Water-borne transmission of *Cryptosporidium* and *Giardia* in Belgium and Bangladesh

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I dedicate this thesis to
my family, my wife, Zakia, my mother and my sweet daughter, Momo
for their constant support and unconditional love.
I love you all dearly

Table of Contents

List of Figures

List of Tables

List of Abbreviations

18S rDNA	18S ribosomal DNA
β-giardin	Beta-giardin
AFNOR	Association Française de Normalisation
CDC	Centers for Disease Control and Prevention
CFC	Continuous flow centrifugation
CFU	Colony-forming units
CI	Confidence interval
COWP	*Cryptosporidium* oocyst wall protein
CPG	Number of *Giardia* cysts per gram of faeces
DAPI	4', 6-diamidino-2-phenylindole
DIC	Differential interface contrast
DNA	Deoxyribonucleic acid
DWTP	Drinking water treatment plant
FACS	Fluorescence activated cell sorter
FCM	Flow cytometry
FISH	Fluorescent *in situ* hybridization
FITC	Fluorescein isothiocyanate
GDH	Glutamate dehydrogenase
GP60	60kDa glycoprotein
GPS	Global positioning system
HCT	Human colonic tumor
HFUF	Hollow-fiber ultrafiltration
HMSO	Her Majesty's Stationery Office
HSP-70	70kDa heat shock protein
IFA	Immunofluoresence assay
IMS	Immunomagnetic separation
ISO	International Organisation for Standardisation
mAbs	Monoclonal antibodies
MF	Membrane filter
mRNA	Messenger RNA
NCBI	National Center for Biotechnology Information
NTU	Nephelometric turbidity units
OPG	Oocysts per gram of faeces
PCFC	Portable continuous flow centrifugation

PCR	Polymerase chain reaction
PI	Propidium iodide
qPCR	Quantitative polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT-PCR	Reverse transcriptase PCR
SCA	Standing Committee of Analysts
SSU	Small subunit
TPI	Triose phosphate isomerase
UCP	Ungar *Cryptosporidium parvum*
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
WASA	Water Supply and Sewerage Authority
WIV	Wetenschappelijk Instituut Volksgezondheid
WHO	World Health Organization
WSP	Water Safety Plan
WWTP	Wastewater treatment plant

Chapter 1

Water-borne transmission of *Cryptosporidium* and *Giardia*: A literature review

1.1 General introduction

Access to safe drinking water and basic sanitation is a fundamental human right, but currently more than one billion people worldwide do not have access to either safe drinking water or adequate sanitation. It is estimated that almost 60% of deaths following diarrhoeal diseases in developing countries are attributable to lack of access to safe drinking water and basic sanitation, children under 5 being at the highest risk. Important water-borne diarrhoeal diseases are cryptosporidiosis and giardiosis (Cacciò and Pozio, 2001; Xiao and Fayer, 2008; Geurden *et al*., 2009). These diseases are caused by the protozoan parasites *Cryptosporidium* and *Giardia*, respectively, which are able to cause disease in humans and animals. Because of their impact on socio-economic development, especially in developing countries, both *Cryptosporidium* and *Giardia* are since 2004 included in the 'Neglected Disease Initiative' of the World Health Organization (WHO) (Savioli *et al.*, 2006).

However, *Cryptosporidium* and *Giardia* also pose an important risk on the safety of drinking water in developed countries. For example, in 1993 more than 400,000 people were affected by cryptosporidiosis in Milwaukee (Wisconsin, USA) due to an ineffective filtration process in the production of drinking water. Since this outbreak screening of tap water for the presence of *Cryptosporidium* has become compulsory in the UK, The Netherlands and the USA, but water-borne outbreaks are still reported on a regular basis.

The aim of this literature review is to first introduce *Cryptosporidium* and *Giardia* by addressing their taxonomy, life cycle, prevalence and clinical importance for both human and animals. Second, we will focus on the transmission of *Cryptosporidium* and *Giardia*. For this, we will discuss the different transmission cycles of both parasites (zoonotic versus anthroponotic transmission). Subsequently, we will elaborate more on their water-borne transmission through drinking and recreational water. An overview is given of water-borne outbreaks, sources of water contamination and current systems to monitor the occurrence of *Cryptospordium* and *Giardia* in drinking and recreational water in different countries. We will end this chapter with a summary of the different methods for detecting and quantifying *Cryptosporidium* and *Giardia* in water.

1.2 Water-borne parasites: *Cryptosporidium* and *Giardia*

1.2.1 *Cryptosporidium*

1.2.1.1 Taxonomy

Cryptosporidium (subphylum Apicomplexa) is a cluster of parasites that infects the microvillus region of epithelial cells in the digestive and respiratory tract of humans and animals. *Cryptosporidium* spp. infect a wide range of hosts, including 155 mammalian species (Fayer, 2004). Currently 27 different species of *Cryptosporidium* (Table 1.1) and over 40 genotypes are recognized (Ng *et al.*, 2011). Most human infections are caused by *C. hominis* (previously known as *C. parvum* human genotype or genotype 1 or H) and *C. parvum* (previously known as *C. parvum* bovine genotype or genotype 2 or C) (Cacciò *et al.*, 2005). Slapeta has proposed to rename *C. parvum* as *C. pestis* (Slapeta, 2006), but this new nomenclature has not yet been widely accepted, mainly due to lack of taxonomic description (Xiao *et al*., 2012). In addition to those, *C. meleagridis*, *C. felis*, *C. canis*, *C. suis*, *C. muris*, *C. andersoni*, *C. ubiquitum*, *C. viatorum*, *C. cuniculus* and the *Cryptosporidium* horse, skunk and chipmunk I genotypes also have been detected in stool of immunocompetent and immunocompromised humans (Fayer, 2010; Xiao, 2010; Elwin *et al*., 2012; Kváč *et al*., 2013). *C. parvum* is the major zoonotic species causing cryptosporidiosis in livestock, which has a major contribution to environmental contamination (Smith *et al.*, 1995). Different molecular diagnostic tools have been used in the differentiation of *Cryptosporidium* species/genotypes and *C. parvum* and *C. hominis* subtypes (see section 1.4.5.1).

Table 1.1: Currently recognised species of *Cryptosporidium*, along with their major and minor host range and location (Jirků *et al.*, 2008; Fayer *et al.*, 2010; Robinson *et al*., 2010; Smith and Nichols, 2010; Elwin *et al*., 2012; Ren *et al*., 2012; Kváč *et al*., 2013).

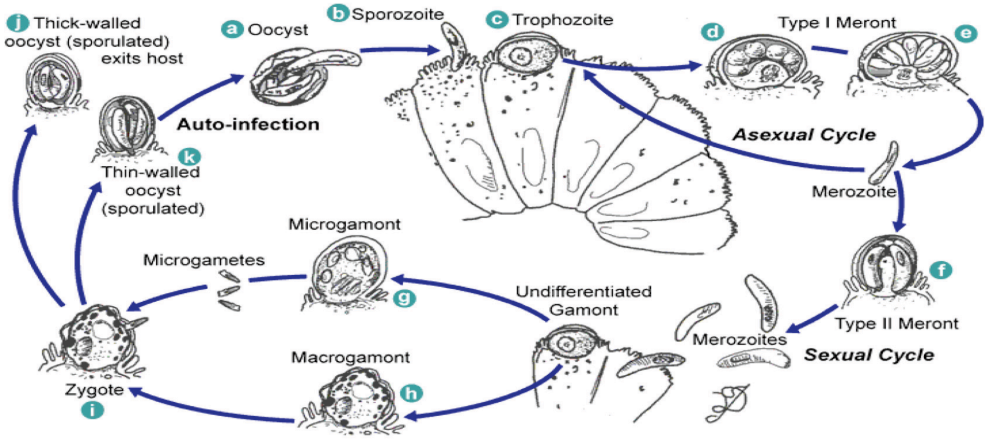
Species	Major hosts	Minor hosts	Location
C. hominis	Humans	Dugong, sheep	Small intestine
C. viatorum	Humans	Not known	Not known
C. parvum	Cattle, humans	Deer, mice, pigs	Small intestine
C. andersoni	Cattle	Sheep	Abomasum
C. bovis	Cattle	Sheep	Small intestine
C. ryanae[1]	Cattle	Not known	Not known
C. ubiquitum[2]	Cattle	Humans, sheep, deer	Small intestine
C. xiaoi[3]	Sheep	Yak, goat	Not known
C. suis	Pigs	Humans	Small and large intestine
C. scrofarum[4]	Pigs	Pigs	Small intestine
C. canis	Dogs	Humans	Small intestine
C. felis	Cats	Humans, cattle	Small intestine
C. meleagridis	Turkey, humans	Parrots	Small intestine
C. baileyi	Poultry	Quails, ostriches, ducks	Small intestine
C. galli	Finches, chicken	Not known	Proventriculus
C. wrairi	Guinea pigs	Not known	Small intestine
C. fayeri	Red kangaroo	Not known	Small intestine
C. macropodum	Eastern grey kangaroo	Not known	Not known
C. serpentis	Lizards, snakes	Not known	Stomach
C. varanii[5]	Lizards	Snakes	Stomach and intestine
C. molnari	Fish	Not known	Stomach and intestine
C. scophthalmi	Fish	Not known	Stomach and intestine
C. fragile	Toads	Amphibians	Stomach
C. cuniculus[6]	Rabbit	Humans	Small intestine
C. tyzzeri[7]	Mouse	Human, ruminants	Small intestine
C. muris	Rodents	Humans, hyrax, goat	Stomach

[1]previously known as deer-like genotype, [2]previously known as cervine genotype, [3]previously known as *C. bovis*-like genotype, [4]previously known as pig genotype II, [5]previously known as *C. saurophilum*, [6]previously known as rabbit genotype, [7]previously known as mouse genotype I

1.2.1.2 Life cycle

Cryptosporidium has a complex life cycle with an asexual and a sexual developmental phase. The life cycle can be completed within 3 to 5 days in the small intestine of the host. The life cycle (Figure 1.1) initiates with the ingestion of the infective oocyst (a) and the excystation of 4 motile sporozoites (b) (Hijjawi *et al.*, 2002; Smith *et al.*, 2005), which subsequently infect the apex of the epithelial cells of the small intestine. In the cells, the sporozoites lie protected between the cell membrane and the cell cytoplasm, the so-called parasitophorous vacuoles. After cell invasion the sporozoite forms a trophozoite (c) which undergoes asexual development with two successive generations of merogony, resulting in the formation of meronts (d, e and f). Merozoites develop into sexual developmental stages known as the micro- and macrogamonts (g and h), which further develop into micro- and macrogametes. The microgametes are released from the host cells and penetrate cells harbouring macrogametes. Their union results in a zygote (i), which further develops into an oocyst with resistant oocyst wall. Approximately 80% of the oocysts (j) have a thick wall and are excreted in the faeces. These thick-walled oocysts are the environmentally resistant stage of the parasite. The remaining 20% are known as thin-walled oocysts (k) and are believed to contribute to autoinfection through the continous recycling of sporozoites from ruptured thin-walled oocysts (Hijjawi *et al.*, 2001).

Figure 1.1: *Cryptosporidium* life cycle with (a) the infective oocyst; (b) the sporozoite released from the oocyst; (c) the trophozoite; (d) and (e) type I meront; (f) type II meront; (g) microgamont; (h) macrogamont; (i) zygote (Source accessed on November 4th 2011: http://www.dpd.cdc.gov/dpdx/HTML/Cryptosporidiosis.htm)



1.2.1.3 *Cryptosporidium* in humans

1.2.1.3.1 Prevalence

In developed countries, the prevalence of *Cryptosporidium* generally is low in asymptomatic people (<1%) and in patients with diarrhoea (1-2%) (Current and Garcia, 1991; Guerrant, 1997; Geurden *et al.*, 2009). In developing countries, high rates of asymptomatic carriage (10-30%) are common in comparision to patients with gastroenteritis (3-20%) (Current and Garcia, 1991; Haque *et al.*, 2003). Among the common *Cryptosporidium* species in humans, *C. parvum* and *C. hominis* are responsible for >90% of human cases of cryptosporidiosis in developed nations (Xiao and Feng, 2008). The distribution of *C. parvum* and *C. hominis* in humans differs between geographic regions. In Europe, both *C. parvum* and *C. hominis* are common in humans (Leoni *et al.*, 2006; Chalmers *et al.*, 2009; Zintl *et al.*, 2009). In the Middle East, *C. parvum* is the dominant species in humans (Sulaiman *et al.*, 2005; Pirestani *et al.*, 2008). Geographic variations in the distribution of *C. parvum* and *C. hominis* can also occur within a country. For example, *C. parvum* is more common than *C. hominis* in rural areas in the United States and Ireland (Feltus *et al.*, 2006; Zintl *et al.*, 2009). In the rest of the world, especially developing countries, *C. hominis* is usually the predominant species in humans, responsible for 70-90% of the infections (Xiao and Feng, 2008). This suggests that zoonotic infection is much less common in developing countries than in developed countries.

Temporal and age-associated differences in the distribution of *C. parvum* and *C. hominis* infections have been reported. Peaks in *Cryptosporidium* infections have been observed in spring and late summer (Casemore, 1990; van Asperen *et al.*, 1996). *C. parvum* was more prevalent in spring (in Ireland, the United Kingdom and New Zealand) and *C. hominis* was more prevalent in autumn (in the Netherlands, the United Kingdom and New Zealand) (McLauchlin *et al.*, 2000; Learmonth *et al.*, 2003, 2004; Smerdon *et al*., 2003; Hunter *et al.*, 2004; Wielinga *et al.*, 2008; Chalmers *et al*., 2009; Zintl *et al.*, 2009). In The Netherlands, *C. hominis* was more commonly found in children and *C. parvum* more in adults (Wielinga *et al.*, 2008). In the UK, *C. hominis* was more prevalent in infants less than one year, females aged 15-44 years and international travelers (Chalmers *et al.*, 2008, 2009). *C. viatorum* was identified among travellers with gastro-intestinal symptoms returning to Great Britain from the Indian subcontinent (Elwin *et al*., 2012). In South American countries, a relatively high

proportion of *C. meleagridis* infections has been identified in children and in immunocompromised patients (Cama *et al*., 2007, 2008; Meireles, 2010).

1.2.1.3.2 Clinical importance

Cryptosporidium is reported to infect people in at least 106 countries (Fayer, 2008). The most common clinical feature of cryptosporidiosis is diarrhoea. Characteristically, the diarrhoea is profuse and watery; it may contain mucus but rarely blood and leucocytes and it is often associated with weight loss. Other less common clinical features include abdominal pain, nausea, vomiting and low-grade fever. Occasionally, nonspecific symptoms such as myalgia, weakness, malaise, headache and anorexia occur (Current and Garcia, 1991).

The severity of a *Cryptosporidium* infection can vary from an asymptomatic shedding of oocysts to a severe and life-threatening disease. The duration and the severity of the symptoms and the outcome may vary with host factors such as the immune status of the person. Most immunocompetent persons experience a short-term illness with complete and spontaneous recovery (Current and Garcia, 1991). However, for immunocompromised patients, cryptosporidiosis can be a critical illness with persistent symptoms leading to dehydration and wasting (O'Donoghue, 1995; Chen *et al*., 2002; Blackburn *et al*., 2004), and eventually leading to death (Juranek, 1995; Manabe *et al*., 1998). In addition, *Cryptosporidium* infections can cause atypical manifestations in immunocompromised patients, such as biliary tract disease, respiratory tract disease and pancreatitis (Hunter and Nichols, 2002).

The severity of the infection is also related to the age of the patient. Diarrhoea is a leading cause of illness and death among children aged <5 years in developing countries and *Cryptosporidium* is one of the most important diarrhoeal pathogens (Shirley *et al*., 2012; Kotloff *et al*. 2013). Children are more likey to be infected with *Cryptosporidium,* which can be explained by a lack of an effective immunity at this age. In a study of 191 children with *C. parvum* in Uganda, 13% died, compared with 6% for children without *C. parvum* (Tumwine *et al.*, 2003). Wielinga *et al.* (2008) found that the majority (80%) of the human cases were children aged between 0 and 9 years and >70% of these were caused by *C. hominis*. *C. hominis* is more common than *C. parvum* in children and is associated with heavier infections and greater growth shortfalls, even in the absence of symptoms (Bushen *et al.*, 2007). Patients >25 years of age were infected mainly with *C. parvum*.

The clinical symptoms may also depend on the parasite species involved. Infections with *C. hominis* are associated with diarrhoea, nausea, vomiting, malaise and non-intestinal sequelae such as joint pain, eye pain, recurrent headache and fatigue, whereas infections with *C. parvum*, *C. meleagridis*, *C. canis* and *C. felis* cause only diarrhoea (Bouzid *et al*., 2013).

1.2.1.4 *Cryptosporidium* in animals

1.2.1.4.1 Prevalence

Cattle are commonly infected with *C. parvum*, *C. andersoni, C. bovis* and *C. ryanae* (Xiao, 2010). In dairy cattle, *C. parvum* is mostly found in pre-weaned calves, *C. bovis* and *C. ryanae* in weaned calves and *C. andersoni* in yearlings and adult cattle (Fayer *et al.*, 2006b, 2007; Santín *et al.*, 2008). Parasite prevalence varies from 1% (Kváč *et al.*, 2006) to 59% (Olson *et al.*, 1997) in individual calves and up to 100% on farm level (Santín *et al.*, 2004). The highest prevalence is observed in calves under the age of 5 weeks (Quílez *et al.*, 1996).

The prevalence of *Cryptosporidium* in flocks of small ruminants varies considerably, ranging from 5% to 70% for **sheep** and from 5% to 35% for **goats**. This difference in prevalence results can be explained by differences in age of the animals, management, and diagnostic methods applied (Robertson, 2009). *C. xiaoi*, *C. ubiquitum* and *C. parvum* are the predominant species in small ruminants (Ryan *et al.*, 2005; Goma *et al.*, 2007; Santín *et al.*, 2007; Geurden *et al*., 2008a; Mueller-Doblies *et al.*, 2008; Pritchard *et al.*, 2007, 2008; Quílez *et al.*, 2008; Yang *et al.*, 2009; Díaz *et al.*, 2010;Wang *et al*., 2010; Tzanidakis *et al*., 2014).

In **pigs**, herd prevalences range from 8% to 100% with individual animal infection rates of between 1% and 34%. *C. suis* (36-83%) and *C. scrofarum (*formerly *Cryptosporidium* pig genotype II) (9-61%) are the major *Cryptosporidium* spp. (Wieler *et al*., 2001; Kváč *et al.*, 2009; Chen *et al.*, 2011; Budu-Amoako *et al.*, 2012). *C. suis* preferentially infects suckling piglets, whereas *C. scrofarum* is more frequently found in weaners (Langkjaer *et al.*, 2007; Johnson *et al.*, 2008; Kváč *et al.*, 2009; Yin *et al*., 2013; Zhang *et al*., 2013). Occasionally, *C. muris* (Kváč *et al*., 2009; Budu-Amoako *et al.*, 2012; Němejc *et al*., 2013) or *C. tyzzeri* (Kváč *et al.*, 2012) are found in pigs. With the exception of one study where *C. parvum* was the predominant species (Farzan *et al*., 2011), this species is less frequently found in pigs, suggesting that pigs are not an important source of zoonotic transmission (Wieler *et al*., 2001; Chen and Huang, 2007; Zintl *et al.*, 2009; Budu-Amoako *et al.*, 2012; de la Fé Rodríguez *et*

al., 2013; Němejc *et al*., 2013).

Low prevalences of *Cryptosporidium* have been reported in **horses** in the USA (7%) and Italy (8%) (Burton *et al.*, 2010; Veronesi *et al.*, 2010). Both *C. parvum* and *Cryptosporidium* horse genotype were found in horses (Ryan *et al.*, 2003a; Chalmers *et al.*, 2005; Grinberg *et al.*, 2008; Veronesi *et al.*, 2010) and rarely the hedgehog genotype (Laatamna *et al*., 2013). These findings support a potential role of infected horses in zoonotic transmission.

A *Cryptosporidium* prevalence ranging from 0% to 13% has been reported in privately owned and stray **dogs** (Chermette and Blondel, 1989; Grimason *et al.*, 1993; Diaz *et al.*, 1996; Giangaspero *et al.*, 2006; Claerebout *et al*., 2009; Yoshiuchi *et al.*, 2010; Bajer *et al.*, 2011). Most infections in dogs are caused by the host-specific *C. canis*. In addition to *C. canis*, other *Cryptosporidium* spp. were detected occasionally in dogs such as *C. muris* (Lupo *et al*., 2008; Ellis *et al*., 2010), *C. parvum* (Hajdušek *et al*., 2004; Giangaspero *et al*., 2006; Sotiriadou *et al*., 2013) and *C. meleagridis* (Hajdušek *et al*., 2004). Thus the risk of zoonotic transmission from *Cryptosporidium*-infected dogs is low (Lucio-Foster *et al.*, 2010; Uehlinger *et al*., 2013).

Cryptosporidium has been detected in **cats** with a range of 2% to 25% (Rambozzi, *et al*., 2007; Hoopes *et al*., 2013). In addition to the cat specific species, *C. felis, C. parvum* and *C. muris* were also identified (Palmer *et al*., 2008; Yoshiuchi *et al*., 2010; FitzGerald *et al*., 2011; Scorza *et al*., 2011; Sotiriadou et *al*., 2013).

Cryptosporidium can also infect a wide range of wildlife including **artiodactyls**. *Cryptosporidium* was found in different species of deer with prevalence rates between 0% and 27% (Heuschele *et al*., 1986; Rickard *et al*., 1999; Sturdee *et al*., 1999; Gomez *et al*., 2000; Perz and Le Blancq, 2001; Heitman *et al*., 2002; Siefker *et al*., 2002; Delgado *et al*., 2003; Hamnes *et al*., 2006; Paziewska *et al*., 2007; Castro-Hermida *et al.*, 2011a). *Cryptosporidium* oocysts have also been detected in buffalos, elephants, wild boars, impalas, lowland anoas, prairie bison, bongos, greater kudus, gemsboks, black wildebeests, tule elks, sable antelopes, caribous, springboks and oryxes (Van Winkle, 1985; Heuschele *et al*., 1986; Mtambo *et al*., 1997; Deng and Cliver, 1999; Delgado *et al*., 2003; Hamnes *et al*., 2006; Abu Samra *et al.*, 2011; Castro-Hermida *et al.*, 2011a). *C. ubiquitum*, the deer genotype and *C. parvum* were found most frequently in these animals (Perz and Le Blancq, 2001; Siefker *et al*., 2002; Xiao *et al*., 2002; Alves *et al*., 2003; Ryan *et al*., 2003b; Hajdušek *et al*., 2004; Feng *et al*., 2007; Karanis *et al*., 2007b; Abu Samra *et al.*, 2011).

Cryptosporidium has been detected in a wide range of **wild carnivores** including foxes, wild dogs, dingo's, mink, raccoon, river otter, coyote, grey wolf, feral cat, bobcat, badger, onchilla, striped skunk and ermine (Mtambo *et al*., 1991; Sturdee *et al*., 1999; Nutter *et al*., 2004; Bednarska *et al*., 2007; Feng *et al*., 2007; Gaydos *et al*., 2007; Paziewska *et al*., 2007; Ziegler *et al*., 2007b; Wang *et al*., 2008; Holsback *et al*., 2013). Wild canids are most frequently infected with *C. canis*. However, *C. hominis* was identified in dogs in Sydney (Ng *et al*., 2011) and some carnivores are infected with multiple *Cryptosporidium* species and genotypes (Feng, 2010). For example, foxes are naturally infected with the *Cryptosporidium* fox genotype, *C. canis* fox subtype and *C. canis* dog subtype (Xiao *et al*., 2002).

Cryptosporidium prevalence rates vary greatly between different **rodent and lagomorph** species in different countries. *Cryptosporidium* has been detected in voles, shrew, muskrats, squirrels, chipmunks, woodchucks, rabbits, beavers, capybaras and porcupines (Klesius *et al*., 1986; Yamura *et al*., 1990, Laakkonen *et al*., 1994; Webster and Macdonald, 1995; Chalmers *et al*., 1997; Sinski *et al*., 1998; Torres *et al*., 2000; Perz and Le Blancq, 2001; Bajer *et al*., 2002; Xiao *et al*., 2002; Atwill *et al*., 2004; Fayer *et al*., 2006a; Bednarska *et al*., 2007; Feng *et al*., 2007; Foo *et al*., 2007; Meireles *et al*., 2007; Paziewska *et al*., 2007; Ziegler *et al*., 2007a,b). Host adapted as well as potentially zoonotic *Cryptosporidium* species and genotypes were found in rodents such as C. *parvum*, *C. ubiquitum* and *C. meleagrides* (Perz and Le Blancq, 2001; Feng *et al*., 2007).

Cryptosporidium was detected in **non-human primates** such as lemurs, langurs, sakis, macaques, guenon monkeys, siamangs, vervets, mangabeys, spider monkeys, baboons and gorillas, (Heuschele *et al*., 1986; Miller *et al*., 1990; Nizeyi *et al*., 1999; Gomez *et al*., 2000; Graczyk *et al*., 2001; Hope *et al*., 2004; Legesse and Erko, 2004; Ekanayake *et al*., 2006; Rasambainarivo *et al*., 2013). Genotype analysis revealed *C. ubiquitum* in lemurs (da Silva *et al*., 2003), *C. parvum* in mountain gorillas (Nizeyi *et al*., 1999; Graczyk *et al*., 2001) and *C. hominis* monkey genotype in captive rhesus monkeys (Xiao *et al*., 2002).

Cryptosporidium prevalences of 5%-7% have been reported in **wild birds** (Ng *et al*., 2006; Nakamura *et al*., 2009; Sevá Ada *et al.*, 2011). *Cryptosporidium* species included *C. galli*, *C. baileyi* and avian genotype II (Santos *et al*., 2005; Meireles *et al*., 2006; Amer *et al*., 2010; Molina-López *et al*., 2010; Sevá Ada *et al.*, 2011). Although *C. parvum* infections have been detected in wild birds, they were thought to be the result of mechanical transmission rather than an established infection (Dieter *et al*., 2001; Zhou *et al*., 2004; Majewska *et al*., 2009).

C. meleagridis was not found in wild birds (Ng *et al*., 2006; Nakamura *et al*., 2009; Sevá Ada *et al.*, 2011).

1.2.1.4.2 Veterinary importance

C. parvum is a well-known cause of diarrhoea in neonatal ruminants. Clinical symptoms are most frequently observed in **calves** between the age of 5 days and 1 month and include profuse watery diarrhoea with acute onset, lethargy, anorexia and dehydration, which is usually self-limiting within 2 weeks (O'Handley *et al.*, 1999, Schnyder *et al*., 2009). Mortality is variable and is most often observed in calves with multiple infections and in certain beef breeds, such as the Belgian Blue and White (de Graaf *et al.*, 1999) and it can be as high as 30% (Olson *et al.*, 2004). However, morbidity in endemic herds can be as high as 100% (Santín *et al*., 2008). Abomasal cryptosporidiosis, caused by *C. andersoni*, does not result in any visible clinical signs (Kváč *et al*., 2008). Infections with *C. andersoni* do not cause diarrhoea and follow a more chronic course than infections with *C. parvum* (Kváč and Vitovec, 2003). *C. andersoni* infections may result in a decrease in daily weight gain, decreased feed efficiency and less milk production (Anderson, 1987; Esteban and Anderson, 1995; Ralston *et al.*, 2003).

Cryptosporidium is a major cause of neonatal diarrhoea in **lambs**, usually within the first 2 weeks of life and diarrhoea can be mild to severe. Cryptosporidiosis outbreaks in lambs are most common in crowded flocks and are associated with a decrease in liveweight, dressing percentage, growth rate and carcass productivity (Angus *et al*., 1982; Alonso-Fresán *et al*., 2005; Sweeny *et al*., 2011; de Graaf *et al*., 1999; Sari *et al*., 2009). Caprine cryptosporidiosis is characterised by diarrhoea and mortality in **kids**. Morbidity can reach 100% and mortality 50% in some herds (Vieira *et al*., 1997; Johnson *et al*., 1999; Sevinc¸ *et al*., 2005; Paraud *et al*., 2010; Santín, 2013).

Diarrhoea is the major clinical sign in **foals** affected by cryptosporidiosis. Foals are more susceptible to the infection than older animals (Majewska *et al*., 1999; Grinberg *et al*., 2003; 2009; Veronesi *et al*., 2010) and most *Cryptosporidium* infections in adult horses are asymptomatic (Majewska *et al*., 1999, 2004; Sturdee *et al*., 2003).

Inappetance, depression, vomiting and/or diarrhoea developed in **piglets** experimentally infected with *C. parvum,* whereas mild or no clinical signs developed with *C. suis* (Enemark *et al*., 2003). However, an association between diarrhoea and infections with *C. suis* and *C.*

scrofarum in nursing piglets has been described (Hamnes *et al*., 2007). In contrast, other studies did not find any significant association between diarrhoea and cryptosporidial infections (Quilez *et al*., 1996; Maddox-Hyttel *et al*., 2006, Vitovec *et al*., 2006).

Cryptosporidiosis in **dogs** has been reported in both asymptomatic and diarrhoeic dogs (Santín and Trout, 2008). Infections with *C. canis* are usually asymptomatic but severe diarrhoea, malabsorption, weakness and weigth loss have been reported (Irwin, 2002; Miller *et al*., 2003). Dogs infected with *C. muris* showed chronic vomiting and profuse diarrhoea in one study (Ellis *et al*., 2010) but in another study no gastrointestinal signs were observed (Lupo *et al*., 2008).

Cryptosporidium oocysts were detected more frequently in **cats** without diarrhoea than in cats with diarrhoea (Sabshin *et al*., 2012) and shedding of *Cryptosporidium* oocysts without the presence of clinical signs was reported in experimentally and naturally infected cats (Mtambo *et al*., 1991; Nash *et al*., 1993; Fayer *et al*., 2006c). However, oocysts were also detected in the faeces of cats with persistent diarrhoea (Goodwin and Barsanti, 1990; Lent *et al*., 1993; Morgan *et al*., 1998).

1.2.2 *Giardia*

1.2.2.1 Taxonomy

Members of the genus *Giardia* are eukaryotic organisms and belong to the Class of Zoomastigophorea, Order Diplomonadida and Family Hexamitidae. The Hexamitidae are diplozoic flagellated protozoa that possess paired organelles, including two equivalent nuclei. The ventral adhesive disc delineates *Giardia* from other Hexamitidae. The taxonomy and host specificity of this organism are still a matter of debate. Six species of *Giardia* have been distinguished on the basis of light microscopic (shape of trophozoit and median body) and electro-microscopic (feature of ventrolateral flange, marginal groove, ventral disc, flagellum) characteristics (Adam, 2001). Five species are represented by isolates from amphibians (*G. agilis*), birds (*G. ardeae*, *G. psittaci*), mice (*G. muris*) and voles (*G. microti*) (McRoberts *et al.*, 1996; Adam, 2001; Cacciò *et al.*, 2005). The sixth species includes *Giardia* strains isolated from a large range of other mammalian hosts, grouped by Filice (1952) into a single species, *G. duodenalis,* because they share morphological features and in particular, have similar median body structures.

