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Cryptosporidium and *Giardia* in calves in Belgium

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List of abbreviations

18S rDNA	18S ribosomal DNA
β-giardin	beta giardin
Bayes-p	Bayesian p-value
BBW	Belgian blue and white
BZD	benzimidazoles
CI	confidence interval
COWP	*Cryptosporidium* oocyst wall protein
C-PCR	*Cryptosporidium* diagnostic PCR
CPG	cysts per gram (of faeces), for *Giardia*
D	day
DIC	deviance information criterion
gp60	60kDa glycoprotein
HSP-70	70kDa heat shock protein
IFA	immunofluoresence assay
ME	microscopical examination
OPG	oocysts per gram (of faeces), for *Cryptosporidium*
PCR	polymerase chain reaction
p_D	number of parameters
Techlab	Techlab ELISA
Tetra	Tetrakit ELISA
tpi	triose phosphate isomerase

Chapter 1

Cryptosporidium and *Giardia* in calves:

a literature review

1.1 General introduction

Cryptosporidium was first described in 1907 in the gastric mucosa and later in the small intestine of laboratory mice. The first detailed description of *Giardia* dates from 1859, although Antonie van Leeuwenhoek already described protozoan trophozoites looking like *Giardia* in 1681. Studies of coprolites from Peru indicate that both *Cryptosporidium* and *Giardia* were already prevalent in ancient human populations (Ortega and Bonavia, 2003), but the clinical relevance of both parasites in human patients was not acknowledged until late in the 20th century. Cryptosporidiosis is nowadays considered as a life threathening disease in AIDS patients and as a common cause of diarrhea in human patients, both in sporadic cases and in outbreaks, such as the large waterborne cryptosporidiosis outbreak in Milwaukee in 1993 (Mac Kenzie *et al*., 1994; Fayer, 2004). *Giardia* is recognised as the most common parasitological cause of human diarrhea, with an estimated 280 million infections worldwide per year. In children chronic giardiosis can result in long-term growth retardation (Fraser *et al.*, 2000) and poor cognitive functions (Berkman *et al.*, 2002). Both giardiosis and cryptosporidiosis are a frequently diagnosed waterborne infection and are a major concern to water utilities. Because of the impact on socio-economic development, especially in developing countries, both *Giardia* and *Cryptosporidium* are since 2004 included in the 'Neglected Disease Initiative' of the World Health Organization (WHO) (Savioli *et al.*, 2006).

In veterinary medicine *Cryptosporidium* and *Giardia* are at present considered as important pathogens in the aetiology of diarrhea in housed calves. However, the pathogenicity of both parasites was not recognised until the end of the last century. In calves *Cryptosporidium* was associated with diarrhea in 1971 (Panciera *et al.*, 1971), although the primary pathogenic effect of *Cryptosporidium* was only later confirmed (Tzipori *et al.*, 1980b). Since then *Cryptosporidium* has been identified in 40 to 70% of the faecal samples collected from diarrhetic calves younger than 1 month both in Europe and in the USA (Moore and Zeman, 1991; Otto *et al.*, 1995; de Graaf *et al.*, 1999b; de la Fuente *et al.*, 1999; Constant, 2001; Tartera, 2002). Therefore, *Cryptosporidium* is considered as the most important pathogen in the aetiology of neonatal calf diarrhea (de Graaf *et al.*, 1999b). Although the first case of bovine giardiosis was reported in 1921 (Fantham, 1921) and despite the high prevalence in farm animals, the clinical importance of giardiosis in cattle was not described until

quite recently (St. Jean, 1987). Since then *Giardia* has increasingly been recognised as an important pathogen in young calves and considered as an important differential diagnosis for coccidiosis in the aetiology of diarrhea in calves older than 1 month. Based on high prevalence of both parasites, cattle have been considered as potential sources for human infection, although in the last decade molecular tools have provided a more differentiated and detailed insight in the epidemiology of *Cryptosporidium* and *Giardia*.

The aim of the literature review is to present an overview of the current knowledge on *Cryptosporidium* and *Giardia,* with emphasis on molecular epidemiology, zoonotic aspects, diagnosis, treatment and control. In chapters 2, 3 and 4 the experimental work of this thesis is presented. In chapter 5 the general outcome, the conclusions and future perspectives are discussed.

