground	284	1 g	IF microscopy, ELISA, PCR	V

8.2 Detection of *Cryptosporidium* and *Giardia* in environmental and faecal samples (I-V)

8.2.1 Sampling and treatment of sewage sludge samples (I, IV)

Untreated 500-ml sewage sludge samples were collected on three subsequent days from 12 Finnish municipal WTPs during spring-summer 2000 (Table 8), whereas treated sludge samples (1-4 l) were obtained from 22 WTPs after composting or stabilization by quicklime and peat addition during 2001. The composted samples were studied after 10 and 30 weeks of hygienization. In total, 24 WTPs participated in studies I and IV. The characteristics of the 24 participating WTPs are listed in Table 9. Test-

Table 9. Properties of the 24 Finnish wastewater treatment plants investigated in studies I and IV.

Treatment method	Treatment plant	Population equivalent	Treatment conditions	
			Sanitary phase	Curing phase
Windrow composting	1	35 000	Open windrow	Open windrow
	2	10 000	Open windrow	Open windrow
	3	55 000	Open windrow	Open windrow
	4	30 000	Open windrow	Open windrow
	5	100 00	Open windrow	Open windrow
	6	40 00	Open windrow	
Mesophilic anaerobic digestion and windrow composting	7	815 000	Anaerobic digestion	Open windrow
	8	25 000	Anaerobic digestion	Open windrow
	9	145 000	Anaerobic digestion	Open windrow
	10	55 000	Anaerobic digestion	Open windrow
			Anaerobic digestion	Thermal drying
Air-ventilated closed reactor composting and windrow composting	11	10 000	Drum composting	Open windrow
	12	20 000	Drum composting	Open windrow
	13	9 000	Drum composting	Open windrow
	14	23 000	Drum and tunnel composting	Open windrow
	15	10 000	Tunnel composting	Open windrow
	16	95 000	Tunnel composting	Open windrow
	17	30 000	Tunnel composting	Open windrow
	18	4 000	Tunnel composting	Open windrow
Lime stabilisation	19	5 000	Quicklime addition	
	20	53 000	Quicklime addition	
	21	8 500	Quicklime addition	
	22	7 500	Quicklime addition	
Peat addition	23	15 000	Peat addition	
	24	95 000	Peat addition	

ing of untreated sludge samples was undertaken within 2 weeks and treated sludge samples within 2 months after sampling. The samples were stored at 4°C until analysis. The untreated sludge samples were analysed using the IC-PCR assay described in Materials and methods 8.1.3-, whereas 150 mg of treated sludge were distributed directly on three microscope slide wells for IF microscopy.

8.2.2 Sampling and treatment of surface water samples (II, III)

Grab samples of surface water (10 l) were taken from seven lakes and 30 rivers in southern and southwestern Finland from autumn 2000 to autumn 2001 (Table 8). Samples were collected on five consecutive seasons from 15 rivers (III). Capsule samples of surface water (6-50 l) were obtained during winter 2000-2001 from six drinking water treatment plants located in western Finland (II). The samples were stored at 4°C until analysis within 2 days after sampling. The surface water grab samples were treated and analysed using the IC-PCR assay described in Materials and methods 8.1.3-. The capsule samples of surface water (II) were filtered through an Envirochek Sampling Capsule (Pall Life Sciences Inc., Ann Arbor, MI, USA) and eluted from the capsule according to the manufacturer´s instructions. The contents were further filtered through a polycarbonate filter and treated as the surface water grab samples. Additionally, the immunobead-parasite complex suspension (50 µl) was divided before addition of Chelex 100, and the 25-ml aliquots were used for IF microscopy and PCR (III). A sample was considered as positive if either IF microscopy, PCR or both were positive.

8.2.3 Sampling and treatment of faecal samples (V)

Canine faecal samples were taken from the ground during 2003-2004 by dog owners (Table 8). The samples were placed in small plastic bags immediately after defecation. Another sample from the same dog was taken 5-7 days after the first sample from 133 out of 150 dogs. After arrival in the laboratory, the samples were divided in two aliquots: one for IF microscopy and the other for ELISA and PCR analyses. The samples intended for IF microscopy were stored at 4°C for no longer than 1 day and then concentrated using formalin-ethyl acetate sedimentation as described by Sloss et al. (1994). Briefly, 1 g of faeces was mixed with 7 ml of 10% formalin. The mixture was then filtered, mixed with 3 ml of ethyl acetate and centrifuged for 1 min at 3000 rpm. Finally, 15 ml of the sediment were distributed on a microscope slide well. The samples intended for ELISA and PCR were frozen at -70°C. A dog was considered infected if one or both successive samples were positive for *Cryptosporidium* or *Giardia*. The samples positive for *Cryptosporidium* or *Giardia* by IF microscopy and/or ELISA were further analysed with PCR.

8.2.4 Identification of Cryptosporidium and Giardia

8.2.4.1 IF microscopy (III-V)

The (oo)cysts were detected using a direct immunofluorescence assay (IFA; Aqua-Glo G/C Kit and Cyst-a-Glo Kit; Waterborne). Microscopic examination and enumeration were carried out with Nikon type 115 (Tokyo, Japan; III, IV) and Olympus BH2 (Tokyo, Japan; V) epifluorescence microscopes. The slides were screened with 200x magnification, and the size and appearance of apple-green fluorescing particles were compared with the control organisms at a magnification of x400. When the Olympus BH2 equipped with a ColourView 12 digital camera (Soft Imaging System, Münster, Germany) and AnalySIS 3.0 image analysis software (Soft Imaging System) for measurement of the (oo)cysts was available (V),

ovoid or spherical objects with diameters of 4-6 µm were recorded as *Cryptosporidium* and round to oval particles with diameters of 5-18 µm were recorded as *Giardia*.