Based on genetic analysis *G. duodenalis* can be considered as a species complex with eight distinct assemblages (A to H) (Monis *et al.*, 2003; Lasek-Nesselquis *et al.*, 2010). Assemblages A and B are responsible for human infection and are also found in a wide range of mammals. Assemblages are subsequently further classified into sub-assemblages, including AI to AIII for assemblage A and BIII, BIII-like, BIV and BIV-like for assemblage B. Sub-assemblage AI is predominantly found in livestock and companion animals whereas AII is found mostly in humans. AIII is predominant in wild livestock and never found in humans. Sub-assemblage BIII is predominantly found in humans whereas BIV is mainly found in wildlife and dogs but also in humans, albeit less frequently than BIII (Levecke *et al*., 2009; Sprong *et al*., 2009). The remaining assemblages show more restricted host ranges: C and D are found in canids, E in livestock, F in cats and G in rodents (Cacciò and Ryan, 2008). Since the genetic distance separating these assemblages within *G. duodenalis* is at the same level as that separating *G. duodenalis* from other recognised species of *Giardia* (Mayrhofer *et al.*, 1995; Monis *et al.*, 1996, 1998), species names for each of these assemblages have been proposed (Thompson and Monis, 2004). However, this novel nomenclature is currently not widely accepted, and hence it will not be discussed in further details in this thesis. Table 1.2 summarizes the host range for the 6 esthablished *Giardia* species.

Table 1.2 The esthablished *Giardia* species and *G. duodenalis* assemblages with their corresponding host range

***Giardia* species**	**Host range**
G. agilis	Amphibians
G. ardeae	Birds
G. psittaci	Budgerigar
G. microti	Rodents
G. muris	Rodents
G. duodenalis	
Assemblage A	Man, dog, cat, livestock,...
Assemblage B	Man, dog, cat, rodents,...
Assemblage C / D	Dog, coyote, mouse, pigs
Assemblage E	Livestock
Assemblage F	Cat, pigs
Assemblage G	Rat
Assemblage H [1]	Seal

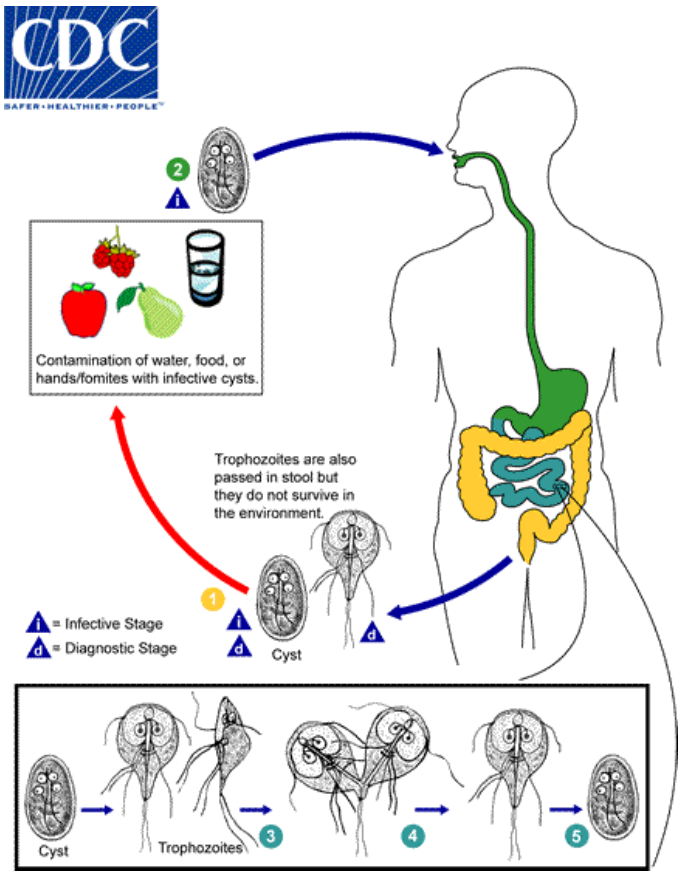
[1](Lasek-Nesselquis *et al*., 2010)

1.2.2.2 Life cycle

Giardia has a simple and direct life cycle (Figure 1.2) consisting of two stages: an infectious cyst (1, 2), which is resistant to many environmental stressors and a trophozoite stage, which colonizes the intestinal lumen of the host and is responsible for the clinical symptoms. After oral ingestion, the trophozoite emerges from the cysts and completes a mitotic division to produce two trophozoites (3) that attach to the epithelial cells by their adhesive disc and feed on the epithelial cell. For the colonisation of the duodenum and the jejunum, attachment to epithelial cells is essential. Unlike *Cryptosporidium*, the trophozoites of *Giardia* are not invasive. They multiply (4) asexually by binary fission in the lumen of the small intestine, although a sexual phase has been suggested (Meloni *et al.*, 1989). Finally, exposure to biliary salts leads to encystation (5) of trophozoites in the jejunum. Cysts are elliptical and 8-12 µm long and 7-10 µm wide. Cysts are passed in the faeces and are immediately infectious upon excretion (Thompson *et al.*, 1993; Adam, 2001). After ingestion of *Giardia* cysts, there is an

incubation period that typically lasts 3 to 25 days, although this can range widely, from 1 to 45 days (Ortega and Adam, 1997).

Figure 1.2 Life cycle of *Giardia* spp. with infective cyst (1, 2, 5), two trophozoites in excystation stage (3) and two trophozoites in asexual multiplication stage (4). (Source accessed on March 23, 2015: http://www.cdc.gov/dpdx/giardiasis/).



1.2.2.3 *Giardia* in humans

1.2.2.3.1 Prevalence

In developed countries *Giardia* is detected in up to 14% of symptomatic patients and 2% in asymptomatic **humans** (Geurden *et al.*, 2009; Homan and Mank, 2001). In developing countries, the prevalence of giardiosis in patients with diarrhoea is around 20%, ranging from 5-43% (Islam, 1990; Haque *et al.*, 2005). *Giardia* infections are very common in children in developing countries (Farthing, 1994; Rabbani and Islam, 1994). *Giardia* assemblages A and B are considered more infectious for humans, with the latter being more prevalent. Sub-

assemblage AII is more prevalent in humans than AI and is distributed globally, except in Asia and Australia. Assemblage AIII has not yet been detected in humans (Sprong *et al.*, 2009). The geographic distribution of sub-assemblages BIII and BIV in human shows marked difference between continents. In Africa, infection with BIII is more prevalent (81%) than with BIV, whereas the opposite is found in North America where 86% of infections are associated with BIV and 14% with BIII. A more balanced distribution was found in Australia and Europe (Sprong *et al.*, 2009). To a much lesser extent, assemblage C, D, E and F were identified in human samples (Gelanew *et al.*, 2007; Foronda *et al.*, 2008). However, it remains unclear whether the presence of these assemblages in human stool is due to patent infections or merely represents passage through the intestinal tract.

1.2.2.3.2 Clinical importance

Approximately 200 million people in Asia, Africa and Latin America have symptomatic infections with about 50,000 cases reported each year (Xiao and Fayer, 2008). In symptomatic patients, mostly children, the severity of symptoms and the duration of *Giardia* infection are highly variable. In some patients, symptoms last for only 3 or 4 days, while in others the symptoms last for months. Higher prevalence of chronic *Giardia* infection in patients with immunodeficiency supports that the failure to develop an effective immune response against *Giardia* may account for the chronicity of the infections (O'Handley *et al*., 2003). In developed countries, the incidence rate peaks at the age of 1-4 years (Flannagan, 1992); a second peak is observed at the 20-40 age groups, partly due to the care for the young children and partly due to travelling (Medema, 1999). The main symptoms include diarrhoea, abdominal pain, nausea, vomiting, flatulence, anorexia and fever (Nash *et al*., 1987; Farthing, 1996; Katz *et al*., 2006). In most instances the diarrhoeal illness is short-lived and self-limited. However, a proportion of individuals develop persistent diarrhoea (Farthing, 1996; Katz *et al*., 2006), sometimes accompanied by malabsorption of sugars and fat and by weight loss. There is evidence that infection with *Giardia* results in 'failure to thrive' in children, by impairment of the uptake of nutrients (Farthing, 1994; Hall, 1994). A high prevalence of chronice fatigue syndrome has been reported as a post-infection sequela in patients (Naess *et al*., 2012; Wensaas *et al*., 2012; Mørch *et al*., 2013).

The relation between clinical symptomatology and the *Giardia* genotype is controversial. In a study in The Netherlands, assemblage A isolates were solely detected in patients with

intermittent diarrhoeal complaints, while assemblage B isolates were present in patients with persistent diarrhoeal complaints (Homan and Mank, 2001). A strong correlation between infection with assemblage B and diarrhoea was observed in Saudi children (Al-Mohammed, 2011). An association between assemblage B and flatulence in children was reported by Lebbad *et al*. (2011). In contrast, Read *et al.* (2002) found that assemblage B genotypes were more prevalent in asymptomatic children than those of assemblage A and according to Haque *et al*. (2005, 2009) only assemblage A was an important cause of diarrhoea in children in Bangladesh. A systematic review and meta-analysis confirmed that *Giardia* infections of both assemblages A and B can cause acute or persistent diarrhoea (Muhsen and Levine, 2012).

However, *Giardia* infections are often asymptomatic. In some studies no significant association between *Giardia* and diarrhoea was found (Guerrant *et al*., 1983; Schorling *et al*., 1990; Hollm-Delgado *et al*., 2008; Boeke *et al*., 2010). In both volunteers and outbreak situations, a sizable proportion of the infected subjects are asymptomatic, often exceeding the proportion with manifest clinical illness (Muhsen and Levine, 2012). It has been estimated that between 50% and 75% of *Giardia*-infected persons may be asymptomatic (USEPA, 1998a). Children with asymptomatic *Giardia* infection serve as unidentified carriers and may be responsible for transmission of the infection. Secondary transmission among family members may occur. Asymptomatic infections may last for months or years (ICAIR, 1984).

1.2.2.4 *Giardia* in animals

1.2.2.4.1 Prevalence

In **calves** younger than six months, the prevalence varies between 17% (Muhid *et al*., 2012) and 73% (Olson *et al.*, 1997) and on farm level it can be as high as 100% (Olson *et al.*, 1997; Hunt *et al.*, 2000; Geurden *et al.*, 2010, 2012). In cattle the livestock specific assemblage E is most prevalent, although up to 59% zoonotic assemblage A isolates and mixed infection with both A and E have been reported (Geurden *et al.*, 2008b; Sprong *et al.*, 2009). This suggests that calves should be considered as a potential reservoir for human infections. However, within assemblage A, sub-assemblage AI is predominantly found in livestock and companion animals, while assemblage AII is more prevalent in humans (Sprong *et al.*, 2009).

The prevalence of *Giardia* in **sheep and goats** is reported to range from 10% to 40% with

assemblage E detected in 75%, assemblage A in 27% and assemblage B in 2% of cases (Robertson, 2009).

The prevalence of *Giardia* in **pigs** ranges from 1% to 51% (Armson *et al*., 2009; Farzan *et al*., 2011; Budu-Amoako *et al*., 2012). The herd prevalence was 18%, 22% and 84% for sows, piglets and weaners in Denmark (Maddox-Hyttel *et al*., 2006) and 12% in Zambia (Siwila and Mwape *et al*., 2012). DNA sequencing demonstrated that assemblage E was the most common genotype in Australia and the UK (Armson *et al*., 2009; Minetti *et al*., 2013), while in Canada assemblage B was predominant (Farzan *et al*., 2011). Assemblage A was found in both weaners and piglets in Denmark (Langkjaer *et al*., 2007) and in pigs in Australia (Armson *et al*., 2009). Unexpectedly, the canine-specific assemblages C and D and the feline-specific assemblage F were also found occasionally in pigs in different countries (Langkjaer *et al*., 2007; Armson *et al*., 2009; Minetti *et al*., 2013). As for humans, it remains unclear whether the presence of these assemblages represents a patent infection or merely indicates carriage.

The prevalence of *Giardia* in **horses** was 1% in Brazil (De Souza *et al*., 2009), 9%-23% in Italy (Veronesi *et al*., 2010; Traversa *et al*., 2012) and 17% in Colombia (Santín *et al*., 2013). *G. duodenalis* isolates from horses belonged predominantly to the zoonotic assemblages A and B (Traub *et al*., 2005; Traversa *et al*., 2012; Santín *et al*., 2013) and to a lesser extent to assemblage E (Veronesi *et al*., 2010; Traversa *et al*., 2012).

In **dogs**, the prevalence of *Giardia* infections varies from 1% to 55% (Itoh *et al*., 2005; Jafari Shoorijeh *et al*., 2008). The most prevalent assemblages in dogs are the dog-specific assemblages C and D (Beck *et al*., 2012) but other sub-assemblages such as AI, AII, BIII and BIV are also detected in dogs worldwide (Souza *et al.*, 2007; Palmer *et al*., 2008; Claerebout *et al*., 2009; Sprong *et al.*, 2009), sometimes in higher frequencies than the dog-specific assemblages (*e.g.* Leonhard *et al*., 2007; Claerebout *et al*., 2009; Covacin *et al*., 2011).

Worldwide the prevalence of *Giardia* in **cats** ranges from 1%-40% (De Santis-Kerr *et al*., 2006; Itoh *et al*., 2006; Gow *et al*., 2009; Mohsen and Hossein, 2009; Mircean *et al*., 2011; Sabshin *et al*., 2012). Assemblage F and sub-assemblage AI are predominant but assemblage D and sub-assemblages AII, AIII were also detected in cats (Papini *et al.*, 2007; Souza *et al.*, 2007; Palmer *et al*., 2008; Sprong *et al.*, 2009).

Little information is available on the prevalence of zoonotic *G. duodenalis* assemblages infecting **wildlife**. Assemblage A was identified in wild ruminants in the USA and Europe

(Trout *et al.*, 2003; van der Giessen *et al.*, 2006; Lalle *et al.*, 2007; Robertson *et al.*, 2007) and in one fox and three kangaroos in Australia (McCarthy *et al.*, 2008). Assemblage B was found in beavers, muskrats and rabbits (Sulaiman *et al.*, 2003; Fayer *et al.*, 2006a). Assemblages B, C and D were identified from coyotes in the USA (Trout *et al.*, 2006). In non-human primates the most prevalent assemblages are A (Graczyk *et al.*, 2002; Volotão *et al*., 2008), B (Itagaki *et al.*, 2005) and mixed A and B (Levecke *et al*., 2007, 2009).

Waterfowl are also a potential reservoir for zoonotic infections with *Giardia* (Graczyk *et al.*, 1999b, 2008; Upcroft *et al.,* 1998). In Hungary, *G. duodenalis* was found in 6% of wild birds and 24% of domestic birds (Plutzer and Tomor, 2009). Both assemblages A and B were detected. The results of this study indicated that waterfowl can play a role in the environmental dessimination of human pathogenic cysts.

1.2.2.4.2 Veterinary importance

Although *G. duodenalis* is recognised worldwide as the most common parasitic cause of gastrointestinal disorder in human patients, the relevance of infection in **production animals** is open to debate (Geurden *et al.*, 2010). The clinical signs may vary considerably between animals and animal species due to the involvement in the pathogenesis of giardiosis of both parasite and host factors. This lack of consistency in clinical outcome resulted in the perception that *Giardia* is not a major cause of clinical disease in ruminants. However, several studies reported clinical signs caused by *Giardia* both in natural infections (St. Jean, 1987; Xiao *et al.*, 1993; O'Handley *et al.*, 1999; Aloisio *et al.*, 2006; Geurden *et al.*, 2006b) and in experimental infections (Olson *et al.*, 1995; Koudela and Vitovec, 1998; Geurden *et al.*, 2006a). Infection can result in diarrhoea that does not respond to antibiotic or coccidiostatic treatment. The excretion of pasty to fluid faeces with a mucoid appearance may be indicative for giardiosis, especially when the diarrhoea occurs in young animals.

A study in dairy calves showed that calves did not begin to excrete *Giardia* cysts until approximately 1 month of age. Passive immunity through colostrum may have the potential to provide initial protection against *Giardia* infections as colostrum contains a high level of anti-*Giardia* antibodies. Failure to develop a humoral immune response from natural infections by these calves could account for the high prevalence and chronic duration of the infections (O'Handley *et al*., 2003).

In **pigs**, a significant association was found between the presence of assemblage E and soft to diarrhoeic stool, whereas assemblage A was not correlated with diarrhoea (Armson *et al*., 2009). This is in contrast to previous studies that have reported no association between *Giardia* infections in pigs and diarrhoea (Xiao *et al*., 1994, Koudela *et al*., 1991, Maddox-Hyttel *et al*., 2006, Hamnes *et al*., 2007; Langkjaer *et al*., 2007).

Next to diarrhoea, there is an economic impact of giardiosis for farmers. In **goat** kids and **lambs** an experimental infection resulted in a decreased feed efficiency and subsequently a decreased weight gain (Olson *et al.*, 1995; Koudela and Vitovec, 1998; Sweeney *et al*., 2011, 2012).

Infections with *Giardia* in **dogs and cats** are common. Clinical signs vary from asymptomatic to small bowel diarrhoea and associated discomfort (Fiechter *et al.*, 2012).

1.3 Transmission

1.3.1 Excretion of (oo)cysts and contamination of the environment

Infection caused by *Cryptosporidium* and *Giardia* spp. is due to oral ingestion of infectious (oo)cysts either directly by contact with infected animals or patients or indirectly through a contaminated environment or through the ingestion of contaminated food and water. Mechanical transmission through vectors such as tools, caretakers or even arthropods and birds is possible (Graczyk *et al.*, 1999a; Fayer *et al.*, 2000). The infected hosts, whether humans or animals, shortly after infection, shed in the environment a large number of these infective stages, contributing to an increase of environmental contamination. For example, experimentally infected lambs can excrete up to 10^9 oocysts of *Cryptosporidium* per day (Blewett, 1989) and a mean of 4.8 x10^9 oocysts of *Cryptosporidium* per gram of faeces (Bukhari and Smith, 1997). At the peak of infection, ruminants can excrete 10^6 *G. duodenalis* cysts per gram of faeces (O'Handley and Olson, 2006). Only bovids in the USA excreted 65 x 10^{15} oocysts per annum, which equates to nearly 6,735 oocysts per annum for every square meter surface area in the USA. Humans can excrete up to 10^7 oocysts per gram (Smith and Nichols, 2010).

1.3.2 Zoonotic vs. anthroponotic transmission

Since humans can be infected with host-specific as well as zoonotic species/genotypes, molecular typing is needed to distinguish human from animal sources of infection. Most cryptosporidiosis outbreaks in the United States are caused by *C. hominis* (Dietz *et al.*, 2000). For example, the large Milwaukee outbreak in 1993, human sources of infection have been identified (Peng *et al.*, 1997) and in only one *water-borne* outbreak in Canada livestock was implicated (Fayer *et al.*, 2000). However, outbreaks have been estimated to account for only 10% of the overall number of cryptosporidiosis cases (Dietz *et al.*, 2000). Although in sporadic cases the source of infection is mostly unknown, direct contact with infected farm animals has repeatedly been associated with sporadic cases of cryptosporidiosis. Cattle have been considered to be an important source of zoonotic cryptosporidiosis since the 1980s (Xiao and Feng, 2008). In some developed countries, contact with cattle was considered as a risk factor for human cryptosporidiosis (Robertson *et al.*, 2002; Goh *et al.*, 2004; Hunter *et al.*, 2004; Saha Roy *et al.*, 2006; Hunter and Thompson, 2005). Small outbreaks of cryptosporidiosis were reported in farmers, veterinarians, researchers and children when they came into contact with young calves or lambs (Miron *et al.*, 1991; Lengerich *et al.*, 1993; Preiser *et al.*, 2003; Smith *et al.*, 2004b; Kiang *et al.*, 2006; Gormley *et al.*, 2011; Cacciò *et al.*, 2013). Lake *et al.* (2007) reported a case-control study in the UK on human cryptosporidiosis where they found that cryptosporidiosis cases were strongly associated with areas with a high estimate of *Cryptosporidium* applied to land from manure. In the UK, during large foot and mouth disease outbreaks in 2001, slaughtering of more than 6 million animals and strict restriction to access countryside reduced the incidence of human cryptosporidiosis (Hunter *et al.*, 2003; Smerdon *et al.*, 2003). There were also some epidemiological studies where sheep and companion animals were found as a source of human cryptosporidiosis (Molbak *et al.*, 1994; Duke *et al.*, 1996; Glaser *et al.*, 1998; Katsumata *et al.*, 1998; Robinson and Pugh, 2002; Goh *et al.*, 2004).

Seasonal differences may play an important role in the distribution of *C. parvum* and *C. hominis*. In the UK and New Zealand, the spring peak of cryptosporidiosis was mostly due to *C. parvum* whereas the autumn peak was mainly due to *C. hominis* (McLauchlin *et al.*, 2000; Learmonth *et al.*, 2003, 2004; Hunter *et al.*, 2004). Lambing, calving and farm runoff from spring rains were thought to be responsible for the *C. parvum* spring peak whereas increased recreational water activities and international travel during late summer and early autumn

were responsible for the *C. hominis* autumn peak (Goh *et al.*, 2004; Hunter *et al.*, 2004).

Geographic differences in the distribution of *C. parvum* and *C. hominis* infections occur within a country (Xiao and Fayer, 2008). For example, in the USA, the UK and New Zealand, *C. parvum* is more common in rural areas and *C. hominis* infection is more common in urban areas (McLauchlin *et al.*, 1999, 2000; Learmonth *et al.*, 2004; Feltus *et al.*, 2006).

Molecular analysis of the 60kDa glycoprotein (GP60) gene supports the probable zoonotic transmission in both developed and developing countries. One major GP60 *C. parvum* subtype family, IIa, is common in humans in rural areas in the USA and in Europe and is also found in calves in the same areas (Glaberman *et al.*, 2002; Alves *et al.*, 2003, 2006; Feltus *et al.*, 2006; Thompson *et al.*, 2007). Another less common bovine *C. parvum* subtype family IId, might be responsible for some zoonotic infections in developed nations like Portugal, Hungary and Kuwait (Alves *et al.*, 2003, 2006; Plutzer and Karanis, 2007; Sulaiman *et al.*, 2005). The anthroponotic IIc subtype was found in most human *C. parvum* infections in urban areas in the USA (Xiao *et al.*, 2004b). In the UK and Portugal both IIa and IIc were fairly common in humans (Alves *et al.*, 2003, 2006). In developing countries, *C. hominis* is the predominant species in humans, suggesting that human-to-human transmission is more important (Xiao and Feng, 2008). This is supported by the observation that in the case of *C. parvum* infections, IIc subtypes of *C. parvum* are responsible for most human infections (Leav *et al.*, 2002; Peng *et al.*, 2003; Xiao *et al.*, 2004a; Xiao and Ryan, 2004; Akiyoshi *et al.*, 2006). However, in some developing countries like Malawi, Peru, Lima, subtype IIe is found (Peng *et al.*, 2003; Cama *et al.*, 2007).

Among the eight assemblages (A-H) of *G. duodenalis*, assemblage A and B have zoonotic potential as both genotypes can infect humans as well as animals. According to Thompson (2000) mainly assemblage A has a major zoonotic risk and to a lesser extent assemblage B. Using a multi-locus sequence typing scheme, Sprong *et al.* (2009) argued that only 2 multi-locus genotypes of assemblage A and none of assemblage B have zoonotic potential. Although the major zoonotic risk is from assemblage A, subtyping data showed that animals are mostly infected with subtype AI whereas humans are mostly infected with subtype AII (Xiao and Fayer, 2008; Sprong *et al.*, 2009). Zoonotic transmission of giardiosis to humans is also evidenced by some epidemiological studies. For example, contact with farm animals was associated with increased risk of infection for adults, whereas pets were not identified as a risk factor for infection in children or adults in case control studies in New Zealand (Hoque *et*

al., 2002, 2003). In a few studies dogs were indicated as a reservoir of human infection, based on the presence of assemblage A in dogs and humans living in close contact (Traub *et al.*, 2004; Inpankaew *et al.*, 2007; Salb *et al.*, 2008). In cattle, assemblage A is often reported (Sprong *et al.*, 2009) whereas assemblage B is more common in wildlife (Johnston *et al.*, 2010). Both assemblages A and B have been detected from wild birds and marine animals indicating the potential role of such vectors in the transmission as they can contaminate water that is used by humans for recreation (Dixon *et al.*, 2008, Plutzer and Tomor, 2009). However, in some other studies, humans are considered to be the source of infection in non-human primates, painted dogs, marsupials, beavers and coyotes, house mice, muskoxen and marine mammals in various parts of the world (Graczyk *et al.*, 2002; Sulaiman *et al.*, 2003; Moro *et al.*, 2003; Appelbee *et al.*, 2005, 2010; Kutz *et al.*, 2008; Dixon *et al.*, 2008; Teichroeb *et al.*, 2009; Thompson *et al.*, 2009, 2010a, 2010b; Ash *et al.*, 2010; Johnston *et al.*, 2010).

1.3.3 Water-borne transmission

Water is a major transmission route of *Cryptosporidium* and *Giardia*, where (oo)cysts can survive and remain infective (Thompson, 2000; Fayer *et al.*, 2000). *G. duodenalis* cysts have been shown to survive for up to 11 weeks in water and 7 weeks in soil at 4°C and *Cryptosporidium* spp. oocysts can survive more than 12 weeks at 4°C in fresh water (Olson *et al.*, 1999). Cysts and oocysts not only remain infective for long periods in the environment, but are also resistant to the conventional treatment processes of water, representing a serious problem of public health (Fayer, 2004; Cacciò *et al.*, 2003; Castro-Hermida *et al.*, 2008a, 2008b, 2009; Lobo *et al.*, 2009). This problem is also potentiated by the fact that the number of parasites required to induce infection is small. According to Smith *et al.* (2006b), the infectious dose is 10-100 cysts for *Giardia* and 10-1000 oocysts for *Cryptosporidium*. The Milwaukee outbreak, the largest *Cryptosporidium* outbreak in the world associated with water consumption, indicated an even lower infectious dose from 1 to 10 oocysts (Dillingham *et al.*, 2002).

1.3.3.1 Cases of water-borne outbreaks

Water-borne diseases caused by protozoan parasites occur worldwide and large numbers of consumers of drinking water are affected during outbreaks. At least 325 water-associated

outbreaks of parasitic protozoan disease were documented worldwide until 2004 (Karanis *et al.*, 2007a). *G. duodenalis* and *C. parvum* were responsible for 41% and 51% of 325 outbreaks respectively. Of the reported outbreaks of giardiosis and cryptosporidiosis, 14% and 50%, respectively, were associated with contaminated recreational water (Karanis *et al.*, 2007a). Swimming in contaminated waters and swimming pools is now recognised as an important transmission route for *Cryptosporidium* and *Giardia*. From 2004 to 2010, 199 outbreaks of human diseases due to the water-borne transmission of parasitic protozoa occurred (Baldursson and Karanis, 2011). *Cryptosporidium* spp. was the etiological agent in 60% of the outbreaks, *Giardia* spp. in 35%.

1.3.3.1.1 Drinking water

Cryptosporidium oocysts and *Giardia* cysts are transported to surface waters (such as rivers, canals, ponds and lakes serving as drinking water supplies) by drainage from manure storage areas, runoff from manure-laden soil or direct contamination of water by livestock, wild animals and birds (Fayer *et al.*, 2000; Graczyk *et al.*, 2000; Sischo *et al.*, 2000; Jellison *et al.*, 2002; Bodley-Tickell *et al.*, 2002; Graczyk *et al*, 2008; Xiao and Fayer, 2008). Other potential sources are human waste, *e.g.* sewage drains and wastewater treatment plants (Cacciò *et al.*, 2003; Roberston *et al.*, 2006; Castro-Hermida *et al.*, 2011b). Thus, the presence of infective (oo)cysts in the drinking water supplies and absence of sufficient drinking water treatment may lead to outbreaks of both cryptosporidiosis and giardiosis resulting from consumption of contaminated drinking water.

Between 1984 and 1999, about 49 drinking water related cryptosporidiosis outbreaks were reported mostly in North America, the UK and Japan (Fayer, 2004). The largest water-borne cryptosporidiosis outbreak was recorded in Milwaukee, Wisconsin in the spring of 1993 with approximately 403,000 persons affected due to an ineffective filtration process, which led to the inadequate removal of *Cryptosporidium* oocysts in one of the two municipal water treatment plants (MacKenzie *et al.*, 1994). Cryptosporidiosis-associated deaths were reported for 54 residents in 2 years following the outbreak (Hoxie *et al.*, 1997). Both *C. hominis* and *C. parvum* were identified in 3 drinking water-associated cryptosporidiosis outbreaks in Northern Ireland in 2000 (Glaberman *et al.*, 2002). In September 2001, a water-borne outbreak of *C. hominis* infection occurred in eastern France (Dalle *et al.*, 2003). The Battlefords area of Saskatchewan (Canada) experienced an outbreak of gastroenteritis in

2001. An estimated 5,800 to 7,100 people from the Battlefords were affected along with hundreds of visitors from other parts of Canada. *C. parvum* infection was confirmed in 275 people and no other pathogens were identified (Stirling *et al.*, 2001). An outbreak in the autumn of 2005 resulted in 218 confirmed cases of *C. hominis* in northwest Wales. A case-control study demonstrated a statistically significant association between drinking unboiled tap water and *C. hominis* infection (Mason *et al.*, 2010). An outbreak of cryptosporidiosis in Korea in 2012 was described by Moon *et al*. (2013). Diarrhoea was the most common clinical symptom in 126 patients and lasted for 5 days on average. The tap water was the only common exposure of the patients. *C. parvum* was detected in stool specimens and *Cryptosporidium* oocysts were also detected in the water specimens from the water tank. It is presumed that the tap water was contaminated by a sewage leak from the aged pipelines.

In recent years water-borne giardiosis outbreaks have been reported from the USA and different countries in Europe. In 2007, a giardiosis outbreak affected attendees of a recreational camp in California. Twenty-six persons had laboratory-confirmed giardiosis; another 24 had giardiosis-like illness with no stool test. A retrospective cohort study determined that showering was associated with illness. Two days before the outbreak began, the camp had installed a slow-sand water filtration system that included unsterilized sand (Karon *et al.*, 2011). In 2007, a giardiosis outbreak occurred in New Hampshire, USA where consuming tap water was found significantly associated with illness (Daly *et al.*, 2010). In 2004, the largest water-borne outbreak of giardiosis described to date occurred in Bergen, Norway affecting around 1500 people. This outbreak was caused by leaking sewage pipes combined with insufficient water treatment and *G. duodenalis* assemblage B was identified (Nygård *et al.*, 2006; Robertson *et al.*, 2006b). In Sweden, giardiosis outbreaks linked to contaminated drinking water have also been reported (Neringer *et al.*, 1987; Andersson and de Jong, 1989). A water-borne outbreak of giardiosis occurred when about 3,000 persons were exposed to contaminated water following a sewage overflow into the drinking water system. Another severe sewage contamination of a drinking water distribution network was reported in Finland in November 2007-February 2008 (Rimhanen-Finne *et al.*, 2010). One of the pathogens found in patients and environmental samples was *Giardia*. In Belgium, a small outbreak of giardiosis due to contaminated drinking water occurred in 2008 (RTBF, 2008).