8.2.4.2 ELISA (V)

Commercial ELISAs, ProSpect *Cryptosporidium* Microplate assay (Remel, Lenexa, KS, USA) and ProSpect *Giardia* Microplate assay (Alexon-Trend, Ramsay, MN, USA), were used to test for *Cryptosporidium* and *Giardia* infection in canine faecal samples according to the manufacturers' instructions. A dog was determined to be infected if one or both subsequent samples were positive for *Cryptosporidium* or *Giardia*.

8.2.4.3 Determining detection limits of IF microscopy and ELISA in canine faeces (V)

Canine faeces tested negative for *Cryptosporidium* and *Giardia* by IF microscopy and ELISA was inoculated with stock solution dilutions of approximately 1.0 x 10^2, 1.0 x 10^3, 1.0 x 10^4 and 1.2 x 10^5 oocysts, as well as 2.5 x 10^2, 2.5 x 10^3, 2.5 x 10^4 and 3.2 x 10^5cysts/g. The spike stocks were mixed by vortexing before each sample was spiked. One unspiked sample served as negative control in the dilution series. Sensitivity testing was repeated three times.

8.2.4.4 Molecular identification techniques (I-III, V)

The untreated sewage sludge and surface water samples were analysed using IC-PCR assays as described in Materials and methods 8.1.3.- 8.1.6 (I-III); in study III, however, southern hybridization was not used. To determine the species and genotypes, 100-200 µl of *Cryptosporidium* amplification product were purified with the QIAquick PCR Purification Kit Protocol according to he manufacturer´s instructions (Qiagen; I). Due to the presence of nonspecific amplification products, the *Giardia* fragments were cut from 0.8% agarose gel and the DNA was purified by the QIAquick Gel Extraction Kit Protocol as described by the manufacturer (Qiagen). Ten units of the restriction enzyme *Rsa*I (BioLabs Inc., Ipswich, MA, USA) for *Cryptosporidium*, and *Dde*I (BioLabs) for *Giardia,* in the relevant digestion buffer, was added to the purified product. Digestion was allowed in a total volume of 50 µl for 1 h at 37ºC. The digestion products were visualized under UV light after electrophoresis in an ethidium bromide-stained 3% agarose gel and the digestion patterns were compared with published patterns (Spano *et al.*, 1997; Homan *et al.*, 1998) and with patterns from control (oo)cysts. In study II, primers LaxA/LaxB were used to identify the zoonotic *Cryptosporidium* species (Balatbat et al., 1996). In study V, 300 mg of faeces were treated using QIAamp DNA Stool Mini Kit (Qiagen) to extract and purify the DNA for PCR analysis. The primer sequences and PCR temperature cycles used (I-III, V) are listed in Table 7. In study V, the amplified PCR products were further purified with a High Pure PCR Product Purification Kit (Roche Molecular Biochemicals, Munich, Germany), sequenced in both directions using a commercial sequencing service (MWG Biotech AG, Ebersberg, Germany) and analysed using a BioEdit Sequence Alignment Editor (Ibis Therapeutics, Carlsbad, CA, USA). Finally, the sequences were compared with those available in GenBank (National Institutes of Health, Bethesda, MD, USA).

8.3 Indicator parameters

8.3.1 Treated sludge (IV)

For analysis of indicator bacteria, 11-g treated sludge samples were suspended in 99 ml of peptone (0.1%)-saline (0.85%) solution, homogenized in a rotary shaker and serially diluted for plating out. *Clostridium perfringens* was analysed accord-

ing to the International Organization for Standardization (ISO) method 7937 (1997) using tryptose sulphite cycloserine (TSC) agar. Agar plates were incubated under anaerobic conditions at 37±1°C for 20 h. Typical colonies were isolated and inoculated onto sheep (7%) blood agar plates (Oxoid, Basingstoke, Hampshire, UK) and incubated under anaerobic conditions at 37±1°C for 20 h. Five haemolytic colonies were then Gram-stained and a single typical colony was confirmed using an Analytical Profile Index 20 (API 20 A; (bioMeriéux, Marcy l'Etoile, France). *Escherichia coli* was analysed according to the Nordic Committee on Food Analysis (NCFA) method 147 (1993). Briefly, Petrifilms (3M Microbiology Products, St. Paul, MN, USA) were incubated for 46-50 h at 37±1°C. Blue colonies were counted and a single typical colony was confirmed with the API 20 E test. Enterococci were analyzed according to the NCFA method 68 (1992) using Slanetz & Bartley agar (Oxoid). The plates were incubated at 44±0.5°C for 48 h and red colonies were counted. Atypical colonies were confirmed by Gram staining, a catalase test and the API 20 Strep test. The samples were stored at 4°C until analyses were performed within 2 days.

8.3.2 Surface water (III)

Escherichia coli counts were analysed from 100 ml of surface water sample using the Colilert-18/Quanti-Tray 2000 most probable number (MPN) test according to the manufacturer´s instructions (IDEXX Laboratories Inc., Westbrook, ME, USA). *Clostridium perfringens* was analysed from 100 ml of sample according to the ISO 6461-2 (1986) standard, but Shahidi Ferguson agar (identical with TSC agar) containing D-cycloserine was used. Turbidity was measured using a Eutech Cyberscan WL TB1000 turbimeter (Eutech Instruments, Singapore) on the day after sampling; the results were expressed in NTUs. Analyses were initiated within 24 h of sample arrival in the laboratory, the samples were stored at 4°C.