1.3.3.1.2 Recreational water

Swimming is one of the most popular recreational activities worldwide with over 350 million person-events estimated to take place annually in the USA alone (Fayer, 2004). *Cryptosporidium* and *Giardia* were the most frequently identified protozoal causal agents of outbreaks associated with recreational water during 1971-2000 in the USA. Important sources of contamination for both treated and untreated recreational waters were the bathers themselves. Contamination from sewage discharges and wild or domestic animals were also important sources for untreated waters (Craun *et al*., 2005). From 1984 to 1999, over 10,000 people were identified in 31 locations as acquiring cryptosporidiosis from recreational waters (Fayer *et al.*, 2000). In the USA, a substantial increase was observed in the number of nationally reported cryptosporidiosis outbreaks associated with treated recreational water venues (*e.g.* pools, water parks and interactive fountains) (CDC, 2008). A cryptosporidiosis outbreak was found to be associated with swimming at the local swimming pool in Minnesota in 1998. Twenty-six persons had illnesses with as most common symptoms diarrhoea, abdominal cramps and nausea. Four cases of cryptosporidiosis were confirmed by stool analysis (Lim *et al*., 2004). In 2001, an Illinois hospital reported a cryptosporidiosis cluster potentially linked to a local waterpark. There were 358 case-patients identified. *Cryptosporidium* was found in stool specimens and pool water samples (Causer *et al.*, 2006). In 2004, a cryptosporidiosis outbreak affected >250 persons who visited a California waterpark. *Cryptosporidium* oocysts in sand and backwash from the waterslides' filter and environmental investigations uncovered inadequate water-quality record keeping and a design flaw in one of the filtration systems (Wheeler *et al*., 2007). A cryptosporidiosis outbreak was reported in a swimming pool in Atlanta despite supplemented disinfection of *Cryptosporidium* oocysts with ultraviolet (UV) radiation. Risk factors for illness included swimming, getting water in mouth and swallowing water (Boehmer *et al.*, 2009). Recently, an outbreak of *Cryptosporidium* at a recreational park in Niagara region, Canada was reported where the ultraviolet disinfection system was offline (Hopkins *et al*., 2013).

In Europe, an outbreak of pool-associated cryptosporidiosis occurred in Sweden in the late summer of 2002 and affected an estimated 800-1,000 individuals (Insulander *et al.*, 2005; Mattsson *et al.*, 2008). *C. parvum* was identified (Mattsson *et al.*, 2008). Smith *et al.* (2006a) reviewed the epidemiological and microbiological characteristics of 89 reported outbreaks of water-borne infectious intestinal disease affecting 4,321 people in England and Wales over the period 1992-2003. *Cryptosporidium* was found in 69% of the outbreaks and *Giardia* in

2%. Swimming pools were involved in 35 outbreaks (39%). In October 2007 an increase in cryptosporidiosis cases in England was associated with swimming in 13 local public swimming pools. One large swimming pool was most frequently visited by swimmers and considered a significant contributor to transmission because of substandard filtration and maintenance systems (Coetzee *et al.*, 2008).

A water-borne outbreak of cryptosporidiosis occurred in Japan in 2004 associated with swimming pools. In total, 288 clinical cases with digestive symptoms, including watery diarrhoea, vomiting, abdominal cramps and tenesmus, were reported to local authorities and 74 were positive for *Cryptosporidium* (Takagi *et al.*, 2008).

In Australia, swimming in the 2 weeks before onset of illness was identified as a likely risk factor for sudden increase of cryptosporidiosis in Brisbane in 1998. Fifty-two eligible cases and *Cryptosporidium* oocysts in three pools were identified (Stafford *et al*., 2000). There have been few water-borne outbreaks detected in Australia between 2001-2007 and most of those reported (78%) have been associated with recreational exposure (Dale *et al*., 2010). Thirty-one cases with cryptosporidiosis and 8 *C. hominis* subtype IbA10G2 isolates were identified in an outbreak of cryptosporidiosis in Sydney in 2008. Cryptosporidiosis was associated with swimming and animal contact (Mayne *et al*., 2011). A large outbreak of cryptosporidiosis was reported in New South Wales, where 1,141 individuals became infected with *Cryptosporidium*. Health authorities in New South Wales indicated that public swimming pool use was a contributing factor in the outbreak and the anthroponotic *C. hominis* IbA10G2 subtype was identified as the causative pathogen (Waldron *et al*., 2011).

Although hardly any reports can be found on *Giardia* infections related to recreational water, a marked seasonality in the onset of giardiosis occurs in early summer through early fall in the USA and a twofold increase in transmission of giardiosis occurs during the summer. This increase coincides with increased outdoor activities (*e.g.* swimming and camping) and might reflect heavy use of community swimming venues by younger children (Hlavsa *et al.*, 2005). In 2006, an outbreak of giardiosis and cryptosporidiosis was associated with exposure to a neighborhood interactive water fountain in central Florida (Eisenstein *et al.*, 2008).

1.3.3.2 Sources of water contamination

The introduction of pathogens such as *Cryptosporidium* and *Giardia* in the environment can come from multiple sources (Dyble *et al*., 2008). These can be subdivided into point sources

and diffuse sources (Burnet, 2012). In the case of a point source, the origin of the contamination can be easily traced. Examples of point sources where (oo)cysts were found both from human and animal origins are water treatment plants, wastewater and effluent treatment plants (Xiao *et al*., 2001; Cacciò *et al*., 2003; Bertrand and Schwartzbrod, 2007; Castro-Hermida *et al*., 2008a; Robertson *et al*., 2008; Kistemann *et al*., 2012). In contrast, diffuse pollution comes from a number of independent households or farms spread over a surface that makes tracing the source of the contamination more difficult. Sources of diffuse pollution can come from livestock grazing areas or the introduction of faeces via manure or sewage sludge on land, or from swimmers or other recreational activities near the water resource (Tyrrel and Quinton, 2003; Sunderland *et al*., 2007). The land drainage following heavy rainfall is a key factor of contribution to faecal cotamination of water. The impact of rainfall events on the burden of *Cryptosporidium* and *Giardia* in surface water has indeed been demonstrated repeatedly (Atherholt *et al*., 1998; Kistemann *et al*., 2002; Lemarchand and Lebaron, 2003).

1.3.3.3 Monitoring of *Cryptosporidium* and *Giardia* in drinking water and surface water

Surface fresh water is widely used for recreational activities and/or as a resource for drinking water production. However, this resource is often contaminated by pathogenic bacteria, viruses and/ or protozoa (e.g. *Cryptosporidium* and *Giardia*) that can pose significant health problems. As a consequence of this, monitoring of *Cryptosporidium* and *Giardia* occurrence is conducted both in recreational reservoirs (swimming pools) and surface water reservoirs used for production of drinking water in several countries.

The third edition of the World Health Organisation (WHO) Guidelines for Drinking Water Quality describe in detail the water safety plan (WSP) approach to ensure water quality between source and consumers' tap (WHO, 2004). The WHO has set out three essential components to the water safety plan: a risk assessment of the water supply; effective operational monitoring and effective management. The WHO has no law-making authority but has established guidelines for the use of individual countries. According to their laws, directives and regulations, it is now compulsory for water companies to carry out an assessment of risk of all sources containing *Cryptosporidium* oocysts in the UK (HMSO, 2000), Scotland (The *Cryptosporidium* (Scottish Water) Directions, 2003), the Netherlands (the Dutch Drinking Water Decree Act, 2001), France (AFNOR, 2001) and Brazil (Federal

Legislation Law 1649). In Canada, a guideline has been established for *Cryptosporidium* and *Giardia*, but because the current detection methods are not very reliable the guideline does not give a maximum acceptable concentration value for these parasites in drinking water. In Australia, no guideline value is set for *Cryptosporidium* because of the lack of a method to identify human infectious strains in drinking water. In New Zealand, there is no requirement to monitor sources for protozoa that supply fewer than 10,000 populations (EPA, 2011). In the USA, the USEPA introduced in 1998 an Interim Enhanced Surface Water Treatment Rule (USEPA, 1998b). One part of this requires that systems supplying populations larger than 10,000 that are required to filter under the Surface Water Treatment Rule must achieve a 2-log (99%) removal of *Cryptosporidium*. According to EU Drinking Water Directive EU/98/83/EC, screening of *Cryptosporidium* is compulsory when *Clostridium perfringens* is detected in water intended for human consumption.

Cryptosporidium and *Giardia* have been detected in different rivers, lakes and reservoirs (surface/raw water used for drinking water production), drinking water plants and sewage plants in different countries of the world as a part of the monitoring of drinking water contamination (Table 1.3). It is evident from these results that high levels of contamination of *Cryptosporidium* and *Giardia* were found in different types of water samples in European countries and in Canada. In general, *Giardia* concentrations were higher than *Cryptosporidium*. Contamination of water samples apparently depended on the water sources. Water from waste water treatment plants often contained high levels of *Cryptosporidium* and *Giardia,* indicating that waste water treatment often does not effectively remove (oo)cysts and that effluent from waste water treatment plants can be a point source of surface water contamination. However, no or very low levels of *Cryptosporidium* and *Giardia* were found in tap water/finished products in most studies, indicating that the applied water management procedures were effective and that these study sites fulfilled the demand of drinking water of the respective countries. Nevertheless, outbreaks have occurred occasionally, due to failure of filtration in drinking water treatment plants, as described above (see section 1.3.3.1).

Several species and assemblages of *Cryptosporidium* and *Giardia* have been detected during monitoring surveys or studies worldwide, of which some are zoonotic. Zoonotic *C. parvum* was identified in the USA and Canada along with *C. hominis*, *C. ubiquitum* and *C. andersoni*. In Europe, several studies confirmed the ubiquitous presence of *Cryptosporidium* oocysts in surface waters. Eight *Cryptosporidium* genotypes, including *C. parvum*, *C. hominis*, *C. muris*

and *C. baileyi* were detected in Germany and Switzerland (Ward *et al*., 2002). *C. parvum, C. andersoni* and *C. hominis* were detected in drinking and waste water treatment plants in Spain (Castro-Hermida *et al*., 2008a, 2008b). A high occurrence of human pathogenic *Cryptosporidium* genotypes and subtypes were found in raw and treated water samples in Portugal (Lobo *et al*., 2009).

Table 1.3 *Cryptosporidium* and *Giardia* detected in various types of water samples worldwide (percentage of positive water samples) (adapted from Burnet, 2012).

Country	**Sample**	***Cryptosporidium* (%)**	***Giardia* (%)**	**Study period**	**Reference**
Finland	RSW	10	14	1 year	Hörman *et al*., 2004
Norway	Sewage	80	93	5 months	Robertson *et al*., 2006a
Germany	WWTP RSW	31 10	65 4	2 years	Gallas-Lindemann *et al*., 2013
	WWTP	100	100	18 - 24 months	Ajonina *et al*., 2012, 2013
France	River	46	94	30 months	Mons *et al*., 2009
Luxemburg	River	81-53	81-53	2 years	Helmi *et al*., 2011
The Netherlands	Canal, Lakes	0-75	0-100	1 year	Schets *et al*., 2008
Poland	RSW DWTP Treated water	85 59 16	61 6 19	1 year	Bajer *et al*., 2012
Spain	River Reservoir WWTP	64 33 15-23	92 56 27-45	30 months	Carmena *et al*., 2007
	River DWTP WWTP	58 41 33	61 42 37	1 year	Castro-Hermida *et al*., 2010
	River Sewage	100 100	-	2 years	Montemayor *et al*., 2005
Portugal	River	10	8	2 years	Almeida *et al*., 2010
	RSW	46	38	-	Lobo *et al*., 2009
	River	83	85	2 years	Júlio *et al*., 2012
Italy	WWTP RSW	100 48	100 71	2 years	Briancesco and Bonadonna, 2005
	RSW	0	80	5 months	Vernile *et al*., 2009
Iran	River	38	77	1 year	Mahmoudi *et al*., 2013
Malaysia	River	23	51	-	Lee *et al*., 2013
China	RSW	86	65	8 months	Xiao *et al*., 2013
	River	32	18	8 months	Feng *et al*., 2011
South Korea	River	-	35	10 years	Lee *et al*., 2011
Japan	RSW	41	36	2 years	Haramoto *et al*., 2012
Brazil	Spring water	8	6	1 year	Branco *et al*., 2012
	Wells	-	63	-	Razzolini *et al*., 2011
	Watersheds	9	50	2 years	Sato *et al*., 2013

	RSW	-	42	-	Fernandes *et al*., 2011
Argentina	RSW	92	31	22 months	Abramovich *et al*.,
	Recreational	100	72		2001
USA	Watershed	15	-	1 year	Jellison *et al*., 2002
Canada	Watershed	77	-	10 weeks	Ruecker *et al*., 2007
	River	88	97	30 months	Van Dyke *et al*., 2012

*RSW=Raw surface water; WWTP= Wastewater treatment plant; DWTP=Drinking water treatment plant

C. parvum was the most common species followed by *C. hominis*, *C. andersoni* and *C. muris*. Subtyping revealed the presence of *C. parvum* subtypes IIaA15G2R1, IIaA16G2R1 and IIdA17G1.

G. duodenalis assemblages AI, AII and E were detected in drinking and waste water treatment plants in Spain and assemblage B in Poland (Castro Hermida *et al*., 2008a, 2008b; Adamska, 2015). Assemblages AII and E were detected in wastewater in China and assemblages A and B in South Africa and only assemblage A in Taiwan and Australia (Li *et al*., 2012; Liang *et al*., 2012; Nolan *et al*., 2013; Samie and Ntekele, 2014). In Brazil, *G. duodenalis* assemblages AII and B were found which are commonly associated with human giardiosis (Fernandes *et al*., 2011).

No significant differences were observed between seasons for infection with *Giardia* and/or *Cryptosporidium* in studies in Portugal and Spain (Montemayor *et al*., 2005; Carmena *et al*., 2007; Júlio *et al*., 2012). However, seasonal variation was found in the presence of *Cryptosporidium* and/or *Giardia* in several other studies. The seasonal distribution showed that (oo)cysts were predominant during autumn and winter in WWTP in Germany (Ajonina *et al*., 2012, 2013) and during the autumn in Spain (Carmena *et al*., 2007) and France (Mons *et al*., 2009) and in winter in Luxemburg (Helmi *et al*., 2011). Only *Giardia* showed a seasonal trend with higher concentrations at cold water temperatures in South Korea (Lee *et al*., 2011) and Canada (Van Dyke *et al*., 2012). In contrast, higher numbers of (oo)cysts were recorded in spring and summer in both WWTP and DWTP in Spain by Castro-Hermida *et al*. (2008a, 2008b) and in flood period in China (Xiao *et al*., 2013). These different seasonal patterns were possibly linked to land application of cattle manure (Thurston-Enriquez *et al*., 2005) and heavy rainfalls, which contribute to parasite runoff from contaminated soils (Atherholt *et al*., 1998; Carmena *et al*., 2007). However, low rainfall in winter is also contributing to winter peaks in South Korea (Lee *et al*., 2011).

Microbial faecal parameters are used to determine the water quality in some countries especially in EU states where screening of *Cryptosporidium* is compulsory when *Clostridium perfringens* is detected in water. A possible association of concentrations of *Cryptosporidium* and *Giardia* with microbial faecal parameters was investigated in several studies worldwide. The presence of *Giardia* and/or *Cryptosporidium* was significantly associated with total coliform and/or entercocci and/or faecal coliforms/*C. perfringens* in several studies (Abramovich *et al*., 2001; Brookes *et al*., 2005; Carmena *et al*., 2007; Mons *et al*., 2009; Vernile *et al*., 2009; Abd El-Salam *et al*., 2012; Júlio *et al*., 2012; Van Dyke *et al*., 2012; Xiao *et al*., 2013). However, other studies did not find any significant correlation between occurrence of these protozoa (both or any one of them) and faecal indicator bacteria (Rimhanen-Finne *et al*., 2004; Hörman *et al*., 2004; Brookes *et al*., 2005; Hänninen *et al*., 2005; Mons *et al*., 2009; Vernile *et al*., 2009; Helmi *et al*., 2011; Van Dyke *et al*., 2012).
Physical parameters such as turbidity, pH, temperature, rainfall and chemical parameters such as chlorine and iron concentrations showed a realationship with the presence of *Cryptosporidium* and *Giardia* in water samples in some studies. Statistically significant relationships were found between these parasites (both or any one of them) and turbidity (Abramovich *et al*., 2001, Carmena *et al*., 2007; Xiao *et al*., 2013). However, in other studies these physical and chemical parameters did not show a significant correlation with (oo)cyst counts (Helmi *et al*., 2011; Lee *et al*., 2013).

1.3.3.4 Monitoring of *Cryptosporidium* and *Giardia* in swimming pools

Swimming pools have been identified as posing some public health risks to users due to either chemical or microbiological contamination. *Cryptosporidium* spp. and *G. duodenalis* have been found in swimming pool filter backwash during outbreaks (see section 1.3.3.1.2). To determine a baseline prevalence, Shields *et al.* (2008) sampled pools not associated with outbreaks in Georgia, USA and found that of 160 sampled pools, 1% were positive for *Cryptosporidium* spp., 6% for *Giardia* sp. and 1% for both. Similar results were obtained in France (0.02% and 0% of swimming pools contaminated with *Cryptosporidium* and *Giardia*, respectively), Italy (9% *Cryptosporidium* and 0% *Giardia)*, the Netherlands (5% *Cryptosporidium* and 6% *Giardia*) and Egypt (3% *Cryptosporidium* and 7% *Giardia*) (Fournier *et al*., 2002; Schets *et al*., 2004; Abd El-Salam *et al*., 2012). Higher contamination rates (29% *Cryptosporidium* and 38% *Giardia*) were found in 7 pools in Italy (Oliveri *et al*.,

2006). Based on, among others, water contamination data, a model of exposure assessment for swimmers in bathing waters and swimming pools was constructed by Schets *et al*. (2011). However, no *Cryptosporidium* and *Giardia* was found in outdoor and indoor swimming pools in Greece (Papadopoulou *et al*., 2008) and two pools in Italy (Maida *et al*., 2008) (Table 1.4). Swimmers, especially young children, are important contributors of pool-associated *Cryptosporidium* and *Giardia* infections worldwide (Stafford *et al*., 2000; Hoque *et al*., 2002; Hlavsa *et al.*, 2005; Waldron *et al*., 2011). Faecal accidents in the pools pose a major risk of contamination (Porter *et al*., 1988; Turabelidze *et al*., 2007; Schets *et al*., 2008). Other contributing factors in swimming pool related outbreaks were inadequate attention to maintenance, operation, disinfection and filtration (Greinert *et al*., 2004; Craun *et al*., 2005; Oliveri *et al*., 2006; Coetzee *et al.*, 2008; Abd El-Salam *et al*., 2012; Hopkins *et al*., 2013), inadequate water-quality record keeping and a design flaw in the filtration systems (Wheeler *et al*., 2007).

Table 1.4 *Cryptosporidium* and *Giardia* detected in swimming pool samples worldwide (percentage of positive water samples) (adapted from Burnet, 2012).

Country	Number sampled	*Cryptosporidium* (%)	*Giardia* (%)	Study period	Reference
The Netherlands	153 filter backwash	5	6	1 year	Schets *et al*., 2004
France	48 samples from 6 pools	0	0	1 year	Fournier *et al*., 2002
Italy	11 pools	9	0	2 years	Briancesco and Bonadonna, 2005
	21 samples from 7 pools	29	38	-	Oliveri *et al*., 2006
	7 pools	0	0	-	Maida *et al*., 2008
Greece	462 water samples	0	0	8 years	Papadopoulou *et al*., 2008
Egypt	30 samples	10	10	6 months	Abd El-Salam *et al*., 2012
USA	160 pools	1	6	7 months	Shields *et al*., 2008

Proper guidelines for pool operators on improving operating standards and on how to manage faecal accidents as well as public information on the importance of hygiene in swimming

pool complexes are recommended tools in controlling the risk of infection (Coetzee *et al.*, 2008; Schets *et al*., 2008).

1.4 Assessment of *Cryptosporidium* and *Giardia* in water samples

The assessment of *Cryptosporidium* and *Giardia* in water samples consists of 5 consecutive steps, including (i) concentration, (ii) separation, (iii) detection, (iv) determination of viability and (v) genotyping (Figure 1.3). The first three steps (concentration, separation and detection) will confirm the presence or absence of (oo)cysts in the water sample, whereas the two remaing steps will provide additional information on the infectiousness and the origin of the (oo)cysts. For each of the different steps there is a wide range of methods available , some of which are included in standardised procedures, such as the USEPA Method 1623 or ISO 15553. We will provide a brief overview of the currently applied methods, highlighting both advantages and disadvantages.

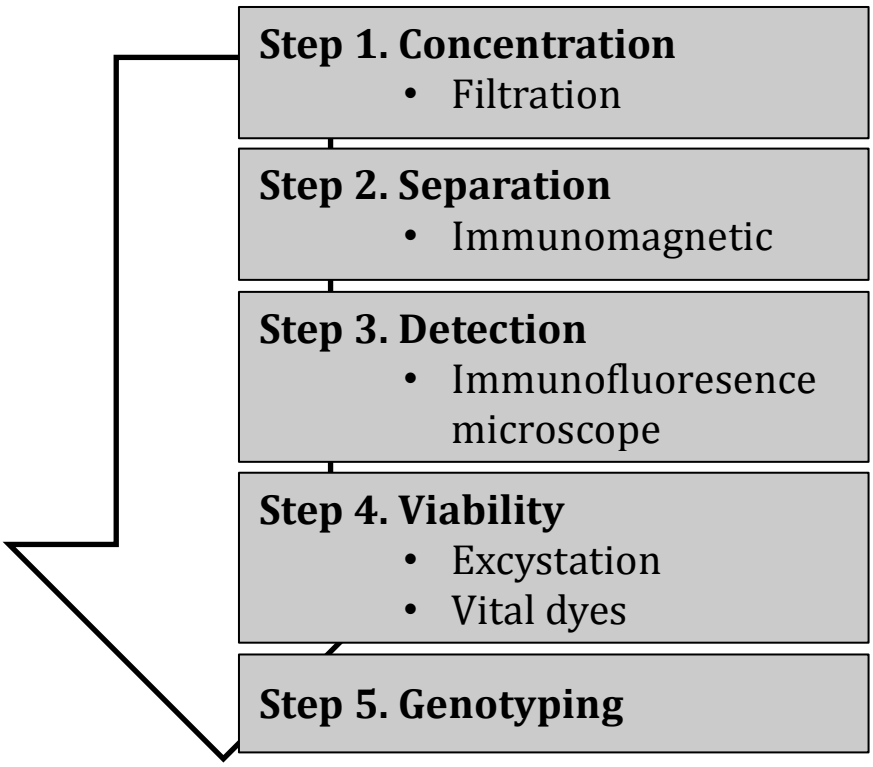


Figure 1.3 Steps of assessment of *Cryptosporidium* and *Giardia* in water samples.

1.4.1 Concentration techniques

Concentration of the (oo)cysts is probably the most crucial step in the assessment of *Cryptosporidium* and *Giardia* in water and its efficiency will largely affect the downstream steps. It will largely determine the recovery rate (% of the initial number of (oo)cysts in the water that can be recovered). The currently applied concentration techniques are filtration, flocculation and continuous flow centrifugation.

1.4.1.1 Filtration

Generally, during filtration (oo)cysts are concentrated on a filter. Depending on the kind of filter used we can apply cartridge, membrane or ultrafiltration. Although difference in recovery rate between filters can be observed (DiGiorgio *et al*., 2002; Lee *et al*., 2004; Wohlsen *et al*., 2004; Helmi *et al*., 2011), the recovery efficiency not only depends on the filtration systems but may also be affected by the water sample. For example, the nature and size of the suspended solids and turbidity and pH of the water samples have been identified as important sources of variable performances of the filtration procedures (DiGiorgio *et al*., 2002; Feng *et al*., 2003).

1.4.1.1.1 Cartridge filtration

Nowadays, a number of filters used in cartridge filtration are commercially available with a nominal pore size of 1 µm through which large volumes of water (10-1000 L, depending on the water type) are passed at a flow rate of 1-5 L per minute. The trapped material is then eluted by washing using a manual or an automatic elution station. Subsequently, the washings are then further concentrated by centrifugation. Filta-Max/Filta-Max *xpress* foam filters or Envirochek capsule filters are listed in the USEPA method 1623 for the concentration of *Cryptosporidium* oocysts and *Giardia* cysts in water.

1.4.1.1.2 Membrane filtration

In membrane filtration (MF) large diameter membrane filters of nominal pore size ranging between 1 and 3 µm are applied (Ongerth and Stibbs, 1987; Shepherd and Wyn-Jones, 1996; Wohlsen *et al*., 2004). Flatbed membranes are generally prone to earlier clogging than cartridge filters when processing turbid samples and therefore suit better for treated water and low-turbidity source water (SCA, 2010). A high risk for (oo)cyst loss was reported with

elution of flatbed membranes and scraping actions were suspected to damage recovered (oo)cysts, thereby complicating their identification under the microscope (Stanfield *et al*., 2000; Wohlsen *et al*., 2004).

1.4.1.1.3 Ultrafiltration

Ultrafiltration is increasingly reported as an effective alternative for simultaneously recovering diverse microbes (*e.g.* viruses, bacteria and parasites) from large volumes of drinking water. This system has recently been shown to be more efficient and more robust at recovering *Cryptosporidium* oocysts and *Giardia* cysts from various water matrices. Moreover, it is less expensive than cartridge filters (Hill *et al*., 2005, 2007, 2009; Rhodes *et al*., 2011, 2012; Francy *et al*., 2013). However, this method is currently not standardized for the detection of *Cryptosporidium* and *Giardia* by relevant authorities.

1.4.1.2 Flocculation

Flocculation concentrates (oo)cysts with calcium carbonate (Vesey *et al.*, 1993). This technique is not recommended as it has a poor recovery rate compared to filtration (Feng *et al*., 2011) and because it affects the viability of the (oo)cysts (Campbell *et al.*, 1995).

1.4.1.3 Continuous flow centrifugation

Portable continuous flow centrifugation (PCFC) was approved by USEPA method 1623 for the concentration of *Cryptosporidium* and *Giardia* (oo)cysts in the different types of water samples. Besides yielding acceptable recovery rates, the technique is considered as costeffective and can be used for the simultaneous concentration of pathogens over a wide size range, including bacteria. The PCFC could be adopted for monitoring of large volumes of source and tap water for contamination with protozoa. It is anticipated that PCFC would also be equal or superior to filtration for protozoa monitoring in wastewater and effluents (Zuckerman and Tzipori, 2006).

1.4.2 Separation techniques

1.4.2.1 Immunomagnetic separation

Immunomagnetic separation (IMS) involves the attachment of (oo)cysts with magnetic beads coated with parasite-specific monoclonal antibodies. Mixing of beads in the water sample

causes separation of (oo)cysts present in the sample from debris. IMS is the only purification technique prescribed by USEPA. The success of this technology depends on many factors. Similarly to the filtration procedure, the turbidity of the water appears to be a critical factor associated with the recovery rate of IMS (Campbell and Smith, 1997; Stanfield *et al*., 2000; Hsu and Huang, 2007). In addition, McCuin *et al*. (2001) observed that recovery of *Giardia* cysts was affected by inorganic debris, recovery rate approaching 80% in water samples containing little, but poor recovery rate of 60% in water samples containing a high amount of clays and silts. Similarly, Yakub and Stadterman-Knauer (2000) showed that recovery rates dropped to almost 0% when dissolved iron concentration was between 4 and 20 mg/L for *Cryptosporidium* and between 100 and 200 mg/L for *Giardia*.

The bead-(oo)cyst complex is dissociated by the addition of acid, which is another critical feature of the IMS procedure. Indeed, some (oo)cysts might still remain attached to the beads after acid treatment (McCuin and Clancy, 2005).

Several modifications to the standard assay have been developed in order to overcome the limitations of IMS and to improve the recovery rate. Oocyst recovery rates were increased by 26% when pH was adjusted to neutrality (Kuhn *et al*., 2002). Some workers suggested increasing both the beads/sample ratio and the incubation time to a certain extent for increased recovery performance for both parasites (Pezzana *et al*., 2000; Hsu and Huang, 2001; Carey *et al*., 2006; Hsu and Huang, 2007). However, the later improvements should be balanced with the high cost of IMS reagents. It was also suggested to rinse the pellet twice with deionized water, in order to wash off possible interfering compounds originating from the elution step (McCuin *et al*., 2001). For wastewater samples, Zhang *et al*. (2008) reported a significant improvement (about 20%) of the recovery rate compared to unwashed pellets. Ware *et al.* (2003) found that heat dissociation (a-10-min incubation at 80°C) instead of acid dissociation improved the average oocysts recovery from 41% to 71% in seeded reagent water and from 10% to 51% in seeded river samples. Alternatively, it was demonstrated that a two-step acid dissociation provided higher recoveries for *Cryptosporidium* oocysts in wastewater samples (Zhang *et al*., 2008).

1.4.2.2 Density gradient centrifugation

Density gradient centrifugation is commonly applied to separate lighter parasites from the heavier debris in faecal material using a gradient or flotation solution (*e.g.* sucrose). This

basic technique has been adopted for use with environmental samples. However, researchers demonstrated that this is an inefficient procedure when trying to detect protozoan parasites in water concentrates or faecal materials (Fricker, 1995; Ward and Wang, 2001).

1.4.3 Detection techniques

1.4.3.1 Immunofluorescence microscopy

Detection of *Cryptosporidium* and *Giardia* (oo)cysts from water samples is done routinely under immunofluorescence microscopy. Staining of the (oo)cyst wall with fluorescein isothiocyanate (FITC)-conjugated anti-*Cryptosporidium* and anti-*Giardia* monoclonal antibodies relies upon the same immune interaction principle as IMS capture. Several commercially available direct antibody labelling reagents are used in existing protocols, using IgG, IgG_1, and/or IgM mAbs. Hoffman *et al*. (1999) tested the binding strength and stability of the antigen-antibody complex for different commercial antibodies. IgM showed a higher strength of binding for both *Cryptosporidium* and *Giardia* than IgG mAbs. Moreover, anti-*Cryptosporidium* IgM mAbs stains *C. parvum*, *C. baileyi* and *C. muris* with similar fluorescence intensities, while IgG mAbs did not.

Non-specific binding of the FITC mAbs may lead to false positives, thus biasing the resulting microbial risk assessment (Clancy, 2000; Zarlenga and Trout, 2004). Depending on the water matrix, organisms with similar morphologies and structures, such as algae, yeasts and fungal spores challenge the screening for *Cryptosporidium* or *Giardia* (Rodgers *et al*., 1995; Vesey *et al*., 1997). The manual counting of the slide is time-consuming and observation errors can occur, mainly due to operator subjectivity and fatigue and the presence of false positives from non-specific binding of FITC mAbs. Clancy *et al*. (1994) reported inconsistencies in immunofluorescence counts in experienced laboratories.