8.4 Statistical analysis

Analysis of variance (ANOVA) was used to test differences in the most probable number of *E. coli,* value of turbidity and prevalence of *Cryptosporidium*, *Giardia* and *C. perfringens* between sampling times and sites (III). The chi-square test was employed to assess the correlation between the presence of *Cryptosporidium* or *Giardia* and that of indicator organisms in treated sludge (IV) and to compare the proportion of infected individuals between young and adult dogs (V). Diagnostic agreement between the detection techniques and between the sample rounds (V) was assessed using kappa (k) statistics. k is a measure of the agreement between detection techniques; a k value of zero represents an agreement produced by chance alone. k values above 0.75 represent excellent agreement, values between 0.60 and 0.75 good agreement, values between 0.40 and 0.60 fair agreement, while values lower than 0.40 indicate poor agreement (Altman, 1991).The p value for a k estimate was used to determine whether a k value differed significantly from zero. In all statistical tests, two-tailed p values lower than 0.05 were considered statistically significant. Statistical analyses were performed using SPSS 12.0 for Windows.

9 Results

9.1 Evaluation of detection techniques (I, II, V)

9.1.1 Detection limits of IF microscopy, ELISA and IC-PCR assays (I, II, V)

The detection limit of the IC-PCR assay for sewage sludge was 625 (oo)cysts/ml (Table 6). Using hybridization of the PCR product, the sensitivity could be increased to 125 (oo)cysts/ml in one attempt out of five for *C. parvum* and in two of five trials for *G. duodenalis* (I). The sensitivity of the assay for surface water was not increased by hybridization (II), remaining at 50 (oo)cysts/ 2 l for both *Cryptosporidium* and *Giardia*. The detection limit of IF microscopy was 10^5 (oo)cysts/g for *Cryptosporidium* and *Giardia* in dog faeces, while 10^5 oocysts and 10^4 cysts/g could be detected in ELISA (V).

9.1.2 Diagnostic accuracy of ELISA (V)

The sensitivity of the ProSpect *Cryptosporidium* Microplate assay was 71% (95% CI 35-91%) and specificity 94% (95% CI 88-97%). The sensitivity of the ProSpect *Giardia* Microplate assay was 100% (95% CI 66-100%) and specificity 96% (95% CI 91-98%). The *Cryptosporidium* ELISA and IF microscopy showed fair agreement ($k = 0.44$, $p < 0.001$), whereas the *Giardia* ELISA and IF microscopy showed good agreement beyond chance ($k = 0.71$, $p < 0.001$).

9.2 Detection of *Cryptosporidium* and *Giardia* in environmental and faecal samples (I-V)

In untreated sewage sludge, *Cryptosporidium* was found in 3/12, and *Giardia* in 8/12 wastewater treatment plants (WTP) studied (I). Of the total of 44 samples studied, three (7%) were positive for *Cryptosporidium* and nine (20%) for *Giardia* by IC-PCR (Table 10). Using PCR-RFLP, all *Cryptosporidium*-positive samples were identified as *C. parvum*. Genotyping results were not obtained from the samples positive for *Giardia*.

In study IV, *Giardia* was found in 10/22 and *Cryptosporidium* in 8/22 WTPs treated sludge. After various sludge treatment processes, *Cryptosporidium* oocysts were found by IF microscopy in 37.5% (6/16 samples) and *Giardia* cysts in 44% (7/16) of the 10-week-old compost samples. In sludge end-products (either composted for 30 weeks or sanitized by quicklime or peat addition), *Cryptosporidium* was detected in 10% (2/21) and *Giardia* in 33% (7/21) of the samples (Table 10). In all positive samples, (oo)cysts were detected in low numbers; the maximum number was three (oo)cysts/450 mg of treated sludge (Table 11).

In surface water, both *Cryptosporidium* and *Giardia* were detected in 4% (5/116) of the samples studied by IF microscopy (Table 10;

Table 10. Number of environmental and faecal samples positive for *Cryptosporidium* and *Giardia* per samples studied.

Type of sample	*Cryptosporidium*			*Giardia*			Study
	IF microscopy	ELISA	PCR	IF microscopy	ELISA	PCR	
Untreated sewage sludge			3/44			9/44	I
Treated sewage sludge	8/37			14/37			IV
Surface water	5/116		13/149	5/116		16/179	II-III
Canine faeces	6/284	15/284	6/26	15/284	20/284	11/25	V

Table 11. Detection of *Cryptosporidium* oocysts, *Giardia* cysts and indicator bacteria during various wastewater treatment processes in 22 Finnish wastewater treatment plants.