1.4.3.2 FISH

Fluorescent *in situ* hybridization (FISH) is used by several researchers (Lemos *et al.*, 2005; Smith *et al.*, 2004a; Graczyk *et al.*, 2003; Jenkins *et al.*, 2003; Surl *et al.*, 2003) in conjunction with FITC-conjugated mAbs to detect *Cryptosporidium* and *Giardia* (oo)cysts in environmental samples. FISH and FITC-conjugated mAbs provide information about the presence, the number, as well as the morphology and viability of the microorganisms involved in the contamination. Potentially viable (oo)cysts can be differentiated from non-

viable ones by colour. Viable (oo)cysts are represented by intact green shells with red cytoplasm (Graczyk *et al*., 2003). Lemos *et al.* (2005) showed that FISH is a highly specific and a sensitive assay for the detection of viable protozoa in faecal and environmental samples. However, the intensity of the FISH-fluorescence signal is relatively weak, which makes microscopic interpretation difficult (Medema, 1999).

1.4.3.3 Flow cytometry

An alternative technique using flocculation concentration, followed by flow cytometry with fluorescence activated cell sorting (FACS) was described by Vesey *et al*., (1994). The technique was found to be significantly more sensitive and considerably faster than the conventional methods. For flow cytometry the (oo)cysts in the water concentrates are stained in suspension with FITC-labelled antibodies and passed through the FACS. The FACS procedure is not specific and sensitive enough to count sorted (oo)cysts, since other organisms/particles of similar size may cross-react with the monoclonal antibody and have similar fluorescence characteristics. In addition, some water samples contain high numbers of autofluorescent algae which may also mimic (oo)cysts and therefore lead to incorrect conclusions if the FACS is used to directly count (oo)cysts. However, combination of IMS and flow cytometry enabled reliable rapid detection of a single cyst in 10 to 100 L of drinking water (Keserue *et al*., 2011). Hsu *et al*. (2005) showed that flow cytometry has the potential to become a more precise method for the detection of *Cryptosporidium* and *Giardia* in water. The flow cytometry-based method is rapid and could therefore help in disaster management and outbreak prevention in a field deployment (Keserue *et al*., 2012).

1.4.3.4 Polymerase chain reaction

Polymerase chain reaction (PCR) is one of the most extensively tested and widely used techniques to monitor water-borne protozoan parasites. Moreover, it allows to identify the sources of these pathogens in a complex environment. By detecting specific sequences of nucleic acids a variety of PCR assays can distinguish between isolates of the same species (sub-genotypes) or can be used to identify the different species within a genus. For example, nested PCR allows a more precise differentiation between parasite species, simultaneous detection of several parasites is possible by multiplex PCR and real-time PCR can provide quantification of the infection. Although PCR is rapid, highly sensitive and accurate, it has

several limitations. Besides false positives resulting from laboratory contamination, false negatives are also possible. These false negative test results can be explained by either inhibition of the PCR amplification by compounds present in the sample (e.g. humic acids, phenols, potassium dichromate and formaldehyde in the form of methylene glycol) (Guy *et al*., 2003; Skotarczak, 2009) or by low recovery rate of DNA during the extraction. To increase DNA yield, DNA can be extracted directly from the sediment using freeze-thaw cycles and/or sonication (*e.g*. Guy *et al.*, 2003; Jiang *et al.*, 2005; Yu *et al.*, 2009) or by use of a Fast prep DNA kit (Cacciò *et al.*, 2003; Shields *et al.*, 2008) or indirectly after purification of (oo)cysts by IMS (Xiao *et al.*, 2001; Ward *et al.*, 2002; Jiang *et al.*, 2005; Yang *et al.*, 2008; Castro-Hermida *et al.*, 2008a, 2008b, 2009; Daly *et al.*, 2010; Helmi *et al.*, 2011).

1.4.4 Viability of (oo)cysts

Determination of (oo)cysts' viability is important, because low numbers of viable (oo)cysts can cause infection when they pass the filtration system, while dead (oo)cysts have no public health significance as they are not able to establish a patent infection anymore. Currently, USEPA Method 1623 is the standard assay used for simultaneous detection of *Cryptosporidium* oocysts and *Giardia* cysts in various water matrices. However, the method is unable to distinguish between species, genotype, or to assess viability. Therefore, there has been considerable interest in developing methods which can determine (oo)cysts viability. An overiew of different techniques on how to assess the infectivity of *Cryptosporidium* oocysts was provided out by Robertson and Gjerde (2007), where it was concluded that infectivity studies with a neonatal mouse model can be considered as the gold standard.

1.4.4.1 Excystation

The most widely accepted *in vitro* procedure for determining oocyst viability is excystation, but this has not been used in combination with the IFA. This is because excystation is difficult to incorporate in the IFA protocol. Protocols for *in vitro* excystation of oocysts of *C. parvum*, including different chemical pre-incubation steps, were compared by Robertson *et al.* (1993) to examine some of the biochemical triggers involved in excystation and to define an *in vitro* excystation protocol of good reproducibility. This method is also used in combination with (quantitative real-time) PCR to estimate (oo)cyst viability (Bertrand *et al.*,

2009). However, *in vitro* excystation needs relatively high numbers of *Giardia* cysts (Sauch *et al.*, 1991) and it is considered to be subjective due to the presence of partially emerged trophozoites (Labatiuk *et al.*, 1991). Therefore the use of this technique in water samples, especially treated samples containing low numbers of (oo)cysts, is impractical. According to Neumann *et al.* (2000) *in vitro* excystation is not an acccurate measure of viability or infectious potential. Oocysts that failed to excyst *in vitro* were found infectious *in vivo.* Furthermore, sporozoites excysted from oocysts are not necessarily infectious *in vivo* although they are viable (Fayer *et al.*, 1998).

1.4.4.2 Vital dyes

Campbell *et al.* (1992) developed techniques with exclusion or inclusion of vital dyes *e.g.* DAPI (4', 6-diamidino-2-phenylindole) and PI (Propidium iodide) and differential interference contrast microscopy (DIC) for assessing viability of individual (oo)cysts isolated from water. DAPI stains the nucleus of cells blue, while PI is capable of passing through only damaged cell membranes and intercalates with the nucleic acids of injured and dead cells to form a bright red fluorescent complex. Although only cysts with a compromised wall are stained by PI, an intact cyst wall may be insufficient to induce an infection in the host (Bertrand *et al.*, 2009). Nevertheless, PI staining is considered to be a rapid and inexpensive method for detecting viability. Four types of oocysts can be identified using the assay: (i) viable (oo)cysts which include DAPI, but exclude PI, (ii) potential viable (oo)cysts which include neither DAPI or PI but show internal contents, (iii) non-viable (oo)cysts which either include neither DAPI or PI and do not show any internal contents, or (iv) which include both DAPI and PI. Since DAPI/PI staining allows simultaneous direct immunofluorescence with FITC-labelled monoclonal antibodies (Thiriat *et al.*, 1998), the combination of fluorogenic vital dye staining with IFA is the conventional method to detect and to estimate the viability of (oo)cysts in environmental samples (Dowd and Pillai, 1997; Thiriat *et al.*, 1998).

1.4.4.3 Cell culture

Cell culture-based models have rapidly evolved as a valuable alternative for the detection of infectious *Cryptosporidium* and in particular, the human colonic tumor cell line (HCT-8) was shown to be an appropriate cell model (Upton *et al*., 1994; Slifko *et al*., 1997; Rochelle *et al*., 1997, 2002). Many reseachers have used cell cultures in combination with PCR (Alum *et al.*,

2012; Di Giovanni *et al.*, 2006; Rochelle *et al.*, 1997). *Cryptosporidium* oocysts were inoculated onto monolayers of Caco-2 cells and grown on microscope slides and infections were detected by *C. parvum* specific reverse transcriptase PCR of extracted mRNA. A single infectious oocyst was detected by this procedure (Rochelle *et al.*, 1999). Alternatively to PCR, infection of cell monolayers can be estimated by immunofluoresence based assays (Slifko *et al*., 1997; Quintero-Betancourt *et al*., 2003; Carey *et al*., 2006). However, the cell culture method alone cannot be used to enumerate the number of (oo)cysts present in water samples because it is not clear if the presence of one infectious (oo)cyst will infect one or more cells in the cell culture. Although cell culture can detect infectious *C. parvum* and *C. hominis,* it is not certain yet which other *Cryptosporidium* species can infect cell monolayers. Moreover, cell culture cannot be used to assess the infectivity of *Giardia* (Burnet, 2012).

1.4.4.4 Molecular methods

Reverse-transcriptase PCR (RT-PCR) can be used to assess the viability of (oo)cysts. This is because RT-PCR relies on the integrity of mRNA, which usually has a very short half-life (seconds). RT-PCR targeting the heat shock protein 70 (hsp-70) gene has been used to detect viable *Giardia* cysts after heat shock induction (Lee *et al.*, 2009). The rationale behind this choice is that heat shock proteins are known to be synthesized in stressed organisms. Therefore, when (oo)cysts are exposed to a thermal shock, the induction of a heat shock response provides an index of viability (Abbaszagedan *et al.*, 1997). Moreover, with the recent introduction of real-time PCR, that allows the continuous monitoring of amplicon formation throughout the reaction, quantitative aspects of the infection could be studied. Bertrand *et al.* (2009) evaluated the potential of transcript quantification as an indicator of *Giardia* cyst viability during excystation by real-time RT-PCR and compared with the percentages of viability estimated using PI staining and *in vitro* excystation. They found significant correlations between the variations of the selected mRNAs (β-giardin, elongation factor 1 alpha and alcohol dehydrogenase E) and the percentages of viability estimated with staining and excystation methods.

However, these methods do not address human health risks because they are unable to differentiate between the currently recognized assemblages that affect humans. To overcome this limitation, Baque *et al.* (2011) developed a novel qRT-PCR method, targeting β-giardin,

which has the ability to discriminate between *G. duodenalis* assemblages A and B and concurrently assess viability.

1.4.5 Genotyping

1.4.5.1 Detection of *Cryptosporidium* genotypes

Different molecular diagnostic tools have been used in the differentiation of *Cryptosporidium* species/genotypes and *C. parvum* and *C. hominis* subtypes. Small subunit (SSU) rRNA-based tools are generally used in genotyping *Cryptosporidium* in humans, animals and water samples. A review of original studies on *Cryptosporidium* genotyping revealed the use of SSU rRNA tools in 86% of 116 publications, particularly the PCR-RFLP tool (Xiao, 2010). Other molecular diagnostic tools based on other genes *e.g.* oocyst wall proteins (COWP) and hsp-70 were popular previously, but their use in *Cryptosporidium* genotyping has decreased in recent years (Xiao, 2010). In recent years, qPCR assays using fluorescent probes and melting curve analysis are increasingly used in *Cryptosporidium* genotyping (Jothikumar *et al*., 2008; Hadfield *et al*., 2011; Burnet *et al*., 2012; Lalonde *et al*., 2013; Mary *et al*., 2013; Staggs *et al*., 2013; Yang *et al*., 2013). Recently, a genus-specific *Cryptosporidium* qPCR based on the actin gene has been developed (Yang *et al*., 2014).

Subtyping tools have been used extensively in studies of the transmission of *C. hominis* in humans and *C. parvum* in humans and ruminants. One of the popular subtyping tools is the DNA sequence analysis of the 60 kDa glycoprotein (gp60, also called gp40/15). Sequence analysis of the gp60 gene is widely used in *Cryptosporidium* subtyping because of its sequence heterogeneity and relevance to parasite biology (Xiao, 2010). It is based on variations in tandem repeats of the serine-coding trinucleotide TCA, TCG or TCT at the 5' (gp40) end of the gene, which categorize *C. hominis* and *C. parvum* each to several subtype families (see section 1.3.2). The name of gp60 subtypes starts with the subtype family designation (Ia, Ib, Id, Ie, If and Ig for *C. hominis* and IIa, IIb, IIc, IId, IIe, IIf, IIg, IIh, IIi, IIk and IIl for *C. parvum*) followed by the number of TCA (represented by the letter A), TCG (represented by the letter G), or TCT (represented by the letter T) repeats (Sulaiman *et al.*, 2005).

1.4.5.2 Detection of *Giardia* genotypes

For detection of *G. duodenalis* different protocols have been developed. Primers were based on different loci of different genes, such as 18S rDNA, glutamate dehydrogenase (GDH), triosephosphate isomerase (TPI) and β-giardin (BG). Based on intragenotypic variations, closely related genotypes were grouped into assemblages and subassemblages (see section 1.2.2.1). Multi-locus sequence analysis provided sub-assemblages within the A and B assemblages, *i.e.* AI, AII, AIII, BIII, BIII-like, BIV and BIV-like (Sprong *et al.*, 2009; Levecke *et al.*, 2009). To identify multi-locus genotypes among isolates of assemblages A and B, the sequences of the BG, GDH and TPI loci from isolates with matching assignment were merged, a multiple alignment was generated and trees were constructed using complete linkage. To increase the accuracy of the analysis, only multi-locus genotypes found in more than one isolate were selected. These analyses confirmed the existence of different monophyletic sub-assemblages at each marker (Sprong *et al*., 2009). Within the assemblage A, sub-assemblages were clear but this was difficult for assemblage B because of higher genetic diversity.

1.5 Conclusions

Significant risk is associated with the transmission of *Cryptosporidium* and *Giardia* through water-borne outbreaks of drinking and recreational water worldwide. Surface water is the main source of contamination of drinking water either by human or animal sources. Human infection risk depends on the presence of viable and potentially zoonotic (oo)cysts present in the water. For safe drinking water there are several regulations and directives established in the USA, the UK, Canada and New Zealand. The new European Drinking Water Directives (Directive 98/83/CE) establishes the goal that all the state members should provide drinking water supplies with the absence of pathogenic organisms. However, the highly variable sensitivity of the methods available for the detection of *Cryptosporidium* and *Giardia* and problems associated with the determination of the (oo)cysts viability/infectivity make the establishment of maximum acceptable concentrations very difficult (Carmena *et al.*, 2007). Further research is needed to optimise, standardise and validate methods for detecting and identifying *Cryptosporidium* and *Giardia* (oo)cysts in water samples.

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Objectives

In developed countries, even with access to advanced water treatment technologies, water-borne outbreaks of *Cryptosporidium* and *Giardia* through drinking and recreational water occur on a regular basis. Surface water is used both for production of drinking water and recreational activities. In Belgium, up to 30% of tap water is produced from surface water. A pilot study in Belgium indicated that surface water is frequently contaminated with *C. parvum* and *G. duodenalis*. Currently, there is no information on the source, nor on the factors contributing to the spread of the surface water contamination.

In contrast, in developing countries water treatment is not readily performed. Due to poor sanitary conditions and the lack of waste water treatment in developing countries, water is an important vector of *C. parvum* and *G. duodenalis* infection, through water-borne or food-borne transmission.

The general objective of this PhD is to determine the extent of water-borne transmission of *Cryptosporidium* and *Giardia* in two different geographical, cultural, and socio-economic landscapes, *i.e.* in Belgium and Bangladesh. Bangladesh was chosen because ground water in Bangladesh is often contaminated with arsenic. Consequently, people rely on surface water for drinking water, especially in rural areas, and hence water contamination is a constant threat to human and animal hosts.

The four specific objectives are:

1- To monitor the presence of *Cryptosporidium* and *Giardia* in different water catchment sites in Belgium and to discriminate between (oo)cysts from human or animal origin (**Chapter 2**).

2- To investigate whether the applied treatment procedures were efficient in eliminating *Cryptosporidium* and *Giardia* from drinking water (**Chapter 2**).

3- To estimate the infection risk of *Cryptosporidium* and *Giardia* infections associated with swimming and other recreational activities in public swimming pools, recreational water bodies and splash parks in Flanders (Northern Belgium) (**Chapter 3)**.

4- To investigate the transmission of *Cryptosporidium* and *Giardia* between cattle and their handlers and indirect water-borne transmission of *Giardia* and *Cryptosporidium* between the inhabitants and their animals in rural villages in Bangladesh (**Chapter 4)**.

Chapter 2

Occurrence and potential health risk of *Cryptosporidium* and *Giardia* in different water catchments in Belgium

Based on:

Ehsan, A.; Geurden, T.; Casaert, S.; Paulussen, J.; De Coster, L.; Schoemaker, T.; Chalmers, R.; Grit, G.; Vercruysse, J.; Claerebout, E. 2015. Occurrence and potential health risk of *Cryptosporidium* and *Giardia* in different water catchments in Belgium. *Environ. Monit. Assess*. 187(2):6.

2.1 Introduction

The protozoan parasites *Cryptosporidium* and *Giardia duodenalis* are worldwide considered as an important cause of gastrointestinal disease in human patients and in animals. In Belgium, the national reported incidence in 2010 was 2.5 and 10.8 cases per 100,000 inhabitants for *Cryptosporidium* and *G. duodenalis*, respectively (WIV-ISP, 2010) and gastrointestinal symptoms in Belgian patients are frequently associated with these protozoa (Geurden *et al.*, 2009).

Gastrointestinal infection is due to oral ingestion of *Cryptosporidium* oocysts or *Giardia* cysts. The high number of (oo)cysts excreted shortly after infection, together with the low infectious dose, results in an easy spread of infection (Smith *et al.*, 2006a). Since the excreted (oo)cysts remain infectious for several weeks in water, both treated tap water and recreational water are considered as excellent vehicles for infection, especially as *Cryptosporidium* in particular is highly resistant to chlorine disinfection. Since large scale drinking water-borne outbreaks of cryptosporidiosis such as the one in Milwaukee (1993) affecting more than 400,000 people, regulatory monitoring for the presence of *Cryptosporidium* has been a requirement in the UK, The Netherlands and the USA and some provinces of Canada and Australia. The EU/98/83/EC directive only requires an investigation of pathogenic microorganisms, e.g., *Cryptosporidium* if there is a non-compliance for *Clostridium perfringens*. In the UK, *Cryptosporidium* has been implicated in 69 % of water-borne outbreaks of infectious intestinal disease (Smith *et al.*, 2006b). An outbreak of water-borne giardiosis in the town of Saint Hubert in 2008 illustrates the public health threat from intestinal protozoa in Belgium (RTBF, 2008).

Surface water supplies are especially vulnerable to contamination. Contamination with *Cryptosporidium* and *Giardia* has been detected in water catchments in several countries in the EU, for example, in The Netherlands (Ketelaars *et al.*, 1995), Germany (Ajonina *et al.*, 2012, 2013) Luxemburg (Helmi *et al.*, 2011), France (Mons *et al.*, 2009), Italy (Briancesco and Bonadonna, 2005), Spain (Carmena *et al.*, 2007) and Portugal (Júlio *et al.*, 2012). A pilot study in four water catchment areas in 2002 indicated that *Cryptosporidium* and *Giardia* are also prevalent in surface water in Belgium (Aquaflanders, unpublished data). Since up to 30 % of Belgian tap water is produced from surface water, this puts drinking water safety under

pressure. Some of the surface water that is used for production of drinking water in Belgium is abstracted downstream of wastewater discharges. Several studies have shown that human wastewater is often contaminated with high concentrations of *Cryptosporidium* and *Giardia* and have identified wastewater treatment plants as point sources of surface water contamination (Chalmers *et al.*, 2010; Castro-Hermida *et al.*, 2010). Next to human wastewater, livestock is considered to contribute to surface water contamination, directly or through effluent from farm steadings and hard standings or from manure-laden fields (Sischo *et al.*, 2000; Bodley-Tickell *et al*, 2002). Several studies indicated that zoonotic *Cryptosporidium* and *Giardia* genotypes are highly prevalent in livestock in Belgium (Geurden *et al.*, 2007, 2008), suggesting that water contamination by infected farm animals may pose a safety risk to drinking water in Belgium.

The objective of this study was to determine the relative contribution of human waste and livestock to contamination of surface water with *Cryptosporidium* and *Giardia* and to investigate whether the applied treatment procedures were efficient in eliminating these parasites from drinking water. To this purpose, the presence of *Cryptosporidium* and *Giardia* in surface water and treated water was monitored in water catchment sites in a rural area in Belgium and (oo)cysts from human or animal origin were discriminated using (multilocus) genotyping.

2.2 Materials and methods

2.2.1 Sampling sites and water samples

Four water catchment sites were selected in the province of West-Flanders (Belgium), based on their location in an agricultural area or nearby a community. From February 2010 until April 2012, each month, three different water matrices were sampled at the Blankaart water catchment site, i.e., raw surface water (RW), water from the storage basin (after 4 months of sedimentation and prior to treatment, SW) and treated tap water (TW). In the Blankaart water catchment, surface water is collected from the river Yzer downstream of the town of Ypres and two wastewater treatment sites (human waste as possible contamination source) and from the Blankaart nature reserve (livestock grazing area) (Figure 2.1).

Two matrices of water, RW and TW, were also sampled monthly from three other water

catchment sites, i.e., Dikkebus, Gavers and Zillebeke, from September 2010 until August 2011. In the catchment sites of Dikkebus and Zillebeke, surface water is collected from the Ieperlee (a tributary of the river Yzer) upstream of Ypres, in an agricultural area (possible contamination by runoff from farms and fields). In the Gavers catchment site (Harelbeke), water is collected from the river Scheldt, downstream of the urban area of northern France and a wastewater treatment site (human waste as possible contamination source). The volume of water sampled in each catchment site was 60, 15 and 15 L for TW, SW and RW respectively. All water samples were transported to the lab for filtration on the day of sampling.

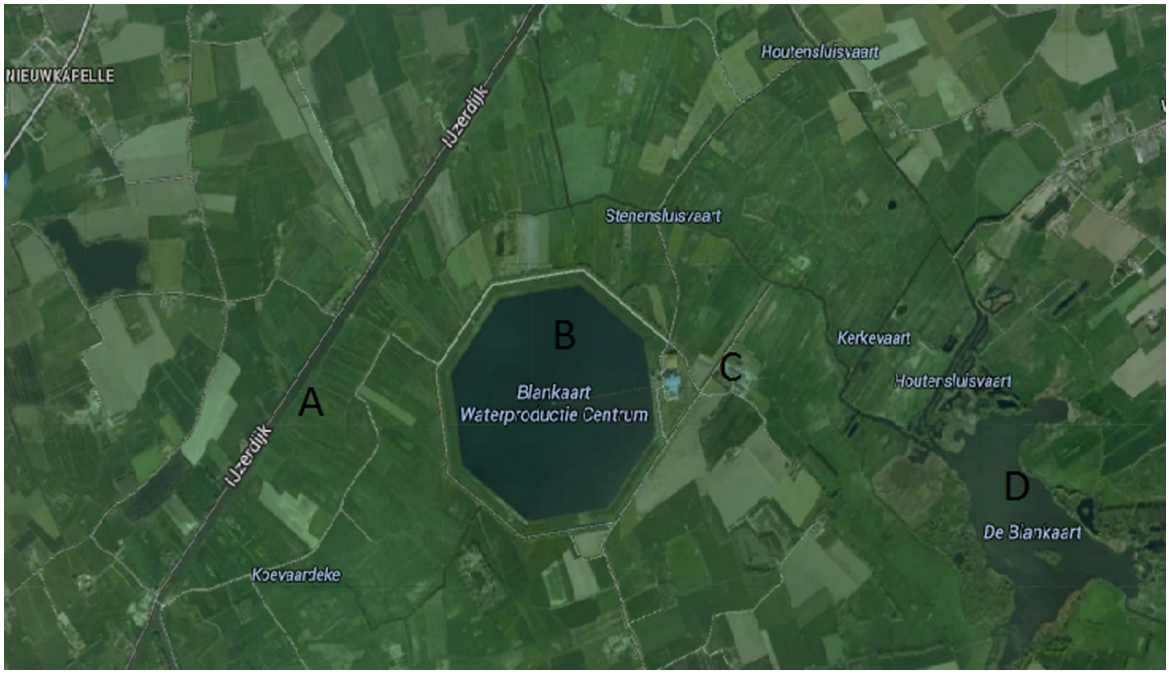

Figure 2.1 Picture of the Blankaart catchment site. A Yzer river, B water storage basin, C water supply channel from the Yzer river and D the Blankaart nature reserve.

The water treatment in Blankaart, Dikkebus and Zillebeke consists of the following steps: storage (for a period of 4 months, in Blankaart only), oxidation, coagulation-sedimentation, rapid sand filtration, granular activated carbon filtration and disinfection. The water treatment in the Gavers treatment plant consists of nitrification, followed by dephosphation in a flocculation step prior to storage in the Gavers reservoir. After storage, the following treatment steps are used: ultrafiltration, granular activated carbon filtration and disinfection.

2.2.2 Detection of *Cryptosporidium* oocysts and *Giardia* cysts

A protocol was optimized to detect *Cryptosporidium* and *G. duodenalis* in water samples, based on the USEPA method 1623 (USEPA, 2005). To validate the protocol, spiking experiments were performed with 15 water samples collected from the Blankaart in February and March 2010 (5 RW samples, 5 SW samples and 5 TW samples) and with RW and TW water samples from each water catchment site in December 2010 or January 2011. A total of 9 RW samples (15 L), 6 SW samples (15 L) and 9 TW samples (60 L) were spiked with 100 inactivated *Cryptosporidium* and 100 inactivated *Giardia* (oo)cysts, permanently labeled with red fluorescent dye (ColorSeedTM, BTF Pty Ltd., North Ryde, Australia). The ColorSeedTM (oo)cysts were added to the water samples prior to filtering to estimate the percent recovery of (oo)cysts according to the manufacturer's instructions.

Both spiked and non-spiked water samples were filtered through Filta-Max Xpress filters (IDEXX Laboratories, Inc., Westbrook, ME, USA) with the aid of a peristaltic pump with recommended flow rates of 2 L/min. The Filta-Max Xpress filters were washed with the Filta-Max Xpress automated washing station for elution of the filters following the manufacturer's instructions. The eluate was centrifuged and the volume of sediment was measured. Between 0.5 ml (TW and SW) and 2 ml (RW) of sediment was used for immunomagnetic separation (IMS) of the (oo)cysts, using *Cryptosporidium* and *Giardia*-specific antibody coated magnetic beads according to the manufacturer's protocol (Dynabeads® GC-Combo, Invitrogen Dynal, A.S., Oslo, Norway). IMS-purified cysts and oocysts were stained on well slides by fluorescein isothiocyanate (FITC)-conjugated anti-*Cryptosporidium* and anti- *Giardia* MAbs FITC-conjugated monoclonal antibodies (EasyStainTM) (BTF Pty Ltd. Australia). Slides were examined using a Leica Leitz DMRB fluorescence microscope. The well surface was scanned at 200 or 400 times magnification using a FITC fluorescence filter (450-590 nm Chroma Technology Corp.) and Texas Red fluorescence filter (530-585 nm, FT600, LP615) to distinguish ColorSeedTM (oo)cysts (which fluoresce red with the Texas Red filter) from unspiked/natural (oo)cysts (which fluoresce bright green with the FITC filter). *Giardia* cysts and *Cryptosporidium* oocysts were identified and counted based on their size, morphology and fluorescence. Results were expressed as recovery percentage for spiked (oo)cysts and as count per liter for naturally occurring (oo)cysts. Slides containing natural (non-spiked) (oo)cysts were kept at 4 °C for DNA extraction.

2.2.3 Bacterial analysis

Faecal bacteria indicators were quantified in all water samples. Coliform bacteria and *Escherichia coli* were quantified in 100 ml water samples by using the international standard method ISO 9308-2 (ISO, 2012), based on the Colilert®-18 Idexx method with a detection limit of 1 colony-forming unit (CFU)/100 ml. Enterococci were detected by the Enterolert®-DW Idexx method conform WAC/V/A/003, with a detection limit of 1 CFU/100 ml. *C. perfringens* was quantified by using standard method ISO 6461-2 (ISO, 1986)-Water quality-Detection and enumeration of the spores of sulphite-reducing anaerobes (Clostridia)- part 2: Method by membrane filtration and conform WAC/V/A/ 007, with a detection limit of 1 CFU/100 ml. Total number of colony counts at 22/37 °C followed the international standard method ISO 6222 (ISO,1999) Water quality-Enumeration of culturable microorganisms-Colony Count by inoculation in a nutrient agar culture medium and conform WAC/V/A/001 with a detection limit of 1 CFU/ml. The bacteriological analyses were done within 12 h after sampling.

2.2.4 Physicochemical analysis

Physicochemical characteristics of the water were determined at each sampling event. The pH of water samples was determined according to the international standard method ISO 10523:1994 Water quality: Determination of pH. The turbidity of water samples was determined following the international standard method ISO 7027:1999 Water quality: Determination of turbidity. The result was expressed as nephelometric turbidity unit (NTU).

2.2.5 DNA extraction and molecular analysis

DNA was extracted from non-spiked water samples that were positive by microscopy for *Cryptosporidium* and/ or *Giardia*. Genomic DNA was extracted from (oo)cysts that were scraped from the microscope slides by the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, incorporating an initial step of three freeze-thaw cycles (freezing in liquid nitrogen for 5 min and heating at 95 °C for 5 min) in the protocol to maximize disruption of (oo)cysts.

Previously described PCR protocols were used to amplify the 18S rDNA gene (Johnson *et al.*, 1995; Xiao *et al.*, 2001) and the heat shock protein 70 (hsp-70) gene (Morgan *et al.*, 2001) of *Cryptosporidium*. For subgenotyping of *Cryptosporidium parvum* and

Cryptosporidium hominis positive samples, the 60 kDa glycoprotein (gp60) was targeted (Peng *et al.*, 2001). For the identification of *Giardia*, the β-giardin gene (Lalle *et al.*, 2005) was used in two-step nested PCR. For assemblage specific amplification of *Giardia*, the triose phosphate isomerase (TPI) gene (Sulaiman *et al.*, 2003) was used (Geurden *et al.*, 2008; Levecke *et al.*, 2009). For all PCR reactions, negative (PCR water) and positive controls (genomic DNA) were included. The PCR products were visualized in agarose gel (1.5 %) stained with ethidium bromide under UV light. PCR products were fully sequenced by the BIG Dye Terminator V3.1 Cycle sequencing Kit (Applied Biosystems). Sequencing reactions were analyzed on a 3100 Genetic Analyzer (Applied Biosystems) and assembled with the programme Seqman II (DNASTAR, Madison WI, USA). To determine the subgenotype, the fragments were aligned with homologous sequences available in the GenBank database, using MegAlign (DNASTAR, Madison WI, USA).

2.2.6 Precipitation data

Daily rainfall data recorded at representative weather stations from the Royal Meteorological Institute were used. The average of 3 days rainfall prior to the collection date of water samples as well as rainfall on each of these individual days and on the day of water sampling was used to investigate a potential association between precipitation and concentration of *Cryptosporidium* and *Giardia* (oo)cysts in water samples.

2.2.7 Statistical analysis

The $\chi 2$ test was used to compare proportions of positive RW samples for *Cryptosporidium* and *Giardia* between different seasons in the Blankaart water catchment. *Giardia* cyst counts and *Cryptosporidium* oocyst counts in Blankaart RW samples were compared between different seasons using the Kruskal-Wallis test. The degree of association between parasite concentrations, the concentration of indicator bacteria, physicochemical parameters and rainfall was determined in all water samples from the Blankaart catchment using the Pearson's correlation test and the nonparametric Spearman rank correlation test. Values of $p<0.05$ were considered statistically significant. All statistical tests were performed using IBM SPSS statistics version 21.

2.3 Results

2.3.1 Prevalence and concentration of *Cryptosporidium* and *Giardia* in water samples

Good recovery rates for both parasites were obtained, with higher recovery rates for *Cryptosporidium* (41-44 %) than *Giardia* (28-45 %, Table 2.1). Recovery rates for both parasites met the standards set by USEPA, 2005 (initial recovery rates of 24-100 %).

Table 2.1 The mean recovery rates ± standard deviation for 100 spiked *Cryptosporidium* oocysts and 100 *Giardia* cysts in raw surface water (RW, n=10), water from the storage basin (SW, n=4) and treated tap water (TW, n=10).

Water Matrices	***Cryptosporidium* oocysts**	***Giardia* cysts**
RW	44 ± 10%	28 ± 22%
SW	41 ± 12%	34 ± 15%
TW	45 ± 16%	45 ± 20%

In Zillebeke, Dikkebus and Gavers, only low numbers of (oo)cysts were recovered occasionally from raw water samples (<1 /L), mainly in winter (December-March, Figure 2.2). In contrast, raw water samples from the Blankaart catchment were frequently contaminated with *Giardia* (92 % positive samples) and *Cryptosporidium* (96 % positive samples). No significant difference was found between different seasons in the proportion of RW samples positive for *Giardia* ($\chi 2 = 5.738$, df=3, $p=0.125$) and *Cryptosporidium* ($\chi 2= 2.898$, df=3, $p=0.408$). However, *Cryptosporidium* oocyst counts ($p<0.01$) and *Giardia* cyst counts ($p<0.01$) were significantly different between different seasons, with higher counts in winter and spring (Figure 2.3). Peak values of 35 *Giardia* cysts/L and 51 *Cryptosporidium* oocysts/L were obtained in April 2010 and February 2011, respectively. (Oo)cyst contamination of water after the storage basin followed a similar seasonal pattern, but (oo)cyst numbers were lower compared to raw water, with peak values of 24 *Giardia* cysts/L (average 5 cysts/L) and 4 *Cryptosporidium* oocysts/L (average 1 oocysts/L (Figure 2.3). All samples from treated tap water in all catchment sites were negative for *Cryptosporidium* and *Giardia*.

2.3.2 Bacteriological indicators of faecal contamination

E. coli, total coliforms, enterococci and *C. perfringens* were detected in all samples of raw water and reservoir water (Table 2.2). In treated water, these organisms were only found in very low numbers in 3 out of 63 samples. At the Blankaart catchment, water samples from the storage basin contained higher concentrations of bacteria than raw water samples from the river (Table 2.2). No significant correlation was observed between faecal bacterial indicators and *Cryptosporidium* and *Giardia* (oo)cyst counts in any sample at any catchment sites ($p>0.05$, results not shown).

2.3.3 Physicochemical parameters

Physicochemical data of the water samples are shown in Table 2.2. Most of the (oo)cysts were detected in winter and spring, when the turbidity was lowest. There was no significant correlation between pH or turbidity and the occurrence of *Cryptosporidium* and *Giardia* ($p>0.05$, results not shown).

2.3.4 Rainfall

Rainfall data of 3 days prior to the collection date of water samples (each individual day as well as cumulative rainfall for 3 days) were plotted against monthly (oo)cyst counts for *Cryptosporidium* and *Giardia*. No significant correlation was observed between rainfall data and the concentration of (oo)cysts in water samples ($p>0.05$, results not shown).

2.3.5 Molecular identification of parasites recovered from water samples

Giardia sequences were obtained from 38 out of 53 microscopy-positive water samples. Most sequences at the Blankaart catchment site belonged to assemblage AI (n=7), AII (n=8) and E (n=10). Assemblages BIV (n=2) and BIV-like (n=1) were detected less frequently (Table 2.3). At the other catchment sites, assemblage AI was predominant (n=6, NCBI accession numbers KF963556, KF963557 and KF963563-KF963566), while one assemblage AII sample was detected at the Gavers catchment site (NCBI accession number KF963577) and one assemblage E sample in Zillebeke (NCBI accession number KF963578).

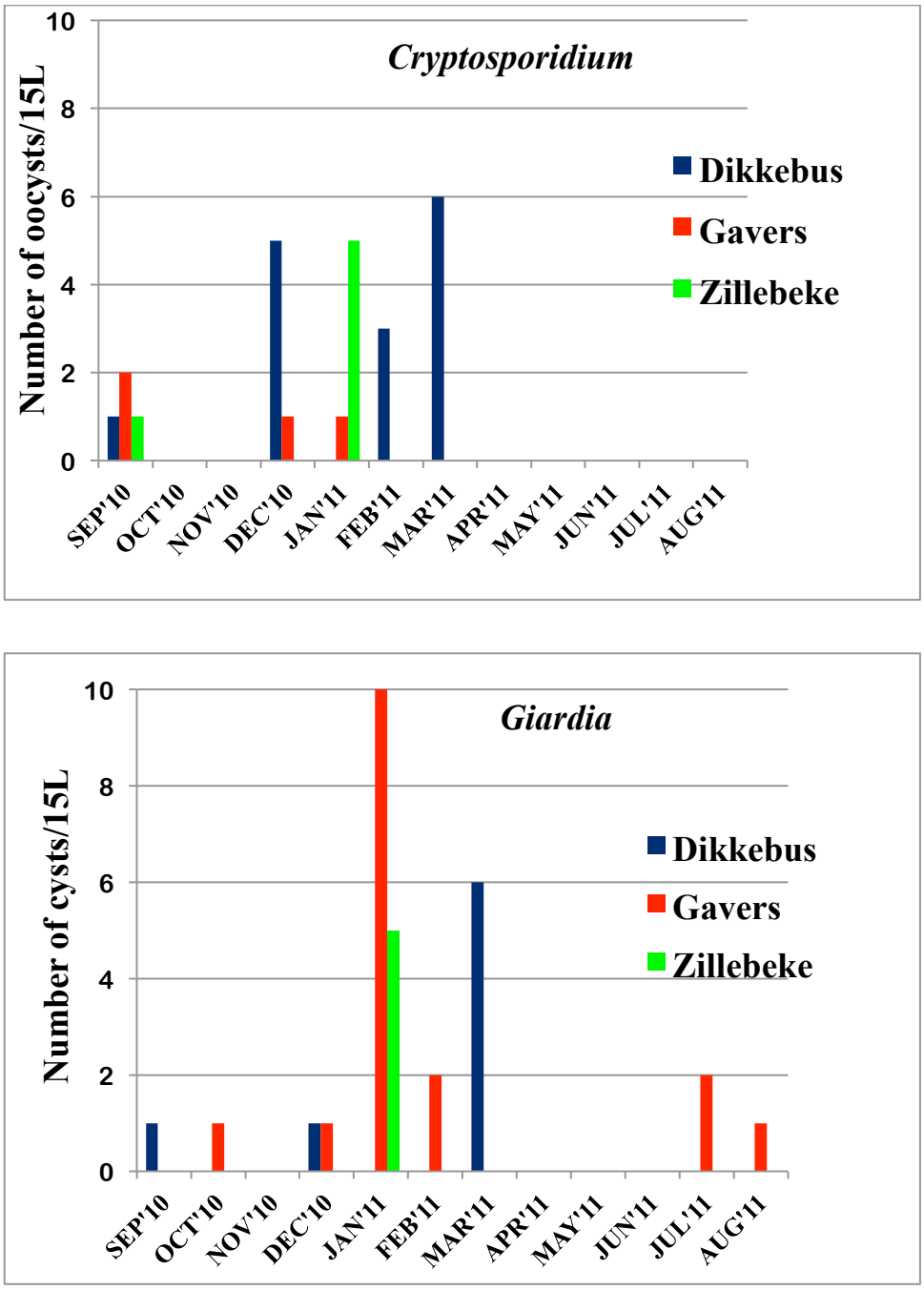

Figure 2.2 Monthly occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in the Zillebeke, Dikkebus and Gavers catchment sites, expressed as total numbers of (oo)cysts seen in raw water (15 L samples).

In water samples from the Blankaart catchment, *C. parvum* (n=7), *C. hominis* (n=2), *C. andersoni* (n=2), *Cryptosporidium* horse genotype (n=4) and *C. suis* (n=1) were detected (Table 3). One *C. parvum* isolate from Blankaart raw water was identified as gp60 subtype

IIdA20G3T5 (NCBI accession number KF944374). No successful gp60 sequence was obtained from *C. hominis* positive samples.

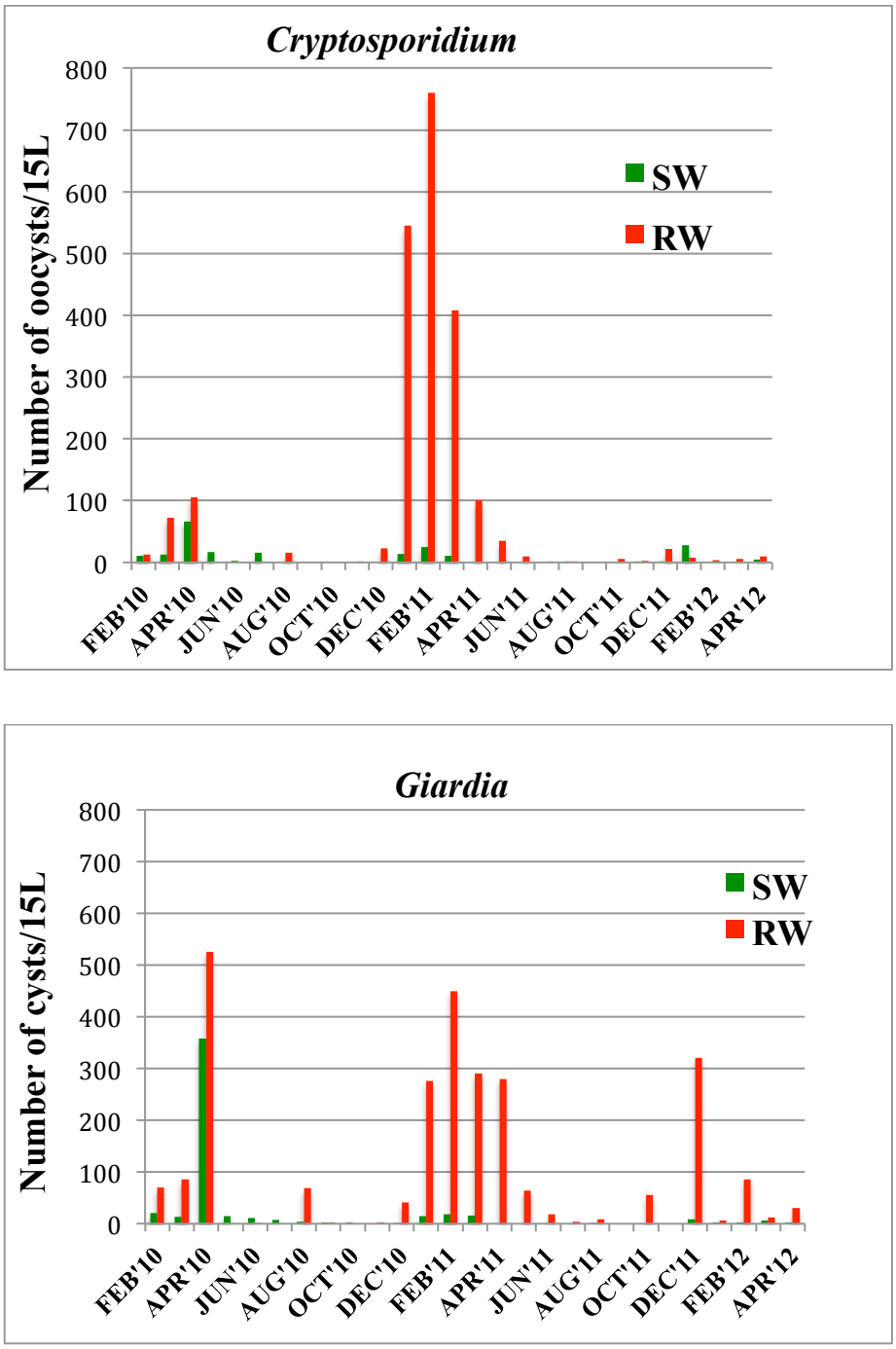

Figure 2.3 Monthly occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in the Blankaart catchment site, expressed as total numbers of (oo)cysts seen in raw water (RW, in red, 15 L samples) and storage basin water (SW, in green, 15 L samples).

2.4 Discussion

In the spiking experiment, higher recovery rates were obtained for *Cryptosporidium* than *Giardia*, which was in an agreement with previous observations (McCuin and Clancy, 2003; USEPA, 2005; Carmena *et al.*, 2007). Recovery rates for both parasites met the standards set by USEPA (2005).

There were large differences in contamination between different water catchment sites. Low levels of contamination were found in Dikkebus, Zillebeke and Gavers, whereas in the Blankaart site, high *Cryptosporidium* and *Giardia* counts were recorded. In the Dikkebus and Zillebeke catchments, water is collected in the basin of the river Ieperlee, a tributary of the river Yzer upstream of the city of Ypres, whereas in the Blankaart site, water is collected from the river Yzer downstream of Ypres and from the Blankaart nature reserve. At first sight, these data suggest a major role of the city of Ypres as a source of contamination and one would expect this contamination to be of human nature. However, the genotyping results suggested that many of the recovered (oo)cysts were from animal origin. Although two *Cryptosporidium* positive samples were identified as *C. hominis*, most *Cryptosporidium* species/genotypes suggested livestock, pigs and horses, as potential sources of water contamination. Similarly, the majority of identified *Giardia* genotypes were associated with livestock (assemblage E) or animals (assemblage AI). Although assemblage AI can infect humans, the most common *Giardia* assemblages in humans are AII and B (Geurden *et al.*, 2009; Sprong *et al.*, 2009). Assemblages AII, BIV and BIV-like were also found in some water samples, suggesting that both human and animal sources contribute to the water contamination in the Blankaart catchment site. *Cryptosporidium* spp. and *G. duodenalis* were found throughout the year, but the highest numbers of (oo)cysts were found in winter and spring. Higher contamination levels of *Cryptosporidium* and/or *Giardia* in surface water in winter and spring were also observed in previous studies (Isaac-Renton *et al.*, 1996; Keeley and Faulkner, 2008; Helmi *et al.*, 2011; Ajonina *et al.*, 2012, 2013; Van Dyke *et al.*, 2012). In other studies, concentrations of *Cryptosporidium* and/or *Giardia* were higher in other seasons (Horman *et al.*, 2004; Carmena *et al.*, 2007; Castro-Hermida *et al.*, 2009; Mons *et al.*, 2009; Wilkes *et al.*, 2009; Ajonina *et al.*, 2012, 2013; Agullo- Barcelo *et al.*, 2013) or no seasonal differences were observed (Robertson and Gjerde, 2001; Carmena *et al.*, 2007; Júlio *et al.*, 2012). The presence of high concentrations of (oo)cysts in surface water in spring and

summer has been associated with the presence of infected cattle (Castro-Hermida *et al.*, 2009). Runoff from manure-laden fields has been suggested as a source of contamination with *Cryptosporidium* oocysts and *Giardia* cysts (Slifko *et al.*, 2000; Fayer, 2004). Other studies have also reported an effect of land use on the contamination of water with *Cryptosporidium* and *Giardia* (Hansen and Ongerth, 1991; Ongerth *et al.*, 1995; Ong *et al.*, 1996; Burnet, 2012). The Blankaart catchment site is situated in an agricultural area and high prevalence of *Cryptosporidium* and *Giardia* have been observed in cattle in Belgium (Geurden *et al.*, 2004, 2006). However, we found no correlation between high concentrations of *Cryptosporidium* and *Giardia* in the surface water and rainfall prior to sampling. Moreover, according to Belgian legislation, spreading of manure onto the fields is only allowed from mid-February until mid-October and high concentrations of *Cryptosporidium* and *Giardia* were sometimes observed outside this period. Therefore, there were no strong indications that runoff from agricultural land was a major source of water contamination in the Blankaart catchment.

Despite the high counts in raw water, the currently applied methods for water treatment seem to be efficient. Sedimentation of the water for 4 months at the Blankaart catchment site strongly reduced the concentration of (oo)cysts in the water and no (oo)cysts were detected in any of the treated water samples at any catchment sites. Although it cannot be excluded that low concentration of (oo)cysts in the treated water remained undetected by the relatively insensitive USEPA method 1623 (Di Giorgio *et al.*, 2002; Zukerman and Tzipori, 2006; Shaw *et al.*, 2008), the results suggest that the applied treatment methods are effective. However, the presence of high numbers of (oo)cysts in surface water in winter and springtime poses a threat in case of failure of the filtration system. Several drinking water-related outbreaks of giardiosis or cryptosporidiosis occurred after failure of the treatment system (Herwaldt *et al.*, 1992; MacKenzie *et al.*, 1994; Kramer *et al.*, 1996; Wallis *et al.*, 1996; Pozio *et al.*, 1997; Levy *et al.*, 1998; Barwick *et al.*, 2000; Stirling *et al.*, 2001; Lee *et al.*, 2002; Webber, 2002; Risebro *et al.*, 2007), including an outbreak of water-borne giardiosis in Belgium in 2008 (RTBF, 2008). Monitoring drinking water for *Cryptosporidium* and *Giardia* is not compulsory in Belgium. According to the European Directive 98/83/CE, no bacterial faecal indicator should be detected in 100 ml of drinking water. The same directive recommends checking the presence of *Cryptosporidium* when *C. perfringens* has been detected in drinking water. Although several studies showed that the use of faecal

indicator bacteria provides relevant information concerning drinking or surface water quality (Payment and Franco, 1993; Obiri-Danso and Jones, 1999; Tallon *et al.*, 2006; Touron *et al.*, 2007; Xiao *et al.*, 2013), others failed to establish correlations between the occurrence of *Cryptosporidium* and/or *Giardia* and the level of faecal contamination (Hörman *et al.*, 2004;

Table 2.2 Descriptive statistics of microbiological and physicochemical parameters of the water samples, collected monthly in four catchment sites in Belgium.

Water samples	n	Turbidity (NTU)			pH			Total Coliform/100ml			Enterococci/100ml			*E. coli*/100ml			*Clostridium*/100ml		
		R	M	SD	R	M	SD	R	M	SD	R	M	SD	R	M	SD	R	M	SD
BPW	27	0.09-0.36	0.23	0.08	6.82-7.44	7.05	0.17	0-179	7.46	36.54	0-1	0.04	0.20	0-0	0	0	0-2	0.2	0.50
BSW	27	0.86-20.00	4.03	4.05	7.32-8.65	8.22	0.30	250-141,400	22,506	35,064	0-5,200	638	1,177	0-11,200	2,123	3,006	10-3,600	710	778
BRW	27	6.87-49.90	25.11	12.19	7.11-9.00	8.11	0.37	0-52,350	4,354	10,983	0-120,000	6,371	25,671	0-11,950	902	2,785	10-1,500	192	353
B7V	8	7.45-52.80	23.14	15.27	7.90-8.87	8.12	0.31	900-173,300	31,724	57,701	0-8,200	1,311	2,799	0-9,400	1,946	3,179	0-3,600	961	1,102
B10	8	5.01-20.20	9.21	4.95	7.57-9.06	8.04	0.56	100-120,300	24,974	45,093	100-400	164	120	0-12,000	1,588	4,208	20-1,000	471	321
DPW	12	0.08-0.89	0.25	0.26	7.52-8.34	7.88	0.24	0	0	0	0	0	0	0	0	0	0	0	0
DRW	12	2.88-34.40	16.63	8.45	7.83-8.64	8.19	0.24	0-6,350	2,323	2,240	0-1,400	283	479	0-1,800	400	783	30-960	290	257
GPW	12	0.07-0.40	0.19	0.12	7.68-7.95	7.80	0.08	0	0	0	0	0	0	0	0	0	0	0	0
GRW	12	0.34-2.86	1.20	0.69	7.93-8.47	8.21	0.15	400-77,700	8,384	21,942	0-180	73	60	50-1,150	288	428	10-290	174	91
ZPW	12	0.09-0.30	0.19	0.06	7.65-8.13	7.81	0.14	0	0	0	0	0	0	0	0	0	0	0	0
ZRW	12	2.54-17.30	7.67	4.37	7.69-9.03	8.20	0.35	0-24,200	4,003	7,676	0-500	133	200	0-100	47	47	10-500	185	187

*n=number of samples; R=Range ; M=Mean ; SD= Standard deviation ; B= Blankaart ; D= Dikkebus ; G=Gavers ; Z= Zillebeke ; PW= Purified weter ; RW= Raw water ; SW= Storage water.

Table 2.3 *Giardia duodenalis* genotypes (assemblages) and *Cryptosporidium* species/genotypes detected in water samples from the Blankaart water catchment.

G. duodenalis	**Species/genotype**	**n**	**NCBI accession numbers**
	AI	9	KF963541, KF963543, KF963544, KF963547, KF963558-KF963562
	AII	8	KF963552- KF963555, KF963567, KF963568, KF963573, KF963575
	BIV	2	KF963569, KF963576
	BIV-like	1	KF963549
	E	10	KF963542, KF963545, KF963546, KF963548, KF963550, KF963551, KF963570-KF963572, KF963574
Cryptosporidium	*C. hominis*	2	KF944361, KF944366
	C. parvum	7	KF944362, KF944365, KF944367, KF944369, KF944371, KF944372, KF944375
	C. andersoni	2	KF944360, KF944363
	C. suis	1	KF944376
	Horse genotype	4	KF944364, KF944368, KF944370, KF944373

Briancesco and Bonadonna, 2005; Hänninen *et al.*, 2005; Helmi *et al.*, 2011; Xiao *et al.*, 2013). Similarly, in our study, no correlation was found between bacterial faecal indicators or *C. perfringens* and *Cryptosporidium* and *Giardia* counts, confirming that bacterial counts are not always a reliable tool to predict the presence/absence of these parasitic protozoa (WHO, 2011).

2.5 Conclusions

In conclusion, our results showed the presence of *Cryptosporidium* and *Giardia*, including human and zoonotic genotypes, in surface water at all investigated catchment sites. Although genotyping results suggested that most of the recovered parasites were from animal origin, surface water contamination could not be associated with runoff from contaminated fields. Further research should aim at identifying (point) sources of contamination, to enable the implication of measures to reduce surface water contamination. In addition, public health regulators should consider continuous monitoring of the treated drinking water for the presence of *Cryptosporidium* and *Giardia* since the presence of these parasites in the surface

water poses a constant threat for drinking water safety. This is of particular importance in many countries where a substantial proportion of drinking water is produced from surface water and where a significant proportion of the water resources are inflows from upstream rivers, e.g., Belgium, The Netherlands, Hungary, Lithuania, Bulgaria and Romania (Eurostat, 2013).

2.6 Acknowledgments

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Chapter 3

Cryptosporidium and *Giardia* in recreational water in Belgium

Based on:

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3.1 Introduction

Cryptosporidium and *Giardia* are important parasites in the aetiology of diarrhoea worldwide. In developed countries *Cryptosporidium* spp. are detected in up to 2% and *Giardia* spp. in up to 14% (Homan and Mank, 2001; Geurden *et al*., 2009) of immunocompetent patients with diarrhoea. In non-outbreak related cases of diarrhoea in Belgian patients, *Giardia* was identified as the second most prevalent pathogen. In the same study *Cryptosporidium* was also within the five most commonly detected pathogens, indicating that both parasites occur frequently in patients with gastrointestinal symptoms (Geurden *et al*., 2009). In Belgium, the national incidence of cryptosporidiosis and giardiosis is 2.5 and 10.8 per 100,000 inhabitants, respectively, with the highest incidence in children (< 5 years of age) and young adults (25-44 years old) (WIV, 2010).

Transmission of *Cryptosporidium* spp. and *Giardia* spp. occurs from either humans or animals by the faecal-oral route. In humans worldwide *C. hominis* and *C. parvum* are the most commonly detected *Cryptosporidium* species. *C. hominis* is human-specific, while *C. parvum* infects ruminants and humans. *Giardia duodenalis* assemblage A and B are responsible for giardiosis in humans and are also found in a wide range of mammals. Within assemblage A, sub-assemblage AI is mostly found in animals, whereas sub-assemblage AII is predominantly found in humans (Sprong *et al*., 2009). Sub-assemblage AIII is almost exclusively found in wild hoofed animals. In contrast, there is no clear subgrouping within assemblage B (Sprong *et al*., 2009; Feng and Xiao, 2011). Next to direct contact with an infected host, *Cryptosporidium* and *Giardia* can be transmitted through faecal contamination of food or water. Because of the parasites' ability to survive in the environment, their relative resistance to disinfection and the low number of (oo)cysts needed for infection, *Cryptosporidium* and *Giardia* are considered as important water-borne infections (Smith *et al*., 2006). From 2004 to 2010, 199 outbreaks of human diseases due to the water-borne transmission of parasitic protozoa were reported worldwide. *Cryptosporidium* spp. were the etiological agent in 60.3% of the outbreaks and *Giardia* spp. in 35.2% (Baldursson and Karanis, 2011). In a third of the outbreaks, recreational water was detected as the source of infection, (Baldursson and Karanis, 2011). Swimming in contaminated waters and swimming pools is now recognised as an important transmission route for *Cryptosporidium* (Karanis *et al*., 2007). Outbreaks of

cryptosporidiosis have been associated with recreational water in the USA (Craun *et al*., 2005; Causer *et al*., 2006; Wheeler *et al*., 2007; Boehmer *et al*., 2009; Cantey *et al*., 2012), Canada (Hopkins *et al*., 2013), Australia (Dale *et al*., 2010; Waldron *et al*., 2011), Japan (Takagi *et al.*, 2008), Sweden (Insulander *et al.*, 2005; Mattsson *et al.*, 2008) and the UK (Smith *et al*., 2006; Coetzee *et al.*, 2008). Although few reports can be found on *Giardia* infections related to recreational water (Porter *et al*., 1988), a marked seasonality in the onset of giardiosis occurs in summer through early autumn in many countries, including Belgium (WIV, 2010). This increase coincides with increased outdoor activities (*e.g.* swimming) (Hlavsa *et al.*, 2005) and with increased travelling during summer holidays.

In Belgium, *Cryptosporidium* and *Giardia* were detected in surface water that is used for drinking water production (Ehsan *et al*., 2015), but no data are available for recreational water and the importance of water recreation in the transmission of *Cryptosporidium* and *Giardia* in Belgium is unknown. The objective of this study was to investigate the presence of *Cryptosporidium* and *Giardia* in public swimming pools, recreational water bodies and splash parks in Flanders (Northern Belgium) and to estimate the infection risk associated with swimming and other recreational activities. Positive samples were genotyped in an attempt to identify the source of infection (human *vs.* animal).

3.2 Materials and methods

3.2.1 Sampling

In total, 101 samples from recreational water bodies in Flanders, Belgium were analysed. From March to October 2010, 36 public swimming pools were sampled. The swimming pools were selected based on an increased risk for faecal accidents or external contamination (*i.e.* paddling pools, therapy pools frequently visited by mentally disabled persons and outdoor swimming pools). Convenience samples (n = 37) were collected from three types of water samples, including pool water (60 L) in 20 swimming pools, filter backwash water (2-60 L) in 16 pools and water from continuous flow centrifugation (2 L) in one of the previous sampled swimming pools.
In August 2011, 40 convenience samples (30 L) were collected from 10 recreational lakes. Each lake was sampled four times with weekly intervals. In July and August 2012, 24 convenience samples (30 L) were taken from neighbourhood water fountains

(n = 16), splash parks (n = 7) and a water fountain in a boating lake (n = 1). All samples were transported to the lab, stored at 4°C and analysed within 72 h.

3.2.2 Detection of *Cryptosporidium* oocysts and *Giardia* cysts

A protocol was optimised to detect *Cryptosporidium* and *G. duodenalis* in water samples, based on the United States Environmental Protection Agency (USEPA) method 1623 (USEPA, 2005). Water samples were filtered through Filta-Max Xpress filters (IDEXX Laboratories, Inc., Westbrook, ME, USA) with the aid of a peristaltic pump with recommended flow rates of 2 L/min. The Filta-Max Xpress filters were washed with the Filta-Max Xpress automated washing station for elution of the filters following the manufacturer's instructions. The eluate was centrifuged and the volume of sediment was measured. Between 0.5 ml and 2 ml of sediment was used for immunomagnetic separation (IMS) of the (oo)cysts (Dynabeads® GC-Combo, Invitrogen Dynal). Oocysts and cysts in the sediment were purified by IMS using *Cryptosporidium* and *Giardia* specific antibody-coated magnetic beads according to the manufacturer's protocol (Dynabeads® GC-Combo, Invitrogen Dynal, A.S., Oslo, Norway). IMS-purified cysts and oocysts were stained on well slides by fluorescein isothiocyanate (FITC)-conjugated anti-*Cryptosporidium* and anti-*Giardia* MAbs FITC conjugated monoclonal antibodies (EasyStain™) (BTF Pty Ltd. Australia). Slides were examined using a Leica Leitz DMRB fluorescence microscope. The well surface was scanned at 200 or 400 times magnification using a FITC fluorescence filter (450-590 nm Chroma technology corp.). *Cryptosporidium* oocysts and *Giardia* cysts were identified and counted based on their size, morphology and fluorescence. Results were expressed as count per litre. Slides containing (oo)cysts were kept at 4°C for DNA extraction.

3.2.3 Risk of *Cryptosporidium* and *Giardia* infection

Risk of *Cryptosporidium* and *Giardia* infection (P_{inf}) during swimming or other recreational water activities (fishing, boating, canoeing and rowing) was based on the equation (Teunis *et al*., 2008) below:

$$P_{inf} = 1 - e^{-Dp_m}$$

In this equation D represents the infection dose and p_m represents the dose-response parameter. The dose D was determined by multiplying the observed number of

(oo)cysts in 1 L by the volume *v* of water swallowed during each of the different water activities, including swimming, limited contact water recreation activities (*e.g.* fishing, boating, canoeing and rowing) and visiting splash parks. For swimming, we applied the *v*-estimates reported by Schets *et al*., (2011b): swimming in a pool: 34.0 $x10^{-3}$ L (male), 23.0 $x10^{-3}$ L (female), 51.0 $x10^{-3}$ L (children); swimming in fresh water: 27.0 $x10^{-3}$ L (male), 18.0 $x10^{-3}$ L (female), 37.0 $x10^{-3}$ L (children). For recreation activities with limited water contact, *v* was set at 3.7 $x10^{-3}$ L (Dorevitch *et al*., 2011). For visiting splash parks, we assumed that a child swallows 75.7 $x10^{-3}$ L during a 3.5 min visit (de Man *et al*., 2014). Recovery rates for *Cryptosporidium* oocysts and *Giardia* cysts obtained with the USEPA 1623 protocol for drinking water (*Cryptosporidium* 44.8%; *Giardia* 45.1%) and surface water (*Cryptosporidium* 44.1%; *Giardia* 27.7%) in Flanders (Ehsan *et al*., 2015) were used to correct *D* in swimming pools, splash parks (drinking water) and recreational lakes (surface water). The p_m was set at 28 $x10^{-3}$ for *Cryptosporidium* (Messner *et al*., 2001) and at 19.9 $x10^{-3}$ for *Giardia* (Teunis *et al*., 1996). Finally, we used Monte Carlo simulations (500,000 iterations) to obtain the median, mean and 95% confidence interval (CI). To this end we considered the variation in each of the parameters inserted in the formula above. We assumed that the dose-response parameter p_m followed a beta distribution with scale parameters α and β (expected value $p_m = \alpha/(\alpha+\beta)$, variance $p_m = \alpha \times \beta/((\alpha+\beta)^2 \times (\alpha+\beta+1))$. For *Cryptosporidium*, α and β were set at 0.53 and 18.45, respectively, for *Giardia* these values were set at 1.83 and 90.05. These values were derived from the estimated p_m and corresponding 90th percentile for *Cryptosporidium* (66.0 $x10^{-3}$; Messner *et al*. 2001) and the 97.5th percentile for *Giardia* (56.6 $x10^{-3}$; Teunis *et al*. 1996). For the variation of number of (oo)cysts per L and the recovery rates of (oo)cyst counts we re-sampled from the original raw data. For the variation in volume *v* swallowed we used distributions described by Schets *et al*. (2011b) (swimming) and de Man *et al*. (2014) (splash parks). For the risk involving limited contact water recreation activities we did not consider the *v*-distribution, as this was not available (Dorevitch *et al*., 2011).