Treatment method		N:o of samples	Mean number of (oo)cysts/ 450 mg of sludge (min-max values)		Median$_{dw}$ 10^{cfu}/g (min-max values)[4]		
			Cryptosporidium	*Giardia*	*E. coli*	*C. perfringens*	Enterococci
Windrow composting	Compost 10 wk	4	0.75 (0–3)	0.5 (0–1)	2.68 (<1.5–3.25)	3.15 (<1.5–5.98)	4.7 (3.47–5.42)
	End-product	4	0.25 (0–1)	0	<2 (<2–<2)	3.16 (2.93–5.12)	2.31 (<2–3.49)
Mesophilic anaerobic digestion and windrow composting	Compost 10 wk	4	0.25 (0–1)	2.25 (1–3)	<1.5 (<1.5–2.87)	5.63 (<1.5–5.71)	2.58 (1.89–3.6)
	End-product	4	0	1.25 (0–2)	1.72 (1.51–2.01)	4.9 (2.89–5.55)	<1.5 (<1.5–3)
	End-product[1]	1	0	0	<1	<1	<1
Drum and windrow composting[2]	Compost 10 wk	4	0.5 (0–1)	0.25 (0–1)	<1.75 (<1.5–<2)	3.36 (<1.5–4.82)	2.36 (<1.5–3.37)
	End-product	4	0	0.25 (0–1)	<2 (<1.5–<2)	<2 (<1.5–4.03)	<2 (<1.5–<2)
Tunnel and windrow composting	Compost 10 wk	4	0.25 (0–1)	0	3.99 (<1–5.44)	<1.25 (<1–4.08)	4.06 (<1–5.31)
	End-product	3	0	0	1.79 (<1.5–3.12)	<1.5 (<1.5–<1.5)	2.09 (<1.5–4.31)
Sanitation by lime or peat addition	End-product	6	0.2 (0–1)[3]	0.6 (0–1)[3]	3.28 (<2–6.00)	4.35 (<2–6.34)	4.26 (<2–5.93)

[1]thermal drying
[2]including plant using combined drum and tunnel composting
[3]n=5
[4]in sludge end-product, cfu counts of *E. coli* and enterococci should be $<10^3$ and *C. perfringens* $<10^{3.48}$/g (Carrington, 2001)

Table 12. Proportion of samples positive for *Cryptosporidium*, *Giardia* and *C. perfringens*, and the mean values of *E. coli* and turbidity in surface water samples.

Sampling time	Number of positive samples/samples studied			Mean values	
	Cryptosporidium	*Giardia*	*C. perfringens*	*E. coli* (MPN/100 ml)	Turbidity (NTU)
September and October 2000	3/19	1/19	11/19	300	42
February and March 2001	0/30	0/30	7/30	213	37
May 2001	0/30	3/30	18/30	93	48
August 2001	7/30	6/30	4/30	72	16
October 2001	4/30	9/30	4/30	129	46

MPN, most probable number
NTU, nephelometric turbidity unit

III). Using IC-PCR, both *Cryptosporidium* (13/149) and *Giardia* (16/179) were found in 9% of the samples (Table 10; II, III). In study III, in total 86 and 116 samples were studied for *Cryptosporidium* and *Giardia,* respectively, by both IF microscopy and PCR. From these, only two *Cryptosporidium* and two *Giardia* samples were positive by both IF microscopy and PCR (III). *Cryptosporidium* and *Giardia* were found in surface water less frequently in winter, than during other seasons ($p < 0.05$; Table 12; III).

In canine faecal samples studied with IF microscopy, both *Cryptosporidium* (7/150) and *Giardia* (8/150) were detected in 5% of the dogs (V). Young dogs were infected with *Cryptosporidium* (17% vs. 1%) and *Giardia* (19% vs. 1%) more often than adults ($p < 0.001$). In total, 284 faecal samples were examined by IF microscopy and ELISA (Table 10). The samples positive for *Cryptosporidium* or *Giardia* by IF microscopy and/or ELISA were studied further by PCR. From these, 4 samples were positive for *Cryptosporidium* and 11 for *Giardia* by all three detection techniques (Table 13). The

Table 13. Correlation between the results of IF microscopy, ELISA, PCR and sequences of 18S rRNA gene in *Cryptosporidium*- and *Giardia duodenalis*-positive dogs.

	Dog number	Sample 1				Sample 2			
		IF microscopy	ELISA	PCR	Species/ assemblage	IF microscopy	ELISA	PCR	Species/ assemblage
Cryptosporidium	9	-	-	-		+	+	+	NI
	52	-	-	-		+	+	+	*C. canis*
	58	+	-	+	NI	-	-	-	
	59	+	+	+	*C. canis*	+	-	-	
	99	+	-	-		-	-	-	
	142	-	-	-		+	+	-	
	146	+	+	+	NI	+	-	-	
	150	-	+	-		-	-	+	*C. canis*
Giardia	12	+	+	-		+	+	+	D
	77	+	+	-		+	+	+	D
	84	+	+	+	D	+	+	-	
	85	+	+	-		+	+	+	C
	101	+	+	+	C				
	141	+	+	+	D	+	+	+	D
	148	+	+	+	NI	+	+	+	C
	150	+	+	+	NI	+	+	+	E

species and genotype identification was carried out by sequencing the 18S rRNA gene region of *Cryptosporidium* and *Giardia*. Three dogs were infected with *C. canis*, whereas *G. duodenalis* assemblages C, D and E were responsible for the *Giardia* infections. Only in two samples the DNA was amplified sufficiently when the GDH gene region was targeted. These sequences were identical with canine *Giardia* isolates from various parts of the world, however, no assemblages were reported in the Genbank. Genotyping results were not obtained from three dogs positive by PCR for *Cryptosporidium* and from two positive for *Giardia* when 18S rRNA gene was targeted.

9.3 Use of indicators for detection of *Cryptosporidium* and *Giardia* in environmental samples (III, IV)

Occurrence of *Cryptosporidium* and *Giardia*, in surface water or in treated sludge did not correlate with the faecal indicator bacteria *E. coli*, *C. perfringens* and enterococci or with water turbidity ($p > 0.05$; Tables 11 and 12).