3.2.4 DNA extraction and molecular identification

DNA was extracted from water samples from recreational lakes that were positive by microscopy for *Cryptosporidium* and/or *Giardia*. Positive samples from indoor swimming pools, neighbourhood fountains and splash parks were not genotyped, as

numbers of (oo)cysts were too low.

Genomic DNA was extracted from (oo)cysts that were scraped from the microscope slides using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) or from sediment using the QIAamp Stool Mini Kit according to the manufacturer's instructions, incorporating an initial step of three freeze-thaw cycles (freezing in liquid nitrogen for 5 min and heating at 95°C for 5 min) in the protocol to maximise disruption of (oo)cysts. Previously described polymerase chain reaction (PCR) protocols were used to amplify the 18S rDNA gene (Ryan *et al*., 2003) and the heat shock protein (*hsp*)-70 gene (Morgan *et al*., 2001) of *Cryptosporidium.* For the identification of *Giardia* the β-giardin gene (Lalle *et al*., 2005) was used in a nested PCR. For assemblage-specific amplification of *Giardia*, the triose phosphate isomerase (TPI) gene was used (Sulaiman *et al*., 2003; Geurden *et al*., 2008; Levecke *et al*., 2009). For all PCR reactions, negative (PCR water) and positive controls (genomic DNA) were included. The PCR products were visualised in agarose gel (1.5%) stained with ethidium bromide under ultraviolet (UV) light. PCR products were fully sequenced by the BIG Dye Terminator V3.1 Cycle sequencing Kit (Applied Biosystems). Sequencing reactions were analysed on a 3100 genetic Analyzer (Applied Biosystems) and assembled with the programme Seqman II (DNASTAR, Madison WI, USA). To determine the genotypes/assemblages the fragments were aligned with homologous sequences available in the GenBank database, using MegAlign (DNASTAR, Madison WI, USA).

3.3 Results

In this study, 3 out of 37 swimming pool samples (8.1%; 2 filter backwash samples and 1 swimming pool water sample) tested at least positive for one of the two protozoa. Backwash water samples from two swimming pools were found positive for *Cryptosporidium*, with 0.23 oocysts/L and 0.03 oocysts/L, respectively. In one of these samples, as well as in one pool water sample from another swimming pool, *Giardia* was detected (0.23 cysts/L and 0.07 cysts/L, respectively). Only the results from the pool water were used for calculating the infection risk. The risk of *Giardia* infection in the investigated swimming pools varied from 1.13×10^{-6} to 2.49×10^{-6} per swim per person (Table 3.1).

Table 3.1 Risk of *Giardia* infection per swim in swimming pools for respectively men, women and children (mean and 95% confidence interval).

		Mean	**95% CI**
Giardia	Men	1.61×10^{-6}	$0 - 8.33 \times 10^{-6}$
	Women	1.13×10^{-6}	$0 - 6.24 \times 10^{-6}$
	Children	2.49×10^{-6}	$0 - 1.90 \times 10^{-5}$

Eight out of 10 sampled recreational lakes were positive for one or both parasites on at least one sampling occasion. In 7/10 lakes *Cryptosporidium* oocysts were detected once (n = 4) or twice (n = 3), with oocyst counts ranging from 0.07 to 0.60 oocysts per L (Table 3.2). Genotyping was only successful for 2/10 samples. Both sequences were identified as *Cryptosporidium andersoni* (NCBI accession numbers KM455082, KM455083). In the other cases either no DNA could be obtained from the slides (n = 1), no PCR product was obtained (n = 6) or no sequence was obtained from the PCR product (n = 1). *Giardia* cysts were detected once (n = 5) or twice (n = 2) in 7/10 lakes. Apart from one lake, these were the same lakes that were also positive for *Cryptosporidium. Giardia* cyst counts ranged from 0.23 to 0.70 cysts/L (Table 3.2). In 7/9 positive samples a PCR product was obtained with the β-giardin gene and/or the TPI gene. Sequencing results showed *G. duodenalis* assemblage AI in 6/7 cases (NCBI accession numbers KM455069-KM455071, KM455074-KM455078, KM455080, KM455081), either as the only assemblage (n = 4) or in combination with assemblage AII (n = 1, NCBI accession number KM455079) or assemblages BIII (NCBI accession number KM455072) and E (NCBI accession number KM455073) (n = 1). In one positive sample assemblage 'BIV-like' was present (NCBI accession number KM455068). The infection risk for men, women and children for swimming in recreational lakes is shown in Table 3.3.

Table 3.2 Number of *Cryptosporidium* oocysts (C) and *Giardia* cysts (G) per L water in recreational lakes in Flanders, Belgium in August, 2011. ND = not done.

Sampling date	**02/08/11**		**08/08/11**		**16/08/11**		**22/08/11**		**28/08/11**	
	C	**G**	**C**	**G**	**C**	**G**	**C**	**G**	**C**	**G**
Lake 1	0	0	0	0	0	0	0	0	ND	ND
Lake 2	0	0	0	0	0	0	0	0	ND	ND
Lake 3	0	0.23	0	0	0.17	0	0	0	ND	ND
Lake 4	0	0	0	0	0	0	0.07	0	ND	ND
Lake 5	0	0	0	0	0	0.70	0	0	ND	ND
Lake 6	0	0.70	0.17	0.47	0	0	0	0	ND	ND
Lake 7	0.30	0	0	0	0	0.23	0.30	0	ND	ND
Lake 8	0.17	0.47	0	0	0	0	0	0.23	ND	ND
Lake 9	0	0	0	0.23	0.17	0	0.17	0	ND	ND
Lake 10	ND	ND	0.07	0.23	0	0	0.60	0	0	0

Table 3.3 Risk of *Cryptosporidium* and *Giardia* infection per swim in recreational lakes for respectively men, women and children (mean and 95% confidence interval).

		Mean	**95% CI**
Cryptosporidium	men	4.13×10^{-5}	$0 - 1.15 \times 10^{-4}$
	women	2.79×10^{-5}	$0 - 1.11 \times 10^{-4}$
	children	5.74×10^{-5}	$0 - 2.35 \times 10^{-4}$
Giardia	men	1.08×10^{-4}	$0 - 9.45 \times 10^{-4}$
	women	7.04×10^{-5}	$0 - 6.37 \times 10^{-4}$
	children	1.46×10^{-4}	$0 - 5.85 \times 10^{-4}$

The estimated infection risk for *Cryptosporidium* varied from 2.79×10^{-5} to 5.74×10^{-5} per swim per person, while for *Giardia* the infection risk was between 7.04×10^{-5} and 1.46×10^{-4} per swim per person. For other outdoor water recreation activities the estimated infection risk was 5.71×10^{-6} for *Cryptosporidium* and 1.47×10^{-5} for *Giardia*. No *Cryptosporidium* oocysts were detected in any of the sampled water fountains or splash parks. However, water samples from three fountains and two splash parks contained *Giardia* cysts. Cyst counts were 0.03, 0.07 and 0.20 cysts/L in the water fountains and 0.13 cysts/L in both splash parks. The infection risk of *Giardia* infection during a 3.5 min visit to a splash park for children equalled 1.68×10^{-4} (95% confidence interval: $0 - 1.57 \times 10^{-3}$).

3.4 Discussion

Swimming is one of the most popular recreational activities worldwide with over 350 million person-events estimated to take place annually in the USA alone (Fayer, 2004). Health risks for swimmers may arise from exposure to bathing waters of poor quality. They may suffer from various diseases such as gastroenteritis caused by bacteria, viruses or parasites of faecal origin (WHO, 2003). Among protozoan parasites, *Cryptosporidium* and *Giardia* are associated with water-borne outbreaks worldwide from recreational water, including swimming pools and recreational lakes. In this study, 3 out of 37 swimming pool samples (8.1%) tested positive for *Cryptosporidium*, *Giardia* or both (2 filter backwash samples and 1 swimming pool water sample). Similar contamination rates were reported in non-outbreak-related pools in France, The Netherlands, the USA and Egypt (Fournier *et al*., 2002; Schets *et al*., 2004; Shields *et al*., 2008; Abd El-Salam, 2012). In Italy, a higher proportion of pools were positive for *Cryptosporidium* and/or *Giardia* (Briancesco and Bonadonna, 2005; Oliveri *et al*., 2006), while in Greece no (oo)cysts were found in five swimming pools (Papadopoulou *et al*., 2008). Based on the observed (oo)cyst concentrations, an attempt was made to estimate the infection risk associated with swimming in these pools. Since oocysts were only detected in filter backwash water, no infection risk for *Cryptosporidium* could be calculated for the investigated swimming pools. Using the methodology of Schets *et al*. (2011b), the infection risk for *Giardia* was estimated as 1.13 to 2.49 $x10^{-6}$ per swim per person. In comparison, in a swimming pool in The Netherlands, an infection risk of 1.3 to 2.8 $x10^{-5}$ for *Giardia* was estimated per swimming event per person (Schets *et al*., 2004, 2011b). The higher infection risk in the Dutch study was due to a faecal contamination incident and filter malfunctioning during the time of sampling. Although no recent faecal contamination incidents were reported in the swimming pools in the present study, the selection of 'high risk' swimming pools may have introduced a bias and the calculated infection risk may not be representative for all swimming pools in Flanders. An increased infection risk has been associated with swimming pools that are frequently visited by young children (Stafford *et al*., 2000; Hlavsa *et al*., 2005). Moreover, as cyst viability was not assessed, total cyst counts were used in our risk assessment, assuming 100% viability. Although *Giardia* cysts can survive in water for a considerable time (Olson *et al*., 1999), the infection risk is possibly overestimated because of this assumption.

However, it should be noted that these are baseline contamination levels and that the infection risk can increase dramatically in the case of a faecal contamination incident or filter malfunctioning.

In 7/10 of the investigated recreational lakes (10/40 samples) *Cryptosporidium* was detected at least once, with relatively low oocyst concentrations (0.07-0.6 oocysts/L). Similar contamination rates and oocyst counts were obtained in recreational waters in France, The Netherlands, Luxembourg and in central Spain (Coupe *et al*., 2006; Schets *et al*., 2008; Helmi *et al*., 2011; Galvan *et al*., 2014), while higher contamination rates and oocyst counts were reported in northern Spain (Castro-Hermida *et al*., 2010) and Canada (Loganthan *et al*., 2012). In most of these studies, contamination rates with *Giardia* were slightly higher, with higher cyst counts, compared to *Cryptosporidium*. Similarly, in the present study *Giardia* cyst concentrations were 0.23-0.7 *Giardia* cysts/L. Based on the observed (oo)cyst counts, the estimated infection risk for *Cryptosporidium* varied from 2.79 to 5.74 $x10^{-5}$ per swim per person, while for *Giardia* the infection risk was between 1.46 $x10^{-4}$ and 7.04 $x10^{-5}$ per swim per person. For the limited contact water recreation activities the estimated infection risk was lower than for swimming for *Cryptosporidium* (5.71 $x10^{-6}$) and comparable for *Giardia* (1.47 $x10^{-5}$). Assuming only one visit to one of the recreational lakes per year, the infection risk for *Giardia* associated with swimming in recreational lakes was already above the generally accepted criterion of < 1 infection per 10,000 individuals (USEPA, 1989) despite the fact that all the investigated lakes had at least good water quality, according to the criteria of Directive 2006/7/EC. In this directive, bathing water quality is defined by threshold values for microbiological parameters, corresponding to four bathing qualities (‘excellent’, ‘good’, ‘average’ and ‘poor’). As parasites are not covered by this directive, they are not routinely monitored. The results of this study and other studies (Schets *et al*., 2008, 2011a, 2011b) suggest that infection risk for *Cryptosporidium* and *Giardia* cannot be extrapolated from the commonly used parameters for bathing water quality. However, it should be noted that the infection risk for *Cryptosporidium* and *Giardia* might be prone to bias. First we used the total (oo)cyst concentrations to estimate this risk, assuming that all (oo)cysts were assumed to be viable in the risk assessment. Second, not all *Cryptosporidium* and *Giardia* species that were detected are infectious to humans. Regarding *Cryptosporidium,* only the *C. andersoni* was identified. Although *C. andersoni* has been reported in human patients (Jiang *et al*., 2014), it is usually

associated with cattle. Similarly, the frequently identified zoonotic *Giardia duodenalis* assemblage AI (6/7 samples) is mostly found in animals (Sprong *et al*., 2009). Other assemblages that were identified are either livestock-specific (E) or found predominantly in animals (BIV-like) and only in two samples were human-specific assemblages (AII, BIII) identified. Although it cannot be excluded that other species or genotypes were overlooked, *e.g.* due to poor recovery of DNA, these data suggest that animals, possibly livestock, were the predominant source of contamination for the investigated recreational lakes, indicating that the risk of these protozoa might be overestimated. Finally, we would like to underscore that the choice of the dose-response parameter p_m has an important impact on the final risk assessment. This is particularly the case when the dose-response varies considerably between different *Cryptosporidium* species and *G. duodenalis* sub-assemblages. For example, within *C. parvum* a large variation in dose-response estimates was observed, ranging from 5.3 $x10^{-3}$ (IOWA-isolate) to 59.0 x 10^{-3} (UCP isolate) (Messner *et al*., 2001). In the present study, we allowed for this variation in the risk assessment of *Cryptosporidium*, but not for *Giardia* as currently little is known about the variation in dose-response between different isolates and sub-assemblages.

Giardia cysts were detected in water samples from 3/17 fountains and 2/7 splash parks. Although no *Cryptosporidium* oocysts were found, it cannot be excluded that low concentrations of oocysts were missed, given the limited sensitivity of the USEPA 1623 method. The infection risk during a splash park visit equalled 1.68 $x10^{-4}$ and hence, of all water related activities, poses the highest risk for a *Giardia* infection. Outbreaks of cryptosporidiosis and giardiasis related to visits to water fountains have been documented in the USA (CDC, 1998, 2000; Eisenstein *et al*., 2008; Kirian *et al*., 2008), suggesting that the presence of *Giardia* in fountains may constitute a real risk for water-borne infection.

3.5 Conclusions

Cryptosporidium oocysts and/or *Giardia* cysts were detected in swimming pools, recreational lakes, splash parks and water fountains in Belgium. Although in recreational lakes (oo)cysts were frequently present, most positive samples belonged to species/genotypes that are either animal-specific or predominantly found in animals, suggesting that the risk of infection during recreation is relatively low. Lower contamination rates were found in swimming pools, splash parks and water

fountains, but assuming that humans are the most probable source of contamination for these water bodies, these findings suggest a risk for human infection.

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Chapter 4

Assessment of zoonotic transmission of *Cryptosporidium* and *Giardia* between cattle and humans in rural villages in Bangladesh

Based on:

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4.1 Introduction

Diarrhoeal diseases are a serious public health problem, affecting mainly children in developing countries (Kosek *et al*., 2003; WHO, 2005). Several bacterial, viral and parasitic agents are responsible for diarrhoeal disease, including *Cryptosporidium* and *Giardia* (Fayer, 2004; Smith *et al*., 2007). Susceptible hosts are infected either through contact with an infected host or indirectly by oral intake of infective (oo)cysts through contaminated water or food. Among the eight assemblages (A-H) of *Giardia duodenalis*, assemblages A and B are found in both humans and animals. The occurrence of the same species/genotypes in human and non-human hosts (dogs, cats, livestock and wildlife) in the same geographical areas supports the potential for zoonotic transmission (Thompson and Smith, 2011). Most cases of human cryptosporidiosis are due to infections with the human specific *C. hominis* or the zoonotic *C. parvum* (Cacciò *et al*., 2005). Other *Cryptosporidium* species have also been detected in humans, although less frequently (Fayer, 2010; Xiao, 2010; Elwin *et al*., 2012). Current evidence indicates that ruminants are a reservoir of zoonotic *Cryptosporidium* from where humans get infected by contaminated food and water or through direct contact with livestock, for example animal handlers (Robertson *et al*., 2010; Gormley *et al*., 2011).

In Bangladesh, both *Cryptosporidium* and *Giardia* have been associated with diarrhoea (Rahman *et al*., 1990; Khan *et al*., 2004; Haque *et al*., 2005, 2009). In cases of giardiosis, mainly *G. duodenalis* assemblage A was associated with diarrhoea (Haque *et al*., 2005, 2009). *Cryptosporidium hominis* as well as *C. parvum* were identified in patients with diarrhoea (Haque *et al*., 2009). The studies mentioned above were all performed in Dhaka, one of the fastest growing megacities of the world. The presence of overpopulated slums and the pollution of neighbouring rivers with untreated wastewater (Karn and Harada, 2001; Rahman and Hossain, 2008) could facilitate (water-borne) human-to-human transmission.

In rural Bangladesh, *Giardia* infections have also been associated with diarrhoea in children (Hasan *et al*., 2006). In rural villages of Bangladesh, there are many smallholder cattle farms consisting of 1-5 animals for milk and draft purposes. A previous study found a high prevalence of *Cryptosporidium* and *Giardia* in cattle in Bangladesh (Rahman *et al*., 1985). Young calves are considered as a reservoir for these parasites and transmission of *Cryptosporidium* and *Giardia* from cattle to cattle

handlers has been suggested in Bangladesh and India (Rahman *et al*., 1985; Khan *et al*., 2011). In addition, people living in rural villages have no or limited access to purified tap water and therefore rely on surface water from ponds or tube wells for their water supply. As water from tube wells is often contaminated with arsenic (UNICEF, 2008), surface water is often the only alternative. These small ponds are used for daily house- hold purposes (*e.g.* swimming, washing of cloths, bathing cattle, cleaning vegetables, fish, utensils etc.). Faeces from cattle that are kept alongside of these ponds are often washed and drained into the ponds. Some households use latrines of which the outlets flow into the ponds. The above-mentioned scenarios indicate that there is a potential for water-borne transmission of *Cryptosporidium* and *Giardia*. However, no studies are available on transmission of *Cryptosporidium* and *Giardia* in these settings.

The aim of this study was to investigate direct transmission of *Cryptosporidium* and *Giardia* between cattle and their handlers and indirect water-borne transmission of *Cryptosporidium* and *Giardia* between the inhabitants and their animals in rural villages in Bangladesh.

4.2 Materials and methods

4.2.1 Sampling design

4.2.1.1 *Cryptosporidium* and *Giardia* in calves and their handlers.

A cross-sectional study was carried out to investigate the prevalence of *Cryptosporidium* and *Giardia* in calves (during March and April, 2009 and from March, 2012 to February, 2013) and their handlers (from July, 2012 to February, 2013) in Sadar Upazilla, Mymensingh District, a rural area in the Northeast of Bangladesh. Since there is no livestock database in Bangladesh, the first step of the sampling process was the digitisation of the map of Mymensingh district using ArcView Version 3.2 (Environmental Systems Research Institute, Inc. Redlands, California) which was previously carried out by Rahman *et al*. (2013). All 13 unions (sub Upazilla) namely Baera, Bhabkhali, Char Ishwardia, Char Nilaxmia, Khagdahar, Kustia, Sirta, Ashtadhar, Bobar Char, Dapunia, Ghagra, Paranganj and Sadar Upazilla, Mymensingh district were selected. One geographical coordinate was randomly selected from 10 previously selected coordinates in each union using the

random number generation function in Microsoft Excel and located by a hand held Global Positioning System (GPS) reader. Livestock farmers within a 0.5 km radius of the selected point were informed about this study (Cringoli *et al*., 2002). To encourage livestock farmers to participate, free anthelmintics and vitamin-mineral premix were supplied to their animals when sampling took place. Rectal faecal samples from 30-40 calves aged between 5 days and 6 months were collected from each union. The specimens (n = 623) were labeled as par ear tag of individual calves and immediately transported to the laboratory of the Department of Medicine, Bangladesh Agricultural University, Mymensingh. The calf handlers (n = 125) were provided with sterile wide-mouthed plastic containers for collection of stool specimens. All specimens were kept at 4°C and processed within 24 hours of collection. All animals and individuals were healthy at the time of sampling.

4.2.1.2 Water-borne transmission of *Cryptosporidium* and *Giardia* in two rural villages.

To investigate water-borne transmission of *Cryptosporidium* and *Giardia*, samples were collected from water ponds in two villages, as well as from inhabitants from households using these ponds and from their cattle. These two villages, Bhabkhali and Digarkandha, are situated in Mymensingh District (Figure 4.1). Both villages have a population of about 4,000 persons in 800 households. Almost every household keeps 1-5 cattle for milk and draft purposes alongside the water ponds. These small ponds are used for household purposes and as main sources of water supply. Like in most of rural Bangladesh, the villages rely mainly on hand-pumped water for drinking and on surface water sources for other domestic and personal purposes. As hand-pumped tube wells are often contaminated with arsenic, villagers rely on surface water as an alternative. The ponds were selected on the basis of availability of water in all seasons and frequent use of the ponds by surrounding village people and their animals. A grab water sample (15 L) was collected from one pond in each village every month at the same place from March, 2012 to February, 2013 and all samples (n = 24) taken immediately to the laboratory.

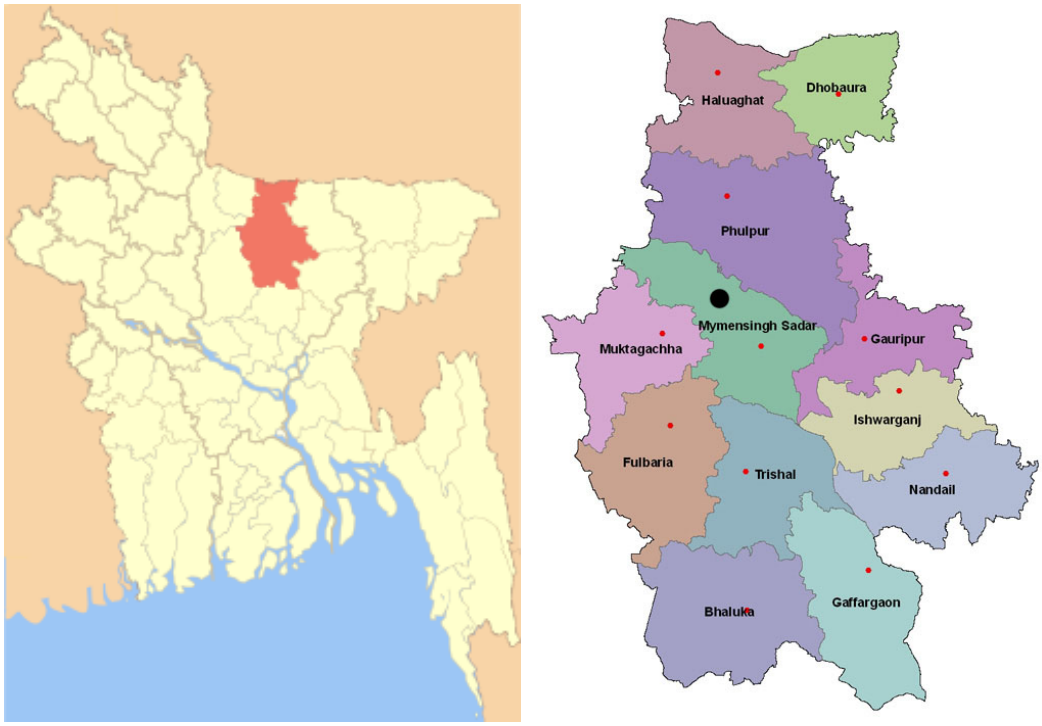

Figure 4.1 Study area. Left: Map of Bangladesh showing Mymensingh district (red). Right: Map of Mymensingh district showing Mymensingh Sadar Upazilla (green with black spot). (Source accessed on May 26th 2015: http://www.bdtradeinfo.com/bangladesh-profile/bd_district1.php?idn=40; http://en.wikipedia.org/wiki/Mymensingh_District).

Rectal faecal samples from 10 cattle and 10 human stool samples were collected without any age and sex restriction per month in each village from March, 2012 to February, 2013 In March 2012, exceptionally 20 cattle and 20 humans were sampled instead of 10, resulting in a total of 130 human and 130 cattle samples. All sampled individuals and animals were healthy at the time of sampling. The specimens were labeled as par ear tag of individual cattle and name of the person and immediately transported to the laboratory. All specimens were kept at 4°C and processed within 24 hours of collection.

4.2.2 Detection of *Cryptosporidium* oocysts and *Giardia* cysts in faecal/stool samples

Faecal samples were examined using a quantitative immunofluorescence assay (IFA; Merifluor *Cryptosporidium*/*Giardia* kit; Meridian Diagnostics Inc.) as described by Geurden *et al*. (2004). In short, 1g of faecal sample was suspended in distilled water and strained through a layer of surgical gauze to withhold large debris. After sedimentation for 1h and centrifugation at 3000g for 5 min, the sediment was resuspended in distilled water up to a volume of 1ml. After thorough vortexing, an aliquot of 20µl was pipetted onto a treated IFA-slide. After staining the slide, as instructed by the manufacturer, the entire smear was examined at a 400x magnification under a fluorescence microscope. The number of *Cryptosporidium* oocysts and *Giardia* cysts per gram faeces was obtained by multiplying the total number of cysts on the smear by 50. A sample was considered to be positive when at least one (oo)cyst was found on the IFA slide.

4.2.3 Detection of *Cryptosporidium* oocysts and *Giardia* cysts in water samples

A protocol was optimised to detect *Cryptosporidium* and *Giardia* in water samples, based on the USEPA method 1623 (USEPA, 2005). To validate the protocol, spiking experiments were performed with water samples collected from Diagarkandha and Bhabkhali in March and November 2012. Water samples were spiked with 100 inactivated *Giardia* cysts and 100 inactivated *Cryptosporidium* oocysts, permanently labeled with red fluorescent dye (ColorSeed, BTF Pty Ltd., North Ryde, Australia). The ColorSeed (oo)cysts were added to each water sample prior to filtering to estimate the percent recovery of (oo)cysts according to the manufacturer's instructions.

Both spiked (15L) and non-spiked water samples (15L) were filtered through Filta-Max filters (IDEXX Laboratories, Inc., Westbrook, ME, USA) with the aid of a motorized peristaltic pump with recommended flow rates of 2L/min. The Filta-Max filters were washed with the Filta-Max manual wash station for elution of the filters following the manufacturer's instructions. The eluate was centrifuged and the sediment was used for immunomagnetic separation (IMS) of the (oo)cysts. Cysts and oocysts in the sediment were purified by IMS using *Cryptosporidium* and *Giardia*

specific antibody-coated magnetic beads according to the manufacturer's protocol (Dynabeads GC-Combo, Invitrogen Dynal, A.S., Oslo, Norway). IMS-purified cysts and oocysts were stained on well slides by fluorescein isothiocyanate (FITC)-conjugated anti-*Giardia* and anti-*Cryptosporidium* MAbs FITC conjugated monoclonal antibodies (EasyStain, BTF Pty Ltd. Australia). Slides were examined using a Leica Leitz DMRB fluorescence microscope. The well surface was scanned at 200 or 400 times magnification for *Cryptosporidium* oocysts and *Giardia* cysts using a FITC fluorescence filter (450-590 nm Chroma technology corp.) and Texas Red fluorescence filter (530-585 nm, FT600, LP615) to distinguish ColorSeed (oo)cysts (which fluoresce red with the Texas Red filter) from natural (oo)cysts (which fluoresce bright green with the FITC filter). *Cryptosporidium* oocysts and *Giardia* cysts were identified and counted based on their size, morphology and fluorescence. Results were expressed as recovery percentage for spiked (oo)cysts and as count per L for naturally occurring (oo)cysts. Slides containing (oo)cysts were kept at 4°C for DNA extraction.

4.2.4 DNA extraction and molecular analysis

DNA was extracted from non-spiked water samples and faecal samples from cattle and humans that were positive for *Cryptosporidium* and/or *Giardia* in immunofluorescence microscopy. Genomic DNA was extracted from water sediment and from faecal/stool samples using the QIAamp Stool Mini Kit (Qiagen GmbH, Hilden, Germany) and from (oo)cysts that were scraped from the microscope slides from water samples by the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, incorporating an initial step of 3 freeze-thaw cycles (freezing in liquid nitrogen for 5 min and heating at 95°C for 5 min) in the protocol to maximize disruption of (oo)cysts.

For the identification of *Giardia* the β-giardin gene (Lalle *et al*. 2005) was used in 2-step nested PCR. In addition, the triose phosphate isomerase (TPI) gene was used for assemblage-specific amplification of *Giardia*, (Sulaiman *et al*., 2003; Geurden *et al*., 2008; Levecke *et al*., 2009). Previously described PCR protocols were used to amplify the 18S rDNA gene (Ryan *et al*. 2003; Xiao *et al*., 2001) and the 70 kDa heat shock protein-70 (hsp-70) gene (Morgan *et al*., 2001) of *Cryptosporidium*. For subgenotyping of *C. parvum* and *C. hominis* positive samples, the 60kDa glycoprotein (GP60) was targeted (Peng *et al*., 2001). For all PCR reactions, negative (PCR water)

and positive controls (genomic DNA) were included. The PCR products were visualized in agarose gel (1.5%) stained with ethidium bromide under UV light. PCR products were fully sequenced by the BIG Dye Terminator V3.1 Cycle sequencing Kit (Applied Biosystems). Sequencing reactions were analysed on a 3100 genetic Analyzer (Applied Biosystems) and assembled with the programme Seqman II (DNASTAR, Madison WI, USA). To determine the subgenotype the fragments were aligned with homologous sequences available in the GenBank database, using MegAlign (DNASTAR, Madison WI, USA).

4.2.5 Data analysis

The prevalence (percentage of samples in which (oo)cysts were detected) and mean (oo)cyst counts were calculated for each of the two parasites for the water samples and per host species. To explore any seaonal differences prevalence and mean (oo)cysts were calculated on a monthly basis. In general, the wet season ranges from mid April to mid October, with a mean rainfall of 278 mm and a mean temperature of 29°C. The dry season ranges from mid October to mid April, with a mean rainfall of 42 mm, and a mean temperature of 23°C. The association in the infections in cattle and their handlers was calculated using odds ratios with their corresponding 95% confidence intervals.

4.2.6 Ethics statement

This study was approved by the Ethical Committees of the University Hospital, Ghent University (EC/2012/604) and Mymensingh Medical College, Bangladesh (MMC/EC/97). All subjects provided written informed consent and a parent or guardian of any child participant provided informed consent on their behalf.