10 Discussion

During the past decade, development of methods for effective detection of *Cryptosporidium* and *Giardia* in environmental samples has been a major area of research interest. IF microscopy, which still remains the most commonly used identification technique for environmental and faecal samples, has been criticized for being too time-consuming, having too low a detection limit and, most importantly, for being unable to differentiate between the species and genotypes of *Cryptosporidium* and *Giardia* (Clancy *et al.*, 1994; Lowery *et al.*, 2000; Fall *et al.*, 2003). Molecular methods make it possible to detect and distinguish between species and genotypes (Sulaiman *et al.*, 1998; Morgan-Ryan *et al.*, 2002; Monis *et al.*, 2003a; Read *et al.*, 2004) and have increased the detection sensitivity compared with IF microscopy in environmental and faecal samples (Webster *et al.*, 1996; Kaucner *et al.*, 1998; Lowery *et al.*, 2000). In some studies, however, more positive samples have been found by IF microscopy than by PCR (Kaucner *et al.*, 1998; Di Giovanni *et al.*, 1999). Several protocols for detection of *Cryptosporidium* from surface water have been published, but only a few methods for simultaneous detection of *Cryptosporidium* and *Giardia* have been described. In the present thesis, IC-PCR assays for simultaneous detection of *Cryptosporidium* and *Giardia* in sewage sludge and surface water were developed. To my knowledge, paper I is the first study published concerning molecular techniques in detection of *Cryptosporidium* and *Giardia* from sewage sludge. The study design in studies I and II simulated a real analysis situation, because the (oo)cysts were inoculated directly into the sample matrix prior to concentration. In most previous studies, the oocysts were inoculated into filtered water pellet suspensions (Johnson *et al.*, 1995; Kostrzynska *et al.*, 1999; Lowery *et al.*, 2000), thus evaluating only part of the detection procedure. However, losses of (oo)cysts have been reported during concentration by filtration (LeChevallier *et al.*, 1995). In many studies, the capacity of the method has been described by determining the recovery efficiency (RE) of the method (Table 4). For detection of *Cryptosporidium* and *Giardia* in surface water, the RE for IMS- and IF microscopy-based protocols can range from < 1 % to 85 % (DiGiorgio et al., 2002; Feng et al.,2003). In studies I and II, IC-PCR assays developed were not validated against IF microscopy. Because IC-PCR assays give no quantitative result, RE could not be determined. The sensitivities of the entire procedure including concentration and separation of the (oo)cysts, DNA purification and PCR amplification were described in studies I and II, but it is not known how many (oo)cysts were lost during the procedure.

In study I, the detection limit of 125-625 (oo)cysts/ml of sewage sludge indicates that the assay is practicable, since the (oo)cysts are known to concentrate in sewage sludge at a level of 10^5-10^7 (oo)cysts/kg in the wastewater treatment process (Straub *et al.*, 1993; Thiriat *et al.*, 1997; Gale, 2005). More recent studies, however, have proved to detect substantially lower numbers of (oo)cysts in sewage sludge. With sedimentation combined with IMS and IF microscopy, an estimate of 40-60 (oo)cysts/ml have been identified by Massanet-Nicolau (2003) and Iacovski et al. (2004). This indicates that IF microscopy continues to be an applicable technique in detection of *Cryptosporidium* spp. and *Giardia* spp. in

sewage sludge, and further development to sensitize the PCR techniques are needed.

The detection limit for surface water samples (50 (oo)cysts/2 l; II) appears high when considering the low numbers (1-18 (oo)cysts/10 l) of (oo)cysts that have been detected in Scandinavia's surface water systems (Hansen *et al.*, 1998; Robertson *et al.*, 2001; Hänninen *et al.*, 2005). This could reflect the fact that the RE of the assay is low, especially as very low numbers (1-15) of oocysts have been detected in some other PCR-based studies (Kaucner *et al.*, 1998; Hallier-Soulier *et al.*, 2000; Sturbaum *et al.*, 2002). The stochastic variation in the distribution of (oo)cysts in seeding experiments with low numbers of (oo)cysts may also influence the output of the assay. In addition to technical and methodological inaccuracy during sample preparation, amplification and detection, also the random distribution of the template molecules affect the sensitivity of PCR (Stenman *et al.*, 2001). However, at the time of experiments in the study II, the detection limit was comparable to that in other PCR-based studies since variable numbers up to 10^5 oocysts were detected previously (Johnson *et al.*, 1995; Kostrzynska *et al.*, 1999; Hallier-Soulier *et al.*, 2000; Lowery *et al.*, 2000).

One possible reason for the high detection limit may also be the turbidity of the water samples. The turbidity of surface water can affect recovery efficiency by reducing the filtration capacity and PCR amplification (Rochelle *et al.*, 1997; DiGiorgio *et al.*, 2002; Feng *et al.*, 2003). The Finnish rivers and lakes typically contain large amounts of humus substances consisting of decomposing organic substances which raise the turbidity measures (Rantakari *et al.*, 2004; Mattsson *et al.*, 2005). The turbidity of the water may have reduced the performance of the IC-PCR assay because at the lowest only 6 l could be filtered before clogging the capsule filter (II). The turbidity of the surface water samples ranged 9-200 NTUs (II) compared with 0.2-77 NTUs in a study done in France, in which a detection limit of 1-5 oocysts was achieved (Hallier-Soulier *et al.*, 2000).