Animal care was approved by the Ethical Committee of the Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh (FVS/EB/2012/01), according to the Animal Experimentation Ethics Committee (AEEC) guidelines of the International Centre for Diarrhoeal Disease Research, Bangladesh and the Prevention of Cruelty to Animals Act, 1890 (Act No. XI 1890). Verbal consent of animal owners was obtained prior to the collection of faecal samples.

4.3 Results

4.3.1 *Cryptosporidium* and *Giardia* in calves and calf handlers

A total of 623 faecal samples from calves were collected during the study period. The prevalence of *Giardia* was 21.7% (95% Confidence Interval (CI): 18-25%), with a mean cyst excretion of 55,038 cysts per gram (CPG) in infected animals (range 50-250,000 CPG). The prevalence of *Cryptosporidium* was 5.0% (95% CI: 3.7%-6.7%) with an average excretion of 1,534 oocysts per gram (OPG) (range 50-25,000 OPG). In the wet season, 21.1% of calves were positive for *Giardia* (83/393), compared to 22.6% (52/230) positive calves in the dry season. Similarly, 4.8% (19/393) calves were positive for *Cryptosporidium* in the wet season, compared to 5.2% (12/230) *Cryptosporidium* positive calves in the dry season.

Giardia sequences were obtained from 60 out of 135 samples that were positive using IFA. The molecular characterization indicated a high prevalence of *G. duodenalis* assemblage E (47/60, NCBI accession numbers KJ188040-KJ188084, KJ363342-KJ363377) in calf samples. Other *G. duodenalis* assemblages found were AI (10/60, NCBI accession numbers KJ188032-KJ188039, KJ363378, KJ363379) and B (3/60, NCBI accession numbers KJ363380-KJ363382), often in mixed infections with assemblage E (5/60). Genotyping of *Cryptosporidium* positive samples (n = 5) revealed the presence of *C. parvum* (2/5, NCBI accession number KJ363386), *C. bovis* (1/5, NCBI accession number KJ363383) and *C. andersoni* (2/5, NCBI accession numbers KJ363384, KJ363385). Both *C. parvum* samples that were successfully amplified in the GP60 PCR belonged to subtype IIdA16G1 (NCBI accession numbers KJ363387, KJ363388).

Giardia cysts were detected in 14 out of 125 calf handler's stool samples (11.7%, 95% CI: 5.7-16.7%) with a mean cyst excretion of 33,168 CPG (range 50-250,000 CPG) and *Cryptosporidium* oocysts in 4 samples (3.3%, 95% CI: 1.1%-6.3%), with an average excretion of 275 OPG (range 50-450 OPG). Out of 14 positive samples, 6 samples were successfully sequenced for *Giardia*. Assemblages AII (2/6, NCBI accession numbers KJ188085, KJ188091), BIII (1/6, NCBI accession number KJ188086), BIV (2/6, NCBI accession numbers KJ188087, KJ188094) or a combination of AII, BIII and BIV (1/6, NCBI accession numbers KJ188088,

KJ188089, KJ188092, KJ188093) were detected. None of the 4 *Cryptosporidium* samples could be amplified in the PCR reactions.

No association was found in *Cryptosporidium* (odds ratio = 0; 95% confidence interval = 0; 28.90) *and Giardia* (odds ratio = 0.81; 95% confidence interval = 0.14; 3.37) infections between cattle and their handlers.

4.3.2 *Cryptosporidium* and *Giardia* in water ponds and faecal samples from village inhabitants and their cattle

4.3.2.1 Water samples

Slightly higher recovery rates were obtained for *Giardia* than for *Cryptosporidium*, which is in agreement with previous observations (McCuin and Clancy, 2003; USEPA, 2005). Mean recovery percentages (with standard deviation) for *Cryptosporidium* and *Giardia* were 40±2% and 41±6%, respectively. Recovery rates for both parasites met the standards set by USEPA (2005).

A total of 24 water samples (12 from Bhabkhali and 12 from Digarkandha) were collected over a period of 1 year. *Giardia* cysts were detected in 14/24 samples, whereas *Cryptosporidium* oocysts were detected in 12/24 water samples. Both ponds were contaminated with *Cryptosporidium* and *Giardia*. *Giardia* cyst counts in positive samples ranged from 0.1/L to 6.5/L in Bhabkhali and between 0.4/L and 11.3/L in Digarkandha. *Cryptosporidium* oocyst counts varied between 0.3/L and 0.7/L and between 0.2/L and 1.1/L in Bhabkhali and Digarkandha, respectively. (Oo)cyst counts appeared to be higher in the wet season than in the dry season (Figure 4.2).

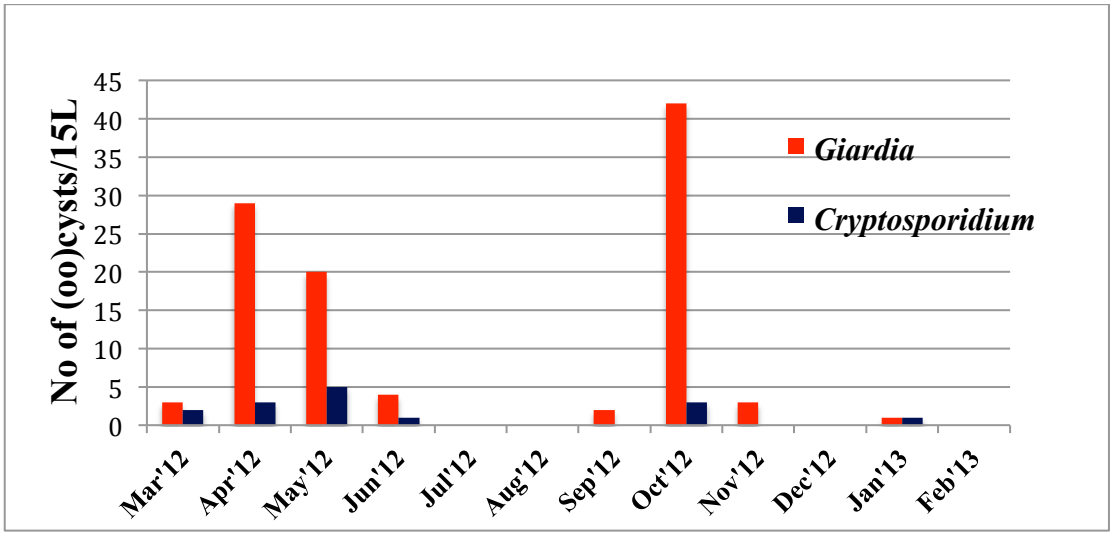

Figure 4.2 Monthly *Cryptosporidium* and *Giardia* (oo)cysts count/15 L in water samples.

Samples that contained at least one (oo)cyst detected by IMS-IFA were selected for genotyping. A total of 11 positive water samples for *Giardia* (6 from Bhabkhali and 5 from Digarkandha) and 9 samples for *Cryptosporidium* (4 from Bhabkhali and 5 from Digarkandha) were selected for PCR. *Giardia* sequences were obtained from 5 positive water samples. Most sequences belonged to assemblage E (n = 4; 2 from each village). One sample from Digarkandha could not be differentiated between assemblage BIV and BIV-like and was possibly a mixed infection. *C. hominis* (NCBI accession numbers KJ363330, KJ363331) and *C. andersoni* (NCBI accession number KJ363329) were identified in 2 and 1 samples from Digarkandha respectively. No successful sequence for *Cryptosporidium* was obtained from Bhabkhali water samples.

4.3.2.2 Cattle samples

The *Giardia* infection rate in cattle in Digarkandha was 13.3% (95% CI: 7.3%-19.4%), with a mean cyst excretion in positive animals of 22,075 CPG (range 50-125,000 CPG), compared to 5.0% (95% CI: 1.1%-8.9%) with a mean cyst excretion of 900 CPG (range 50-3,750 CPG) in Bhabkhali. *Cryptosporidium* infection rates were 9.2% (95% CI: 4.0%-14.3%) with a mean excretion of 150 OPG (range 50-600 OPG) and 5.0% (95% CI: 1.1%-8.9%) with a mean excretion of 8,417 OPG (range 50-35,000 OPG) in Bhabkhali and Digarkandha, respectively. *Cryptosporidium* and *Giardia* infection rates appeared to be higher in the wet season than dry season (Figure 4.3).

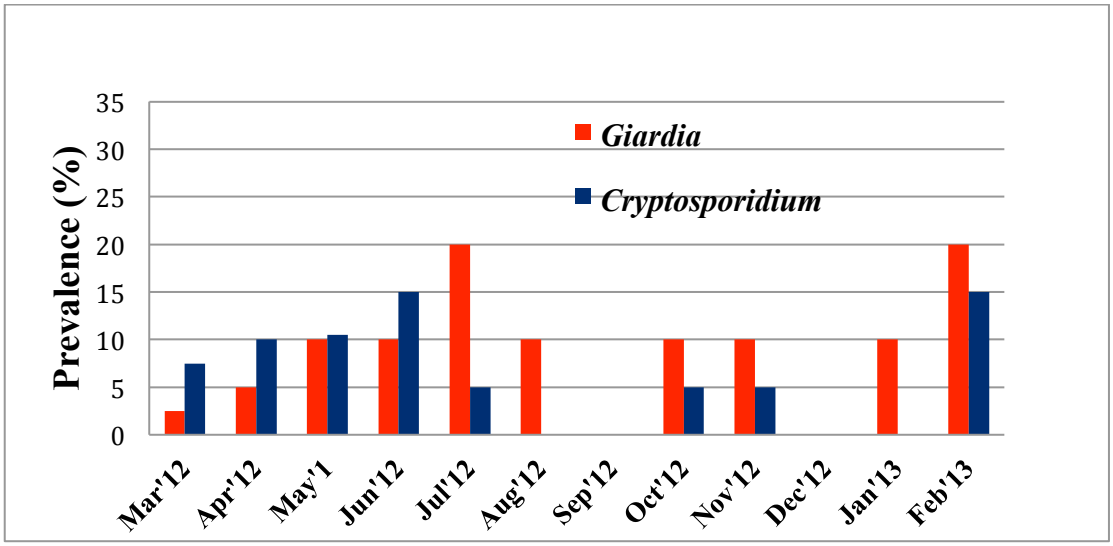

Figure 4.3 Monthly prevalence of *Cryptosporidium* and *Giardia* in cattle samples.

A total of 22 positive samples for *Giardia* were selected for PCR (6 from Bhabkhali and 16 from Digarkandha). Most sequences belonged to assemblage E (n = 7; 1 from Bhabkhali and 6 from Digarkandha, NCBI accession numbers KJ363394-KJ363397 and KJ363338- KJ363341). In Digarkandha, one sample was identified as AI (NCBI accession number KJ363336) and one sample had mixed infections of assemblages AI and B (NCBI accession numbers KJ363393, KJ363337). Out of 17 samples that were positive for *Cryptosporidium*, *C. andersoni* was detected in one sample in each village (NCBI accession numbers KJ363327, KJ363328) and *Cryptosporidium* horse genotype in one sample from Bhabkhali (NCBI accession number KJ363326).

4.3.2.3 Human samples

Infection rates with *Cryptosporidium* and *Giardia* were similar in inhabitants of both villages. *Giardia* infection rates were 11.7% (95% CI: 9.3%-22.4%) with a mean excretion of 39,950 CPG (range 50-400,000 CPG) and 15.8% (95% CI: 9.3%-22.4%) with a mean excretion of 67,329 CPG (range 50-1,000,000 CPG) in Bhabkhali and Digarkandha, respectively. *Cryptosporidium* was detected in 6.7% (95% CI: 2.2%-11.1%) of human stool samples in Bhabkhali (mean 11,638 OPG, range 50-80,000 OPG) and 5.0% (95% CI: 1.1%-8.9%) of human samples in Digarkandha (mean 325 OPG, range 50-1,000 OPG). *Cryptosporidium* and *Giardia* infection rates appeared to be higher in the wet season than dry season (Figure 4.4).

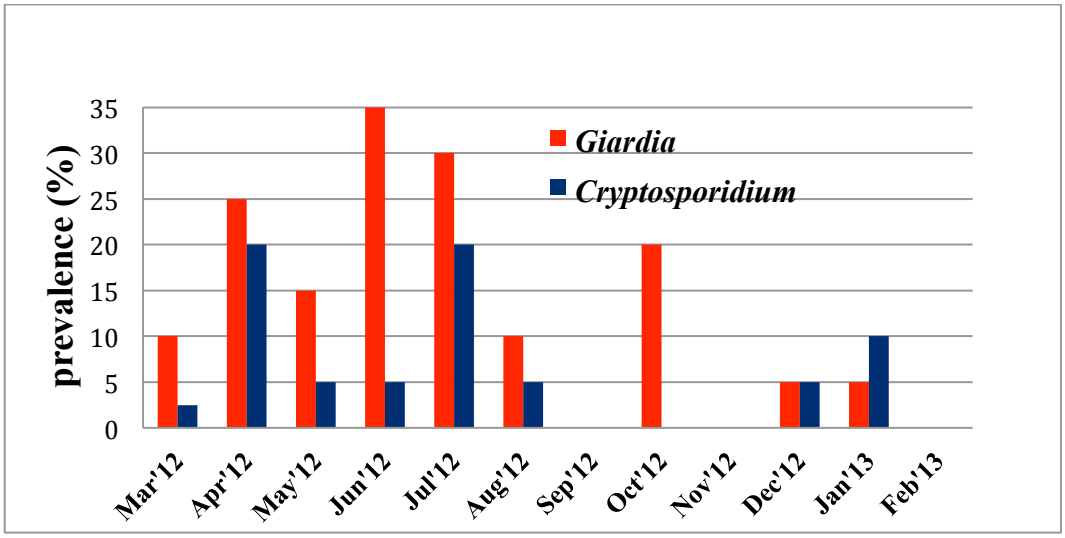

Figure 4.4 Monthly prevalence of *Cryptosporidium* and *Giardia* in human samples.

A total of 33 positive samples for *Giardia* were selected for PCR (14 from Bhabkhali and 19 from Digarkandha). *Giardia* sequences were obtained from 6 positive samples (2 from Bhabkhali and 4 from Digarkandha). All sequences belonged to assemblage BIII (n = 3; all from Digarkandha, NCBI accession numbers KJ363334, KJ363390, KJ363391), BIV (1 sample from Bhabkhali, NCBI accession number KJ363335) or a mixed infection of assemblage BIII and BIV (1 in each village, NCBI accession numbers KJ363332, KJ363333, KJ363389, KJ363392). No PCR products were obtained for *Cryptosporidium*.

4.4 Discussion

The prevalence of *Giardia* in calves was higher compared to *Cryptosporidium* in the cross-sectional study. A possible reason was the age range of the calves, as *Cryptosporidium* infection rates are typically highest in calves younger than one month (Quílez *et al*., 1996; Saha Roy *et al*., 2006) while a large proportion of the calf population in the present study was older than one month. It should be noted that the estimated prevalence in the cross-sectional study could have been affected by intermittent shedding of (oo)cysts, as all subjects were only sampled once.

Higher infection rates in calves were previously observed in the rainy season and in autumn in Bangladesh, India and Pakistan (Rahman *et al*., 1985; Saha Roy *et al*., 2006; Paul *et al*., 2008; Ayaz *et al*., 2012). The warm and humid conditions may favour survival of (oo)cysts and heavy rainfall and flooding may facilitate transmission of the infections. Although our study design did not alow to draw clear inference on a variation in infection rate across seasons, a seasonal pattern in *Cryptosporidium* and *Giardia* infections seemed to be present in water, cattle and village inhabitants, with more positive samples in the wet season. Similarly, higher *Cryptosporidium* infection rates in human patients during the wet season have been reported previously in Bangladesh (Rahman *et al*., 1990).

A significant association between the occurrence of *Cryptosporidium* and *Giardia* infections in calves and calf handlers has been documented in previous studies in Bangladesh and in West Bengal, India (Rahman *et al*., 1985; Khan *et al*., 2011).

However, in this study no evidence was found of direct zoonotic transmission between calves and their handlers. The cattle handlers were almost exclusively infected with *G. duodenalis* assemblage AII and BIII, while the calves were predominantly infected with the hoofed livestock-specific assemblage E and to a lesser extent with assemblage AI. The other assemblages that were occasionally found in cattle (BIV, BIV-like) are also found predominantly in animals (Sprong *et al*., 2009). Furthermore, mainly bovine *Cryptosporidium* species were identified in the calves, although two samples belonged to a *C. parvum* subtype that has also been reported in human cases (IIdA16G1).

In both villages, water samples from the ponds were frequently contaminated with *Cryptosporidium* and *Giardia*. However, since no viability staining was performed on the detected (oo)cysts, it cannot be excluded that some of the detected (oo)cysts were dead.

No evidence for zoonotic transmission of *Cryptosporidium* and *Giardia* between cattle and humans could be found in these environments with high potential for zoonotic transmission. In both villages, cattle were carrying predominantly animal-specific parasite species (*C. andersoni*, *G. duodenalis* assemblage E). Although the zoonotic assemblage AI was also detected in a few bovine samples, this assemblage is mostly found in animals (Sprong *et al*., 2009) and was not identified in any human sample in Bhabkhali or Digarkandha. Similarly, Laishram *et al*. (2012) did not find assemblage AI in children and adults in neighboring India. Only assemblage BIII and BIV were identified in the human population in these villages. Although these assemblages can also infect animals (Sprong *et al*., 2009), they were not detected in any of the cattle samples. Together with the presence of *C. andersoni*, *C. hominis* and *G. duodenalis* assemblage E and (less frequently) BIV (-like) in the water samples, these data suggest that water-borne transmission of *Cryptosporidium* and *Giardia* may occur in Bhabkhali and Digarkandha, but that (water-borne) transmission cycles are predominantly within host species.

4.5 Conclusions

The present study demonstrated the presence of *Cryptosporidium* and *Giardia* in cattle and people in a rural area of Mymensingh, Bangladesh. Water ponds in rural villages were also contaminated with *Cryptosporidium* and *Giardia* and may facilitate

water-borne transmission of these parasites. However, no evidence was found of zoonotic transmission of *Giardia* from cattle to humans, either directly (between cattle and their handlers) or indirectly through the water ponds, suggesting that (water-borne) transmission cycles of *Giardia* in this area are predominantly within host species. Because of the low success rate of *Cryptosporidium* genotyping in human and water samples, no conclusions can be made about transmission patterns for this parasite.

4.6 Acknowledgments

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Chapter 5

General Discussion

5.1 Introduction

The overall aim of this thesis was to determine the extent of water-borne transmission of *Cryptosporidium* and *Giardia* in Belgium and Bangladesh. In Belgium we examined whether *Cryptosporidium* and *Giardia* can be transmitted through drinking water that is produced from surface water (**Chapter 2**) and through water recreation (**Chapter 3**). In Bangladesh, we evaluated zoonotic transmission by cattle and water-borne transmission through village ponds (**Chapter 4**). In this final chapter, we will discuss our overall results and present future studies.

5.2 Water-borne transmission of *Cryptosporidium* and *Giardia* in Belgium

5.2.1 Drinking water

In chapter 2, different water catchments in Belgium were monitored for 24 months for the presence of *Cryptosporidium* and *Giardia* in different water matrices, *i.e.* raw surface water from rivers, reservoir water (after sedimentation) and treated drinking water. There seems to be no immediate risk for consumers of tap water, since all drinking water samples tested negative. Although the results suggest that the applied treatment methods are effective, we cannot rule out false negative test results due to the poor sensitivity of the USEPA method 1623 to detect low number of (oo)cysts. (Di Giorgio *et al*., 2002; Zukerman and Tzipori, 2006; Shaw *et al*., 2008).

In most catchment sites, low numbers of (oo)cysts were only sporadically detected in raw water samples, suggesting a limited risk for drinking water production. However, in one catchment site (Blankaart) seasonal peaks of surface water contamination were observed each winter. This may pose a threat for drinking water contamination in case of filter malfunction. Malfunctioning of the filtration system has been the cause of drinking water related outbreaks in the past (Herwaldt *et al*., 1992; MacKenzie *et al*., 1994; Kramer *et al*., 1996; Wallis *et al*, 1996; Pozio *et al*., 1997; Levy *et al*., 1998; Barwick *et al*., 2000; CCDR, 2001; Lee *et al*., 2002; Webber, 2002; Risebro *et al.*, 2007), including an outbreak of water-borne giardiosis in Belgium in 2008 (RTBF, 2008). As drinking water related outbreaks could affect large numbers of people and the fact that infection doses for *Cryptosporidium* and *Giardia* are very low (Dillingham *et al.*, 2002; Smith *et al.*, 2006; Karanis *et al*., 2007), additional water treatment and monitoring measures should be taken to further reduce the risk of water related outbreaks. However, these measures will come with an operational cost that will need to be compared with the potential costs of waterborne disease outbreaks (Corso *et al*., 2003). For

example, it has been estimated that the total costs for persons with mild, moderate, and severe illness due to *Cryptosporidium* were US$116, US$475, and US$7,808, respectively, leading to a total cost of US$96.2 million for the water-borne *Cryptosporidium* outbreak in Milwaukee, Wisconsin. For a Flemish setting we would like to propose the following water treatment and monitoring measures:

Possible additional water treatment measures that kill *Cryptosporidium* and *Giardia* are ozone and UV treatments. Both ozone and UV light has shown efficacy in inactivating (oo)cysts and can be used for disinfection of *Cryptosporidium* or *Giardia* (oo)cysts in water treatment facilities (Rose *et al*., 1997; Clancy *et al*., 2000; Kanjo *et al*., 2000). UV disinfection is now a well-established method because of its reliability and ease-to-use. It is also highly effective at destroying *Cryptosporidium* and *Giardia* at the dose levels provided by common portable UV units which is a 10-30 times less dose required to destroy other organisms (Campbell and Wallis, 2002; Linden *et al*., 2002; Mofidi *et al*., 2002). Ozone is a powerful oxidant that destroys micro-organisms through an irreversible physicochemical action (Finch, 1999). Ozone's use in the treatment of municipal and industrial drinking water as well as wastewater is well documented (Rice, 1997; Suty *et al*., 2004; von Gunten, 2007; Matilainen and Sillanpää, 2010; Kothavade, 2012; Ngwenya *et al*., 2013; Papageorgiou *et al*., 2014; Wadhawan *et al*., 2014). Ozone offers the highest efficiency in the treatment of water and effluents. Thus the treated water is free of microorganisms, colour, odour and environmental and public health protection are ensured. However, at this stage we would not recommend any additional treatment measures. First, our study results indicated that the applied filtration methods are effectively removing (oo)cysts. For example, the river Yser carries an average load of 20 oocysts/L and 32 cysts/L at the Blankaart catchment, but no (oo)cyst could be detected in the finished water. Although we cannot rule out false negative test results, the residual risk of infection is expected to be low. Based on literature, the total removal efficiency (= $\log_{10}$(number of (oo)cysts prior treatment / number of (oo)cysts post treatment) of the different treatment steps applied is expected to be at least 7 (Table 5.1). Hence, it is expected that the concentration of *Cryptosporidium* and *Giardia* in finished water is not higher than 2.0 $x10^{-6}$ oocysts/L (= $10^{(\log_{10}(20)-7)}$) and 3.2 $x10^{-6}$ cysts/L (= $10^{(\log_{10}(32)-7)}$). In other words, when drinking a glass of tap water (0.25 L), there is a 1.4 $x10^{-8}$ risk to get a *Cryptosporidium* infection, and a 1.59 $x10^{-8}$ risk to get an *Giardia* infection. Note that the removal efficiency of the storing water in the storage basin for 3 to 4 months at the Blankaart

equals 1.3 (= $\log_{10}(20/1)$) for *Cryptosporidium* and 0.8 (= $\log_{10}(32/5)$) for *Giardia*, and that these values support the findings of Medema, 1999.

Table 5.1 The expected removal efficiency of *Cryptosporidium* and *Giardia* for each of the different treatment steps applied at the Blankaart.

Treatment steps	**Removal efficiency (10 log-units)**	**Reference**
Storage	0.5-2	Medema, 1999
Coagulation/flocculation	2.5	Betancourt & Rose, 2004
Sand filtration	2	Betancourt & Rose, 2004
Granular activated carbon filtration	2-2.8	WHO, 2004
Disinfection (chlorine)	0-2	Medema,1999

Second, although additional treatment measures may increase the removal efficiency (ozon: 1-4; UV: 0-4, Medema, 1999), they require a high establishment costs. Therefore, they may not provide a cost-effective solution, particularly for large scale production plants (Dore *et al*., 2013).

Regular monitoring of treated drinking water for the presence of *Cryptosporidium* and *Giardia* currently is not required by Belgian and European legislation. Analysis for *Cryptosporidium* is only required when *Clostridium perfringens* is detected. However, previous studies (Hörman *et al*., 2004; Briancesco and Bonadonna, 2005; Hänninen *et al*., 2005; Helmi *et al*., 2011; Xiao *et al*., 2013) and our results (chapter 2) have shown that faecal bacterial indicators are no reliable indicators for the presence of *Cryptosporidium* and *Giardia*. Therefore, water companies should specifically monitor for *Cryptosporidium* and *Giardia* on a regular basis preferably once per month or once per quarter. This montoring schedule is followed by some water companies in USA (Betancourt and Rose, 2004). Note that water samples used for monitoring bacterial contamination can also be applied for the diagnosis of protozoa, and hence this would already reduce the costs for monitoring for *Cryptosporidium* and *Giardia.* In case (oo)cysts are detected, three actions should be taken. First, the treatment filtration system should be thoroughly checked. Second, the water should be hyperchlorinated. Finally, the consumers should be well informed to take the necessary steps to avoid infection, including boiling and filtering of the water. Where possible, upstream sources of surface water contamination should be identified to enable

measures to reduce further contamination. However, this is not an easy task, as shown by our results (chapter 2). From a comparison of the different catchment sites, it looked like human contamination (sewage, effluent of sewage treatment plants) could be the major source, since only the catchment site downstream of the town of Ypres (Blankaart) was positive, while both catchment sites upstream of Ypres (Dikkebus and Zillebeke) were negative. On the other hand, the seasonal pattern at the Blankaart (peaks of contamination in winter) suggested that runoff from fields could be a source of contamination as the Blankaart catchment site is situated in an agricultural area and high prevalences of *Cryptosporidium* and *Giardia* have been observed in cattle in Belgium (Geurden *et al*., 2004, 2006). However, no relationship was found between rainfall and (oo)cyst concentrations in our study. Moreover, genotyping could not confirm either hypothesis, since both human and animal-related species/genotypes were found.

Water-borne cryptosporidiosis and giardiosis have become a major concern for the sanitary authorities and the water industry responsible for providing safe drinking water supplies for human consumption. This can only be achieved through a combination of efficient water treatment, properly maintained distribution systems and source water protection, as recommended in the water safety plans (WSPs) established by the World Health Organization (WHO) (Betancourt and Rose, 2004; WHO, 2009). The first step of WSPs consists of identification and prioritization of the hazards and their risk assessment. These can be achieved through proper knowledge on the drinking water supply chain (up to the point of consumers). The next step is to identify control measures in drinking water supply systems that will collectively monitor these risks. For each control measure identified, an appropriate means of operational monitoring should be defined. Finally, timely corrective actions to ensure that safe water is consistently supplied; and undertaking verification of drinking-water quality to ensure that the WSP is being implemented correctly and is achieving the performance required to meet relevant national, regional and local water quality standards or objectives. Practices such as restricting access of livestock to water bodies by fencing, access ramps, rotational grazing, or off-site watering, prevention of runoff from animal housing, isolation of ill animals (particularly calves) and active control of herds to prevent extensive surface water contact may significantly reduce (oo)cysts contamination. Intake points from surface water supplies should be kept well away from other contamination sources, such as sewer overflows or discharges of untreated and treated sewage.

European states follow the WHO water safety plan that recommends and promotes the application of multiple barriers including source water protection and appropriate treatment processes, as well as protection during storage and distribution in conjunction with disinfection to prevent or remove microbial contamination. However, no monitoring plan has been introduced in Belgium. Water companies in Belgium perform several parameters of microbial and physico-chemical tests in their laboratories but these tests do not include identification of *Cryptosporidium* and *Giardia*.

5.2.2. Recreational water

5.2.2.1 Swimming pools

The major risks for water contamination are faecal incidents. However, these are difficult to prevent and to detect. Since cases of cryptosporidiosis and giardiosis are not notifiable in Belgium, small water related outbreaks are probably often overlooked. Improved diagnosis and reporting of cryptosporidiosis and giardiosis by medical doctors would help to identify clusters of cases, which may in some occasions provide epidemiological evidence of water-borne transmission. This should then be confirmed by detection of *Cryptosporidium* or *Giardia* in water samples in the suspected swimming pool (Porter *et al*., 1988; Mathieu *et al*., 2004; Turabelidze *et al*., 2007; Schets *et al*., 2008). In practice this often proves to be difficult, for several reasons, a) the cause of diarrhoea is often not determined in individual out-patients, b) underreporting of diagnosed cases, c) sampling of the suspected swimming pool is often several days after the incident and d) detected parasites (in the patients and in the water) are often not genotyped, impeding definite proof of water-borne transmission (Schets *et al*., 2004; Coetzee *et al*., 2008; Shields *et al*., 2008).

Proper guidelines for pool operators on improving operating standards including proper training of all persons involved in maintenance and operation in public pools, frequent monitoring of filter bed and pool filter filtrate and on how to manage faecal accidents as well as public information on the importance of hygiene in swimming pools are recommended tools in controlling the risk of infection (Coetzee *et al.*, 2008; Schets *et al*., 2004, 2008).

5.2.2.2 Recreational lakes

All sampled recreational lakes complied with the EU directives. Despite their (very) good water quality, they were frequently contaminated with *Cryptosporidium* and/or *Giardia*. This resulted in an estimated infection risk of > 1/100,000 based on a single visit to a contaminated lake per year. However, the calculated infection risk was probably overestimated, because

mainly animal-specific or animal-associated species/genotypes were identified and because no viability assessment data was included and, consequently, all detected (oo)cysts were assumed to be infectious. DAPI-PI staining of the (oo)cysts was performed on the slides in an attempt to determine (oo)cyst viability in the present study in chapter 3 as well as in chapters 2 and 4 (results not shown), as described by Schets *et al*. (2008). However, since desiccation of the (oo)cysts on the slides may cause damage to the (oo)cyst wall, this approach may considerably underestimate (oo)cyst viability (Robertson *et al*., 2014). Therefore, the DAPI-PI data were not included. In future studies, (oo)cysts in suspension will be used to determine their viability with DAPI staining, as suggested by Robertson *et al*. (2014). Cell-culture based models along with real-time RT-PCR methods can be used as alternative methods to determine viability (Rochelle *et al*., 1997; Slifko *et al*., 1997; Baque *et al.*, 2011).

Proper guidelines should also be implemented for recreational water. These guidelines include: 1) monitoring of recreational water time for the presence of chemicals (ammonia, nitrates, fluorides) and microorganisms (*Cryptosporidium, Giardia*, faecal bacterial indicators and other organisms for skin and ear infections), particularly in summer, 2) source tracking of the contaminations and taking adequate measures to prevent contamination and 3) informing the swimmers regarding water-borne health hazards, particularly when the water quality is poor according to European bathing water quality directive.