Environmental samples are known to be a difficult matrix for PCR analysis because they contain low numbers of (oo)cysts compared with the massive amounts of other organisms and compounds that may act as inhibitory factors for PCR (Stinear *et al.*, 1996; McIntyre *et al.*, 2000; Jiang *et al.*, 2005). A potential PCR inhibition can be assessed by using internal positive controls in the PCR amplification (Kaucner *et al.*, 1998). Immunomagnetic capture (IC) was used to separate and purify the (oo)cysts from extraneous material, thus reducing the effect of PCR inhibitors (I-III). Immunomagnetic separation (IMS) has proved to be useful for reducing PCR inhibition both in environmental and faecal samples (Webster *et al.*, 1996; Lowery *et al.*, 2000; Jiang *et al.*, 2005). Using IMS, the recovery rates in turbid environmental water samples were raised to 70% compared with < 10% when the samples were concentrated by conventional sugar flotation technique (Bukhari *et al.*, 1998). In studies I-III, additional purification of the DNA released from the immunobead-parasite complexes was necessary to amplify the PCR products successfully. Similarly, IMS combined with Chelex resin purification prior to DNA extraction was needed to increase the detection limit in turbid water samples in another study (Lowery *et al.*, 2000). The low amount of DNA together with nonspecific amplification may have caused the unsuccessful restriction digestion of *Giardia*-positive samples (I). Furthermore, low amount and poor quality of DNA together with freezing of the samples may have been responsible for the failed sequencing results of some canine faecal samples (V).

ELISA was compared with IF microscopy in detection of *Cryptosporidium* and *Giardia* from canine faeces (V). IF microscopy for detection of *Cryptosporidium* and *Giardia*,

and *Cryptosporidium* ELISA showed equal analytic sensitivity, with a detection limit of 10^5 (oo)cysts/g of faeces; the detection limit was 10-fold lower for *Giardia* ELISA. In other studies, detection limits of 10^3-10^4 oocysts/g for IF microscopy (Anusz *et al.*, 1990; Weber *et al.*, 1991; Weber *et al.*, 1992; Webster *et al.*, 1996; Pereira *et al.*, 1999) and 10^3-10^5 (oo)cysts/g for ELISA (Anusz *et al.*, 1990; Johnston *et al.*, 2003) in human or bovine stool have been reported. Compared with the reported level of cyst excretion (up to 2 x 10^3 cysts/g of canine faeces; (Sykes *et al.*, 1989), the detection limits in study V appear high. However, the detection technique used (sugar flotation combined with eosine staining) in the Sykes study probably affected their low counts. It would, therefore, be worthwhile to determine the (oo)cyst excretion rate in dog faeces using the present detection techniques. In study V, the diagnostic sensitivity and specificity of *Cryptosporidium* ELISA were lower than in previous reports, in which the test accuracy was determined with human stool samples (Garcia *et al.*, 1997; Aldeen *et al.*, 1998; Johnston *et al.*, 2003). Ten samples positive for *Cryptosporidium* by ELISA could not be confirmed either by IF microscopy or PCR, while four IF-positive specimens were negative by ELISA (V); thus, *Cryptosporidium* ELISA showed only fair agreement with IF microscopy. In a previous study, a lower accuracy in canine compared with human stool samples was suspected to be a result of different types of *Giardia* isolates present in dog faeces (Hopkins *et al.*, 1993). Similarly, the reduced level of diagnostic sensitivity in study V may have been due to different species of *Cryptosporidium*. The typing results suggested that all the species identified in our study were dog-specific *C. canis*, which could explain why the kits designed for human stools did not perform well. On the other hand, the reduced capacity of *Cryptosporidium* ELISA for detecting low numbers of oocysts as well as presence of false-positive test results were also reported for human stools (Doing *et al.*, 1999; Johnston *et al.*, 2003). Due to the high rate of false-positive results and the low diagnostic sensitivity, the *Cryptosporidium* ELISA evaluated (V) cannot be recommended for detection of *Cryptosporidium* in canine feces. In contrast, the *Giardia* ELISA correlated well with IF microscopy (V). Furthermore, the results showed that ELISA antigens, originally prepared against a human *Giardia* isolate, are also capable of detecting dog- and livestock-specific genotypes.

Cryptosporidium and *Giardia* were frequently found in environmental and faecal samples (I-V). In raw and treated municipal sewage sludge, *Giardia* was detected slightly more often than *Cryptosporidium* (I, IV; Table 10). The finding is in line with previous and later studies, in which more *Giardia* than *Cryptosporidium* in sewage were detected (Robertson *et al.*, 1995; Bukhari *et al.*, 1997; Robertson *et al.*, 2000; Heitman *et al.*, 2002). The finding is also concordant with the reported infection intensity; about 10 cryptosporidiosis and approximately 300 giardiasis cases are annually reported in Finland (Registry of Infectious Diseases, 2004). The number of cases reported, however, must be observed only as an indication of infection intensity since clearly not all cryptosporidiosis and giardiasis cases will be recorded in the National Infectious Disease Registry (NIDR). The statute of infectious diseases (1383/2003) by the Council of State in Finland stipulates that *Cryptosporidium* and *Giardia* belong to a group of microbial findings, which should be recorded in the NIDR by the laboratory. The true prevalence of giardiasis and cryptosporidiosis in a community may be underestimated, because the surveys are usually based on stool examinations from selected populations (Jakubowski *et al.*, 1991). Due to the mild nature of most *Cryptosporidium* and *Giardia* infections (Jokipii, 1971; Jokipii *et al.*, 1974; Jokipii *et al.*, 1983; Jokipii *et al.*, 1985a) probably not all in-

fected individuals seek medical aid. *Cryptosporidium* and *Giardia* were detected both in the sludge end-products and in samples taken during various composting processes, but the level of (oo)cysts remained low (up to 3 (oo)cysts/450 mg) in all positive samples (IV). However, variation in the efficiency of hygienization, as evaluated by indicator bacteria, was also noted within the WTPs employing similar treatment processes (Table 11).

Similar number of samples positive for *Cryptosporidium* (16/179) and *Giardia* (19/179) were detected in surface water samples (II, III). Using PCR, more samples were found to be positive for both *Cryptosporidium* and *Giardia* than by IF microscopy (Table 10). Only two samples were positive for *Cryptosporidium* and *Giardia* by both IF microscopy and PCR. The reason for the low concordance between IF microscopy and PCR results may be due to a possibly uneven division of the sample after immunocapture into two aliquots for IF microscopy and PCR (III) and the high detection level of the methods used. The detection limit in study II implicates that at least 50 (oo)cysts should be present in a surface water sample. Despite the high detection limit, the proportion of positive samples was similar to that obtained in the Norwegian prevalence study (Robertson *et al.*, 2001). This can be partly explained by the fact that 47 % of the *Giardia*-positive, and 14 % of the *Cryptosporidium*-positive samples were obtained from two rivers, Kokemäki and Aura (III), which increased the number of positive findings. In the Swedish study, (oo)cysts were detected somewhat more often than in the Norwegian study and in studies II and III (Hansen *et al.*, 1998; Robertson *et al.*, 2001).

In study II, primers LaxA/LaxB were selected to differentiate *C. parvum* from other *Cryptosporidium* spp. (Balatbat *et al.*, 1996). According to present knowledge, these primers can, in addition to *C. parvum*, also amplify DNA from some other *Cryptosporidium* species such as *C. hominis* and *C. meleagridis* (Guyot *et al.*, 2002). In study II, four water samples were positive for *Cryptosporidium* by targeting the COWP gene region. These samples were not amplified successfully with the primers LaxA/LaxB suggesting that the water samples studied had been contaminated with *Cryptosporidium* spp. other than aforementioned. However, since the sensitivity of the IC-PCR assay to detect *C. hominis* and *C. meleagridis* is unknown, the presence of these species in the water samples in study II can not be excluded. The frequent presence of *Cryptosporidium* and *Giardia* in Finnish surface water samples detected by the method developed in study II indicates that more precise occurrence studies with validated methods are required in the future.

In study III, *Cryptosporidium* and *Giardia* were found less frequently during winter than during spring, summer or autumn. In the case of water system contamination by agricultural discharges, (oo)cysts are usually detected after snowmelt, floods or rainfall during pasture season (Bodley-Tickell *et al.*, 2002). As a result of ice cover, winter runoffs to the water systems appear unlikely. The fact that (oo)cysts are not able to survive winter in Scandinavia (Robertson *et al.*, 2004) may also reduce the springtime (oo)cyst runoffs. Thus, the agricultural runoffs appear to be more likely during the summer and autumn months. However, no consensus regarding the occurrence of *Cryptosporidium* and *Giardia* by season in surface water exists, although a trend towards more infections in humans and calves during warm and wet seasons has been suspected (Tzipori *et al.*, 1983; Mata *et al.*, 1984; Rahaman *et al.*, 1984; Wallis *et al.*, 1996; Hansen *et al.*, 1998; Robertson *et al.*, 2001; Bodley-Tickell *et al.*, 2002).

In study V, a possible animal reservoir for human *Cryptosporidium* and *Giardia* infections was examined. Dogs were chosen since they are common pets and small children, also otherwise susceptible to crypto-

sporidiosis and giardiasis (Meloni *et al.*, 1993; Molbak *et al.*, 1993; Xiao *et al.*, 2001), easily come into contact with dogs or their droppings in the environment. Recent studies have confirmed that dogs may harbour zoonotic species and genotypes of *Cryptosporidium* and *Giardia* (Fayer *et al.*, 2001; Ponce-Macotela *et al.*, 2002; van Keulen *et al.*, 2002; Monis *et al.*, 2003a; Monis *et al.*, 2003b; Berrilli *et al.*, 2004; Hajdusek *et al.*, 2004; Traub *et al.*, 2004).

In study V, the presence of *Cryptosporidium* and *Giardia* in dog faeces was reported for the first time in Finland. Cryptosporidia, however, were sought for but not found in 55 asymptomatic dogs participating in a dog show in the 1980s (Jokipii *et al.*, 1985a). The overall prevalence of 5% for both *Cryptosporidium* and *Giardia* is in line with previous reports from other countries (Castor *et al.*, 1990; Nolan *et al.*, 1995; Fayer *et al.*, 2001; Jacobs *et al.*, 2001; Abe *et al.*, 2002; Capelli *et al.*, 2003; Hackett *et al.*, 2003; Traub *et al.*, 2004). We demonstrated that cryptosporidiosis and giardiasis are mostly infections of young dogs (V), as was also found in other studies (Hahn *et al.*, 1988; Sykes *et al.*, 1989; Castor *et al.*, 1990; Rahman, 1990; Jacobs *et al.*, 2001).