5.3 Transmission of *Cryptosporidium* and *Giardia* in Bangladesh

In chapter 4, the prevalence and transmission of *Cryptosporidium* and *Giardia* in calves and their handlers was studied in rural areas in Bangladesh along with transmission in village inhabitants through water. Echoing results from similar investigations, the current study in Bangladesh found a high prevalence of both parasites in calves younger than 1 month, with high levels of (oo)cyst excretion (Quílez *et al*., 1996; Mtambo *et al*., 1997; Saha Roy *et al*., 2006). It is indicated that this particular age group of calves might be the major reservoir of these parasites (Quílez *et al*., 1996; Saha Roy *et al*., 2006; Geurden *et al*., 2007). Therefore, cattle seem to be the most important reservoir for zoonotic transmission for *Cryptosporidium* (Hunter and Thompson, 2005). On the other hand, Olson *et al*. (2004) proposed that cattle were not a significant *Giardia* reservoir for human infection, as they were most commonly infected with livestock specific assemblage E. However, mixed infections of assemblage E with the potentially zoonotic assemblage A have been reported (Geurden *et al*., 2008; Khan *et al*., 2011). Moreover, associations of giardiosis with exposure to farm animals and pets

suggest they may be a potential reservoir for human infection by *Giardia* spp. (Hoque *et al.*, 2002, 2003; Xiao and Fayer, 2008). In our study, there was no evidence for zoonotic transmission between cattle and humans. This is in contrast to previous studies. Previously, detection of *Cryptosporidium* in calf handlers and their family members and absence of the parasite in healthy individuals indicated calf-to-man transmission in Bangladesh (Raman *et al*., 1985). Moreover, detection of the zoonotic assemblage AI of *Giardia* in calves indicates a public health significance in India (Khan *et al*., 2011). However, in the present study no evidence was found of direct zoonotic transmission between calves and their handlers. The cattle handlers were almost exclusively infected with *G. duodenalis* assemblage AII and BIII, while the calves were predominantly infected with the hoofed livestock-specific assemblage E and to a lesser extent with assemblage AI. These findings support the current trend in thinking that *Giardia* is seldom a zoonotic parasite (i.e. human infections are seldom derived from animals). However, one of the problems with the present study so far has been the general lack of genotyping data from *Cryptosporidium* in human. Recent study (Hira *et al*., 2011) in the children of Bangladesh indicated that 'human-to-human' transmission was more common and *C. hominis* and 'human-adapted' *C. parvum* subtype families were predominate as found in other developing countries (Cama *et al*., 2008; Ajjampur *et al*., 2007; Leav *et al*., 2002).

Similarly, in the villages no evidence for zoonotic transmission was found. However, the presence of (oo)cysts in the ponds showed that water-borne transmission is possible. Measures that can be taken to break water-borne transmission cycles in rural Bangladesh include the use of boiled water for all kinds of house hold purposes, drainage outlets (livestock and human wastes) should be constructed well away from those ponds and ponds should be protected from livestock by putting a fence.

In the cities, transmission patterns may be different from the countryside. Cryptosporidiosis and giardiosis are important diseases in urban Bangladesh (Rahman *et al*., 1985, 1990; Khan *et al*., 2004; Haque *et al*., 2005, 2009). In contrast to rural Bangladesh, the Water Supply and Sewerage Authority (WASA), produces drinking water from surface water that is provided to consumers through a distribution network (*e.g.* in Dhaka and Chittagong). WASA always claims that finished drinking water is free of contamination. However, this finished drinking water get contaminated afterwards by the poor quality of the distribution network, poor quality of the sewage system, flooding and illegal tapping of water from the distribution network, causing damage and leaking of the distribution system (Mahbub *et al*., 2011; Nahar *et al*., 2014).

5.4 Future studies

In chapters 2 and 3, it was demonstrated that raw surface water from rivers, ponds, natural reservoirs, lakes and treated water from swimming pools are contaminated with *Cryptosporidium* and *Giardia*. Among them, anthroponotic and zoonotic species and assemblages are found, indicating a public health threat. Both point and diffuse sources of contamination need to be addressed in the future to implement effective control measures. For example, in chapter 2, samples were collected from one point from each location throughout the year which might introduce a bias because the concentration of (oo)cysts may not be the same in all sampling points of a catchment area. The possible future work can be with more sampling points as well as with other water companies in Belgium. Geographic Information Systems (GIS) can be used to identify *Cryptosporidium* and *Giardia* clusters and their approximate locations. Mapping of the geographical distributions of agricultural and land-use factors including livestock densities and the percentage of agricultural land on which manure is applied can also be done with GIS. Finally, GIS can analyse the spatial and temporal distribution of both parasites in order to identify areas with different degrees of epidemiological risk (Odoi *et al*., 2004).

In Bangladesh, the current study investigated water-borne transmission and did not find solid evidence of zoonotic transmission. However, the present study was conducted only in 2 ponds in 2 rural villages in northern Bangladesh. The result of this pilot study cannot be extrapolated to all rural areas in Bangladesh. Thus, sampling from more villages in future may reveal different patterns of transmission of both parasites in rural Bangladesh. In addition, future work could be done to investigation water-borne transmission of *Cryptosporidium* and *Giardia* in urban areas of Bangladesh. In the biggest city of Bangladesh, Dhaka, several studies indicated the importance of diarrhoea caused by *Cryptosporidium* and *Giardia* (Rahman *et al*., 1990; Khan *et al*., 2004; Haque *et al*., 2005, 2009), but the sources of contamination were never looked at. The drinking water supply company, WASA, in Dhaka and Chittagong, uses surface water from the rivers Shitalakkha and Karnophuli, for the production of drinking water. These rivers are contaminated with both human and livestock wastes (Mahbub *et al*., 2011; Nahar *et al*., 2014). The WASA water intake points from these two rivers might be interesting sampling points for future work regarding water-borne transmission of *Cryptosporidium* and *Giardia* in urban areas of Bangladesh. Similarly, food-borne transmission of *Cryptosporidium* and *Giardia* could be studied in Bangladesh, as food sources of contaminations are well documented worldwide (Said, 2012; Dixon *et al*., 2013;

Olyaei and Hajivand, 2013; Saki *et al*., 2013; Eraky *et al*., 2014; Rahman *et al*., 2014a, b). Previous study in Bangladesh indicated that fresh vegetables from local market would be the source of contamination of *Cryptosporidium* and *Giardia* and posed a health risk to consumers (Rahman *et al*., 2014b). Thus, investigation of these fresh vegetables may reveal a new sight of food-borne transmission of *Cryptosporidium* and *Giardia* in Bangladesh.

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Summary

Access to safe drinking water and basic sanitation is a fundamental human right, but currently more than one billion people worldwide do not have access to either safe drinking water or adequate sanitation. It is estimated that almost 60% of deaths following diarrhoeal diseases in low and middle income countries are attributable to lack of access to safe drinking water and basic sanitation, children under 5 being at the highest risk. Important water-borne diarrhoeal diseases are cryptosporidiosis and giardiosis. These diseases are caused by the protozoan parasites *Cryptosporidium* and *Giardia*, respectively, which are able to cause disease in both humans and animals.

The overall aim of this thesis was to determine the water-borne transmission of *Cryptosporidium* and *Giardia* in Belgium and in Bangladesh. In Belgium we examined whether *Cryptosporidium* and *Giardia* can be transmitted through drinking water that is produced from surface water and through water recreation. In Bangladesh, we evaluated zoonotic transmission by cattle and water-borne transmission over village ponds.

In **Chapter 1** *Cryptosporidium* and *Giardia* were introduced by addressing their taxonomy, life cycle, prevalence and clinical importance for both human and animals. Second, the transmission of *Cryptosporidium* and *Giardia* was described. For this purpose, the different transmission cycles of both parasites were discussed (zoonotic versus anthroponotic transmission). Subsequently, we elaborated more on their water-borne transmission through drinking water and recreational water. An overview was given of water-borne outbreaks, sources of water contamination and current systems to monitor the occurrence of *Cryptosporidium* and *Giardia* in drinking water and recreational water in different countries. Finally, different methods for detecting and quantifying *Cryptosporidium* and *Giardia* in water were described.

Surface waters, such as rivers and lakes, can be contaminated with oocysts of *Cryptosporidium* and cysts of *Giardia* through human wastewater (e.g. sewage water) and farm animals (e.g. slops of stables and runoff from pastures). In Belgium, approximately one third of the drinking water is produced through surface water, which poses an important risk on the safety of drinking water. The objective of **Chapter 2** was to monitor the presence of *Cryptosporidium* and *Giardia* in four water catchment sites in Belgium (Zillebeke, Gavers, Dikkebus en Blankaart) and to discriminated between (oo)cysts from human or animal origin using genotyping. For the water catchments Zillebeke, Gavers and Dikkebus samples were monthly collected from raw surface water (15 L) and purified drinking water (60 L) between

September 2010 and August 2011. For the water catchment in Blankaart, samples were monthly collected from raw surface water (15 L), water from the storage basin (15 L) and purified drinking water between February 2010 and April 2012. *Cryptosporidium* and *Giardia* were detected using USEPA method 1623 and positive samples were genotyped. No contamination was found in purified drinking water at any site. In three catchments (Zillebeke, Gavers and Dikkebus), only low numbers of (oo)cysts were recovered from raw water samples (<1/liter). Raw water samples from the Blankaart catchment were frequently contaminated with *Cryptosporidium* (96 %) and *Giardia* (92 %), especially in winter and spring. Genotyping of positive water samples revealed the presence of *Cryptosporidium andersoni*, *C. suis*, *C.* horse genotype, *C. parvum* and *C. hominis*. Genotyping of *Giardia* identified the presence of *Giardia duodenalis* assemblage AI, AII, BIV, BIV-like and E. The genotyping results suggest that agriculture may be a more important source of surface water contamination than human waste in this catchment. In catchment sites with contaminated surface water, such as the Blankaart, continuous monitoring of treated water for the presence of *Cryptosporidium* and *Giardia* would be justified and (point) sources of surface water contamination should be identified.

The objective of **Chapter 3** was to assess the presence of *Cryptosporidium* and *Giardia* in different recreational water bodies in Belgium and to estimate the infection risk associated with swimming and other recreational activities. *Cryptosporidium* oocysts and/or *Giardia* cysts were detected in 3/37 swimming pools, 7/10 recreational lakes, 2/7 splash parks and 4/16 water fountains. In the swimming pools no infection risk for *Cryptosporidium* could be calculated, since oocysts were only detected in filter backwash water. The risk of *Giardia* infection in the swimming pools varied from 1.13×10^{-6} to 2.49×10^{-6} per swim per person.

In recreational lakes the estimated infection risk varied from 2.79×10^{-5} to 5.74×10^{-5} per swim per person for *Cryptosporidium* and from 7.04×10^{-5} to 1.46×10^{-4} for *Giardia*. For other outdoor water recreation activities (*e.g.* fishing, boating, canoeing and rowing) the estimated infection risk was 5.71×10^{-6} for *Cryptosporidium* and 1.47×10^{-5} for *Giardia*. However, most positive samples in the recreational lakes belonged to species/genotypes that are either animal-specific or predominantly found in animals. No *Cryptosporidium* was found in splash parks and water fountains, but the presence of *Giardia* cysts suggests a risk for human infection. The infection risk of

Giardia infection during a 3.5 min visit to a splash park for children equalled 1.68 $x10^{-4}$.

Cryptosporidium and *Giardia* are important causes of diarrhoea in Bangladesh. The high prevalence of both parasites in humans and cattle in rural Bangladesh and the common use of water ponds by village inhabitants and their animals suggest a potential for zoonotic transmission. Direct transmission of *Cryptosporidium* and *Giardia* between cattle and their handlers and indirect transmission through water ponds was investigated in **Chapter 4.** Faecal/stool samples were collected from 623 calves and 125 calf handlers in a cross-sectional survey. In two villages, water samples were collected monthly from water ponds and faecal/stool samples were collected monthly from inhabitants and their cattle. *Cryptosporidium* oocysts and *Giardia* cysts were detected in water samples and in faecal/stool samples and positive samples were genotyped, to determine their human or animal origin. The prevalence of *Cryptosporidium* and *Giardia* in calves was 22% and 5% respectively. In calf handlers, the prevalence of *Cryptosporidium* and *Giardia* was 11.2% and 3.2% respectively. Both in the cross-sectional survey and in the longitudinal study in the villages, *G. duodenalis* assemblage E was most prevalent in calves, while in humans assemblage AII, BIII and BIV were found. In cattle, *Cryptosporidium parvum*, *C. bovis* and *C. andersoni* were identified, but no *Cryptosporidium* sequences were obtained from humans. *Cryptosporidium* and *Giardia* were detected in 14/24 and 12/24 water samples respectively. *G. duodenalis* assemblage E and BIV (-like), as well as *C. andersoni* and *C. hominis* were identified. Although the presence of *Cryptosporidium* and *Giardia* in both water ponds suggests that water-borne transmission of *Cryptosporidium* and *Giardia* is possible, the genotyping results indicate that there is no significant direct or indirect (water-borne) transmission of *Giardia* between cattle and people in this area of rural Bangladesh. No conclusions could be drawn for *Cryptosporidium*, because of the low number of sequences that were obtained from human and water samples.

In **Chapter 5** conclusions from the results concerning the role of water-borne transmission of *Cryptosporidium* and *Giardia* in Belgium and Bangladesh are drawn. In addition, the limitations of the studies are discussed and opportunities for future research are highlighted.

In Belgium, there seems to be no immediate risk of *Cryptosporidium* and *Giardia* infection by drinking water from the investigated water catchment sites, as (oo)cysts

were found in none of the purified drinking water samples. The high (oo)cyst concentrations in surface water, however, may pose a potential risk for drinking water contamination in the event of malfunctioning of the filter system. Filter malfunctioning has been identified as the source of water-related outbreaks of cryptosporidiosis and giardiosis in several countries, including Belgium. Therefore it is recommended to regularly monitor the purified drinking water for *Cryptosporidium* and *Giardia*. Our research and previous studies have shown that faecal bacterial indicators are not a reliable indicator for the presence of *Cryptosporidium* and *Giardia*. Additionally, efforts should be made to reduce the contamination of the surface water that is used for drinking water production. For example, identification of upstream contamination sources would allow to take preventive measures. However, in **Chapter 2** the sources of surface water contamination could not be identified, because the data remained inconclusive. For future studies, the use of temporal-spatial models and a multi-locus genotyping strategy are recommended.

Contamination of swimming pools is mainly due to faecal incidents or malfunctioning of the filter. In our study, *Cryptosporidium* and *Giardia* were found in the filter water or swimming pool water sampled from a few pools, highlighting that there is a risk of infection. Since cryptosporidiosis and giardiosis are no notifiable diseases in Belgium, small water-related outbreaks are probably often missed. In practice it is sometimes difficult to associate clusters of cryptosporidiosis or giardiosis with a contaminated swimming pool, because (i) the cause of individual cases of diarrhoea is often not been studied, (ii) the identified cases of cryptosporidiosis and giardiosis are not always reported, (iii) the suspicious pools are only sampled after the outbreak has been noticed and (iv) positive water samples and stool samples are often not genotyped.

While the sampled recreational ponds all met the EU directives, they were frequently contaminated with *Cryptosporidium* and *Giardia*. However, the risk of infection (>1 / 100,000 per visit to a contaminated swimming pond per year) was probably overestimated. This is because mainly animal-associated species / genotypes were found and because the viability of the (oo)cysts was not determined.

In Bangladesh, there was no evidence for zoonotic transmission between cattle and humans. There were, however, indications of water-borne transmission, since *Cryptosporidium* and *Giardia* were frequently detected in both ponds and villagers. Possible measures to prevent transmission through these village ponds were

discussed. In this study, water-borne transmission of *Cryptosporidium* and *Giardia* was only studied in a rural area. However, cryptosporidiosis and giardiosis are also a major health problem in the cities. In addition, both parasites can also be transmitted through food (e.g. raw vegetables). Possible measures to reduce risks for water- and food-borne transmission in the cities and further studies on this subject are discussed.

Samenvatting

Toegang tot veilig drinkwater is een fundamenteel mensenrecht, maar momenteel hebben meer dan een miljard mensen geen toegang tot zuiver drinkwater en sanitaire voorzieningen. Naar schatting 60% van de overlijdens ten gevolge van diarree in ontwikkelingslanden is te wijten aan een gebrek aan veilig drinkwater en de afwezigheid van sanitair, waarbij kinderen jonger dan vijf jaar het grootste risico lopen. Cryptosporidiose en giardiose zijn belangrijke watergedragen oorzaken van diarree. Deze ziekten worden veroorzaakt door de parasieten *Cryptosporidium* en *Giardia,* die zowel bij dieren als bij mensen ziekte kunnen veroorzaken.

Het doel van dit doctoraat was de watergedragen transmissie van *Cryptosporidium* en *Giardia* te bestuderen in België en in Bangladesh. In België werd onderzocht of *Cryptosporidium* en *Giardia* overgedragen kunnen worden via drinkwater, gewonnen uit oppervlaktewater en via recreatiewater. In Bangladesh werd mogelijke zoönotische transmissie door runderen en watergedragen transmissie via dorpsvijvers onderzocht.

In **hoofdstuk 1** wordt eerst een introductie gegeven van beide parasieten, waarbij hun taxonomie, levenscyclus, de prevalentie bij mens en dier en hun medisch en veterinair belang wordt beschreven. Daarna verschuift de aandacht naar de transmissie van *Cryptosporidium* en *Giardia.* Eerst worden de verschillende gekende transmissie-cycli beschreven (zoönotische versus anthroponotische transmissie), en vervolgens wordt ingezoomd op de verschillende watergedragen transmissiewegen, nl. via drinkwater en recreatie-water. Er wordt achtereenvolgens een overzicht gegeven van de gerapporteerde uitbraken van watergedragen cryptosporidiose en giardiose, mogelijke bronnen van contaminatie met (oö)cysten en het monitoren van *Cryptosporidium* en *Giardia* in drinkwater en recreatiewater in verschillende landen. Dit hoofdstuk wordt afgesloten met een overzicht van de verschillende methodes om *Cryptosporidium* en *Giardia* op te sporen in water.

Oppervlaktewaters, zoals rivieren en meren, kunnen gecontamineerd worden met (oö)cysten van *Cryptosporidium* en *Giardia* door o.a. afvalwater (bv. rioleringswater) en landbouwdieren (bv. spoelwater van stallen, afvloeiing van weiden). In landen waar een substantieel deel van het drinkwater uit oppervlaktewater wordt gewonnen, zoals België, vormt dit een voortdurende bedreiging voor de veiligheid van het drinkwater. De doelstelling van **hoofdstuk 2** was de aanwezigheid van *Cryptosporidium* en *Giardia* te monitoren in verschillende drinkwaterproductiecentra waar drinkwater geproduceerd wordt uit oppervlaktewater (Zillebeke, Gavers,

Dikkebus en Blankaart). Aanvullend werd ook een onderscheid gemaakt tussen contaminatie van humane en dierlijke oorsprong met behulp van genotypering. In de waterproductiecentra van Zillebeke, Gavers en Dikkebus werden van september 2010 tot augustus 2011 maandelijks watermonsters verzameld van oppervlaktewater (15 L) en gefilterd drinkwater (60 L). In de Blankaart werden van februari 2010 tot april 2012 maandelijks monsters verzameld van oppervlaktewater (15 L), water uit het spaarbekken (15 L) en gefilterd drinkwater (60 L). *Cryptosporidium* en *Giardia* werden gedetecteerd met de USEPA 1623 methode en positieve stalen werden gegenotypeerd. In geen enkel drinkwatermonster werd *Cryptosporidium* of *Giardia* gevonden. In drie van de vier waterproductiecentra (Zillebeke, Gavers en Dikkebus) werden bovendien slechts af en toe lage concentraties (oö)cysten gedetecteerd in oppervlaktewater(<1 per liter). Ruw oppervlaktewater van de Blankaart was echter frequent gecontamineerd met *Cryptosporidium* (96%) en *Giardia* (92%), vooral in de winter en de lente. Een lagere frequentie en lagere concentraties van (oö)cysten werden gevonden in het spaarbekken, wat aangeeft dat bezinking in het spaarbekken een gunstige maatregel is. Genotypering van 38 positieve watermonsters toonde de aanwezigheid aan van *Cryptosporidium andersoni, C. suis, C.* horse genotype, *C. parvum* en *C. hominis*. Genotypering van *Giardia* toonde de aanwezigheid aan van *G. duodenalis* assemblage AI, AII, BIV, BIV-like en E. De dominantie van diersoort-specifieke species/genotypes en species/genotypes die eerder bij dieren voorkomen, suggereert dat landbouw in dit waterproductie-centrum een belangrijkere contaminatiebron was dan menselijk afvalwater. In waterproductiecentra met gecontamineerd oppervlakte-water is continue monitoring van het gezuiverde drinkwater voor de aanwezigheid van *Cryptosporidium* en *Giardia* aan te raden. Bronnen van contaminatie van het oppervlaktewater zouden moeten opgespoord worden.

De doelstelling van **hoofdstuk 3** was de aanwezigheid van *Cryptosporidium* en *Giardia* in verschillende recreatiewaters in België te onderzoeken en het infectierisico geassocieerd met zwemmen of andere recreatie-activiteiten in te schatten. *Cryptosporidium* oöcysten en/of *Giardia* cysten werden gedetecteerd in 3/37 zwembaden, 7/10 recreatievijvers, 2/7 waterparken en 4/16 waterfonteinen. Het infectierisico voor *Cryptosporidium* in zwembaden kon niet geschat worden, omdat *Cryptosporidium* oöcysten enkel in filterwater werden aangetroffen, en dus niet in het zwembadwater zelf. Het risico op infectie met *Giardia* in zwembaden varieerde van

1,13 $x10^{-6}$ tot 2,49 $x10^{-6}$ per zwembeurt per persoon. In recreatievijvers varieerde het infectierisico voor *Cryptosporidium* van 2,79 $x10^{-5}$ tot 5,74 $x10^{-5}$ per zwembeurt per persoon en voor *Giardia* van 7,04 $x10^{-5}$ tot 1,46 x 10^{-4}. Voor andere waterrecreatie-activiteiten (vissen, varen, roeien) was het geschatte infectierisico 5,71 $x10^{-6}$ voor *Cryptosporidium* en 1,47 x 10^{-5} voor *Giardia.* Deze infectierisico's moeten wel genuanceerd worden, omdat de meeste geïdentificeerde *Cryptosporidium* en *Giardia* isolaten behoorden tot dierspecifieke species/genotypes of species/genotypes die vooral bij dieren voorkomen, en omdat de viabiliteit van de (oö)cysten niet werd bepaald. In waterparken en fonteinen werden geen *Cryptosporidium* oöcysten gevonden, maar de aanwezigheid van *Giardia* cysten wijst op een infectierisico. Het geschatte *Giardia* infectierisico geassocieerd met een bezoek van 3,5 minuten aan een waterpark was 1,68 $x10^{-4}$.

In **hoofdstuk 4** wordt de transmissie van *Cryptosporidium* en *Giardia* in een ruraal gebied in het noorden van Bangladesh onderzocht. *Cryptosporidium* en *Giardia* zijn belangrijke oorzaken van diarree in Bangladesh, zowel in de steden als op het platteland. De hoge prevalentie van beide parasieten bij mensen en runderen in ruraal Bangladesh en het gemeenschappelijke gebruik van vijvers door dorpsbewoners en hun dieren doet mogelijke (watergedragen) zoönotische transmissie vermoeden. Daarom werd zowel rechtstreekse transmissie tussen runderen en hun hoeders als indirecte transmissie via dorpsvijvers onderzocht. Fecesstalen werden verzameld van 623 kalveren en 125 veehoeders in een cross-sectionele prevalentiestudie. Daarenboven werden in twee dorpen maandelijks watermonsters genomen van een vijver. Aanvullend werden ook fecesmonsters verzameld van dorpsbewoners en hun runderen (10 mensen en 10 runderen per maand). *Cryptosporidium* oöcysten en *Giardia* cysten werden aangetoond en geteld in water- en fecesmonsters met behulp van een immunofluorescentie test en positieve monsters werden gegenotypeerd met behulp van PCR. De prevalentie van *Cryptosporidium* en *Giardia* in kalveren was respectievelijk 5% en 22%, terwijl respectievelijk 3% en 11% van de herders positief was voor *Cryptosporidium* en *Giardia.* Zowel in de cross-sectionele studie als in de longitudinale studie in de dorpen werd *G. duodenalis* assemblage E het meest frequent aangetoond in kalveren, terwijl bij mensen assemblage AII, BIII en BIV werden gevonden. In runderen werden ook *Cryptosporidium parvum*, *C. bovis* en *C. andersoni* geïdentificeerd, maar er konden geen *Cryptosporidium* DNA sequenties verkregen worden van humane stalen. In watermonsters werd frequent

Cryptosporidium (12/24) en *Giardia* (14/24) gevonden. *G. duodenalis* assemblage E en BIV(-like) en *C. andersoni* en *C. hominis* werden geïdentificeerd. De aanwezigheid van (oö)cysten in beide vijvers toont aan dat watergedragen transmissie van *Cryptosporidium* en *Giardia* in deze dorpen mogelijk is, maar uit de resultaten van de genotypering blijkt dat er geen significante zoönotische transmissie (direct of via water) plaatsvindt tussen runderen en mensen. Over eventuele zoönotische transmissie van *Cryptosporidium* kan geen uitsluitsel gegeven worden, door het lage aantal sequenties dat verkregen werd uit watermonsters en humane fecesstalen.

In de algemene discussie (**hoofdstuk 5**) wordt getracht om uit de resultaten conclusies te trekken over (watergedragen) transmissie van *Cryptosporidium* en *Giardia* in België en Bangladesh. Ook worden de problemen in het uitgevoerde onderzoek en mogelijkheden voor toekomstig onderzoek besproken.

In België lijkt er geen gevaar voor *Cryptosporidium* en *Giardia* infectie verbonden te zijn aan de consumptie van drinkwater uit de onderzochte waterproductie-centra, aangezien in geen enkel monster (oö)cysten werden aangetroffen. De hoge (oö)cyst concentraties in het oppervlaktewater zijn echter een risico voor drinkwatercontaminatie in het geval van een defect filtersysteem. Defecte filtersystemen lagen in het verleden meermaals aan de basis van water-gerelateerde uitbraken van cryptosporidiose en giardiose in verschillende landen, inclusief België. Daarom wordt aangeraden om het geproduceerde drinkwater regelmatig te monitoren voor *Cryptosporidium* en *Giardia*. Ons onderzoek en eerdere studies hebben immers aangetoond dat fecale bacteriële indicatoren geen betrouwbare indicator zijn voor de aanwezigheid van *Cryptosporidium* en *Giardia*. Daarnaast moet ook getracht worden om de contaminatie van het oppervlaktewater dat gebruikt wordt voor drinkwater-productie te verminderen. Identificatie van stroomopwaarts gelegen besmettingsbronnen zou toelaten om preventieve maatregelen te treffen. Uit de resultaten van hoofdstuk 2 konden echter geen duidelijke contaminatiebronnen geïdentificeerd worden, omdat de gegevens (lokalisatie van de productiecentra, precipitatiegegevens, genotypering) niet eenduidig waren. Het gebruik van temporo-spatiale modellen en multi-locus genotypering zou in de toekomst hierover misschien uitsluitsel kunnen geven.

Contaminatie van zwembaden is vooral te wijten aan fecale incidenten of malfunctie van het filtersysteem. In onze studie werden *Cryptosporidium* en *Giardia* aangetroffen in filterwater of zwembadwater van enkele bemonsterde zwembaden,

hetgeen wijst op een infectierisico. Aangezien cryptosporidiose en giardiose geen aangifteplichtige ziekten zijn in België, worden kleine water-gerelateerde uitbraken waarschijnlijk vaak over het hoofd gezien. In de praktijk blijkt het soms moeilijk om clusters van cryptosporidiose of giardiose te associëren met een gecontamineerd zwembad, omdat de etiologie van individuele diarree-gevallen vaak niet onderzocht wordt, geïdentificeerde gevallen van cryptosporidiose en giardiose niet altijd gerapporteerd worden, verdachte zwembaden pas enkele dagen na de uitbraak worden bemonsterd en positieve watermonsters vaak niet worden gegenotypeerd.

Hoewel de bemonsterde zwem- en recreatievijvers allemaal voldeden aan de EU directieven, waren ze frequent gecontamineerd met *Cryptosporidium* en *Giardia*. Het infectierisico (>1/100.000 per bezoek aan een gecontamineerde zwemvijver per jaar) was echter waarschijnlijk overschat, omdat vooral dier-geassocieerde species/genotypes werden gevonden en omdat de viabiliteit van de (oö)cysten niet was bepaald.

In Bangladesh werden geen aanwijzingen gevonden voor zoönotische transmissie tussen runderen en mensen. Er waren wel aanwijzingen voor watergedragen transmissie, aangezien beide bemonsterde vijvers frequent gecontamineerd waren met (oö)cysten, en ook bij de dorpsbewoners *Cryptosporidium* en *Giardia* werd aangetroffen. Mogelijke maatregelen om transmissie via de dorpsvijvers te voorkomen worden besproken. In deze studie werd watergedragen transmissie van *Cryptosporidium* en *Giardia* enkel bestudeerd in een plattelandsgebied. In de steden zijn cryptosporidiose en giardiose echter ook een groot gezondheidsprobleem. Daarnaast kunnen beide parasieten ook overgedragen worden via voedsel, bv. rauwe groenten. Mogelijke maatregelen om de risico's voor water- en voedsel-gedragen transmissie in de steden te verkleinen en verdere studies hierover worden besproken.

Curriculum vitae

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Academic Qualifcation

Degree	Name of Board/University	Major	Division/ Class	Year	Remarks
Ph.D	Ghent University, Belgium	Parasitology	N/A	June, 2015	Expected
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PhD Thesis

Ehsan, M. A. (2015). Water-borne transmission of *Cryptosporidium* and *Giardia* in Belgium and in Bangladesh. Laboratory for parasitology, Department of Virology, Parasitologya and Immunology, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Salisburylaan, 133, 9820, Belgium. (PhD in Veterinary Parasitology).

MSTAH Thesis

Ehsan, M. A. (2008). Effect of ecological parameters on the distribution and phenology of *Culicoides* species in Belgium. Institute of Tropical Medicine Antwerp, Nationalestraat, 151, Antwerpen, Belgium. (MSTAH in Tropical Animal Health).

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Awards / Scholarships / Honours

1. BOF scholarship for PhD programme, Ghent University, Belgium in 2009.
2. DGDC scholarship for MSTAH degree in the Institute of Tropical Medicine Antwerp in 2007
3. Bangladesh Agricultural University Award for securing First class and 1st position in graduation (2003) among 63 students.
4. Professor Dr. Abdus Salam Mia Trust Merit Scholarship (Gold Medal) for securing First class and 1st position in graduation (2002).
5. University Grants Commission (UGC) Scholarship for securing highest score in graduation among the students in 1999.
6. University Talent Scholarship, Bangladesh Agricultural University, Mymensingh, Bangladesh, 1994 to 2000.
7. Kalyan Tahabil Scholarship, Dhaka, Bangladesh, from 1994-2000.
8. Islamic Bank Foundation Scholarship, Dhaka, Bangladesh, 1995.
9. Secondary Junior Scholarship in 1989.

Field of Research Activities and Interest

- Water-borne and food-borne transmission of *Cryptosporidium* and *Giardia*
- Zoonotic diseases
- Molecular Medicine