All *Cryptosporidium* and *Giardia* isolates found in study V were animal-specific. Dog-specific *C. canis* and *G. duodenalis* assemblages C and D were found and, interestingly, livestock-specific *G. duodenalis* assemblage E was also found in one dog. According to the owner, the dog had not had close contact with cattle (unpublished data). In previous studies *G. duodenalis* assemblages A-D have been isolated from canine faeces (Ponce-Macotela *et al.*, 2002; Berrilli *et al.*, 2004; Traub *et al.*, 2004). The fact that all species and genotypes found were animal-specific indicates a restricted risk for zoonotic infection (V). *Cryptosporidium canis* infections, however, have been suspected of causing diarrhoeal illness in young, immunocompetent children (Pedraza-Diaz *et al.*, 2001a; Xiao *et al.*, 2001; Learmonth *et al.*, 2004), in addition to immunocompromised patients (Pieniazek *et al.*, 1999; Gatei *et al.*, 2002; Cama *et al.*, 2003). Therefore, further studies determining the species and assemblages of human infections would be needed to obtain a brighter insight into the transmission epidemiology of *Cryptosporidium* and *Giardia*.

Land application of sewage sludge has increased for economic and environmental reasons. The aim of the European Commission Directive 86/278/EEC and the statute on amending sewage sludge on agricultural land (282/1994) by the Council of State in Finland is to protect the environment from pathogens present in sludge. The hygienic quality of water and sludge hygienization processes have traditionally been evaluated by analysis of indicator microorganisms (deBertoldi *et al.*, 1988; Tillett *et al.*, 2001). *Escherichia coli*, enterococci and *C. perfringens* are bacteria used as indicator organisms for microbial water hygiene worldwide and are present in raw sludge in large numbers (Payment *et al.*, 1993; Shaban, 1999; Scott *et al.*, 2002). Furthermore, the Drinking Water Directive 98/83/EEC proposes *C. perfringens* to be used as an indicator organism for the presence of *Cryptosporidium* (Briancesco *et al.*, 2005).

The most important characteristic of suitable indicator organisms for *Cryptosporidium* and *Giardia* is to show similar or greater resistance to various environmental or treatment conditions than the (oo)cysts do. This is a tough criterion, since *Cryptosporidium* and *Giardia* are known to be environmentally resistant (deRegnier *et al.*, 1989; Robertson *et al.*, 1992; Whitmore *et al.*, 1995). In studies III and IV, the occurrence of *Cryptosporidium* or *Giardia* in surface water or in treated sewage sludge did not correlate with any of the faecal indicator organisms, *E. coli*, enterococci or *C. perfringens*, or the turbidity of the water samples. Similarly, in a recent study, Harwood *et al.* (2005) found that faecal indicators could not predict the presence of

Cryptosporidium and *Giardia* in reclaimed wastewater. In an Italian study, no correlation between *Cryptosporidium* and *Giardia* compared to the presence of *E.coli* or *C. perfringens* could be detected in sewage and surface water, whereas the presence of cysts and oocysts correlated with each other and with enterococci (Briancesco *et al.*, 2005). The absence of correlation between the indicators and (oo)cysts in study IV may reflect the occasional appearance of *Cryptosporidium* and *Giardia* in surface waters and in treated sludge or the different survival, density and recovery rates of the (oo)cysts compared with indicator organisms. In fact, very low numbers of (oo)cysts were detected in treated sludge compared with the counts of indicator organisms in study IV (Table 11). Furthermore, anaerobic conditions in the digestion phase may have enhanced the proliferation of *C. perfringens* at the WTPs using mesophilic anaerobic digestion. Whatever the reason for the absence of correlation between *Cryptosporidium* or *Giardia* and indicator parameters in surface water or in treated sludge may be, the finding indicates that direct analysis is the most reliable way to confirm the presence of (oo)cysts in both surface water and sewage sludge.

Cryptosporidium and *Giardia* are significant waterborne pathogens in both the developed and developing countries, because the infections are endemic in many parts of the world and the parasites are highly infectious. Furthermore, the densities of (oo)cysts excreted by the infected individuals are sufficient to contaminate the environment effectively. The quality of surface water used for drinking water production, irrigation or recreation is a significant factor for human and animal health and wellbeing worldwide. Unfortunately, considering the environmental cycle of *Cryptosporidium* and *Giardia*, breaking the cycle of transmission remains a huge challenge especially in the developing countries, due to the lack of clean water, limited hygienic conditions and high transmission pressure.

11 Conclusions

1. IC-PCR assays for detecting *Cryptosporidium* and *Giardia* in sewage sludge and in surface water were developed. The assays were applied in studies in which the occurrence of *Cryptosporidium* and *Giardia* in Finland's environment were determined.

2. *Cryptosporidium* and *Giardia* could be found both in untreated and treated sewage sludge and in surface water, indicating that these protozoans cycle in the Finland's environment.

3. No statistically significant correlation between indicator parameters (occurrence of *C. perfringens*, *E. coli*, enterococci and water turbidity) and occurrence of *Cryptosporidium* and *Giardia* was detected, indicating that direct analysis is the best way to confirm the presence of *Cryptosporidium* and *Giardia* in sewage sludge and surface water.

4. To detect *Cryptosporidium* and *Giardia* in canine faecal samples, the *Giardia* ELISA performed well (sensitivity 100%, specificity 96%) and was capable of detecting dog- and livestock-specific genotypes. The accuracy of the *Cryptosporidium* ELISA was low (sensitivity 71%, specificity 94%), and due to a high rate of false-positive results and low diagnostic sensitivity, it cannot be recommended for detection of *Cryptosporidium* in canine faeces.

5. *Cryptosporidium* and *Giardia* were frequent findings in asymptomatic dogs. Since all species and genotypes found are considered animal-specific, the zoonotic transmission risk from these dogs appears restricted. However, since *C. canis* is known to cause human infections occasionally, much is still to be learned of the transmission epidemiology of *Cryptosporidium* and *Giardia*.

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