1.2 *Cryptosporidium*

1.2.1 Classification

Cryptosporidium is an apicomplexan protozoan parasite and was until recently classified as a coccidian parasite, despite the many unique characteristics, such as the lack of host specificity, resistance to anticoccidial treatment, ability for autoinfection and the particular location within the host cell (Hijjawi *et al.*, 2004). Phylogenetic analysis on the small-subunit rRNA locus indicated that *Cryptosporidium* is indeed not related to the coccidia (Xiao *et al.*, 1999). Cross-reaction of an anti-cryptosporidial monoclonal antibody with gregarines suggested a close relation (Bull *et al.*, 1998), which was later confirmed by molecular (Carreno *et al.*, 1999) and life cycle similarities (Hijjawi *et al.*, 2001, 2002, 2004; Rosales *et al.*, 2005). It is now believed that *Cryptosporidium* should be placed in a taxonomic group separate from the coccidians and close to the gregarine parasites (Barta and Thompson, 2006).

Cryptosporidium spp. infect a wide range of hosts, including 155 mammalian species (Fayer, 2004). Similar to *Giardia* the traditional taxonomy was largely based on host occurence. Genetically related hosts have related *Cryptosporidium* parasites, although there are two important exceptions to this 'host-parasite co-evolution' theory. The first considers the *C. parvum* bovine genotype, which forms a monophyletic group with the *C. parvum* human, mouse, rabbit and monkey genotypes. Rodents, primates and lagomorphs originate from a common ancestor which is however different from

the ancestor of ruminants. It is therefore possible that the *C. parvum* bovine genotype was originally a parasite of rodents and only recently established in ruminants. This 'host expansion' also occured for *C. meleagridis*, which was originally a mammalian parasite that has established in birds (Xiao *et al.*, 2002). Furthermore, the assumption of a tight host-specificity was rejected based on cross-transmission experiments (Tzipori *et al.*, 1980a; Moon *et al.*, 1982a).

The application of PCR on faecal and environmental samples indicated that *Cryptosporidium* is a heterogeneous cluster of morphologically identical species and genotypes (Monis and Thompson, 2003), with the distinction of a gastric and an intestinal group. To date 16 *Cryptosporidium* species and multiple genotypes have been described (Table 1.1). Although the genotype designation reflects significant genetic differences, not all genotypes differ from each other to the same extent. The challenge is therefore to determine what genotypes can be considered as separate species, based not only on genetic, but also on biological and morphological differences (Xiao *et al.*, 2004). Several new species have thus been described, such as *C. bovis* (Fayer *et al.*, 2005) and *C. suis* (Ryan *et al.*, 2004).

Most research focused on *C. parvum*, which has been identified in a wide range of hosts, including man. Izoenzyme and molecular analysis indicated the existence of at least two distinct genotypes within *C. parvum*: the human genotype (genotype 1 or H) and the zoonotic bovine genotype (genotype 2 or C). The human genotype was shown to be largely human specific and was reclassified as *C. hominis* (Morgan-Ryan *et al.*, 2002). The bovine genotype is refered to as *C. parvum*. Recently, *C. pestis* was proposed as a new species name, although still not formerly accepted (Slapeta, 2006; Xiao *et al*., 2006).

Based on highly polymorphic microsatellite and minisatellite markers, intra-species variation has been described, especially within *C. parvum* (Caccio *et al.*, 2000; Feng *et al.*, 2000). Within *C. parvum* the level of polymorphism is high, with indications of genetic exchange (panmixia). The occurence of panmixia is due to the high transmission between hosts, which is supported by the high prevalence of *C. parvum* in human patients and farm animals (Mallon *et al.*, 2003a). Despite the existence of panmixia, discrete lineages of both human specific and zoonotic subgenotypes specific to geographical regions have been identified within *C. parvum*. This might indicate the existence of both host and geographical specific alleles (Caccio *et al.*, 2001), although a rather limited number of these lineages has been described so far.

Table 1.1: Currently recognised species and genotypes of *Cryptosporidium*, along with their former name and major host range. For the recognised species the reference of species description is provided.

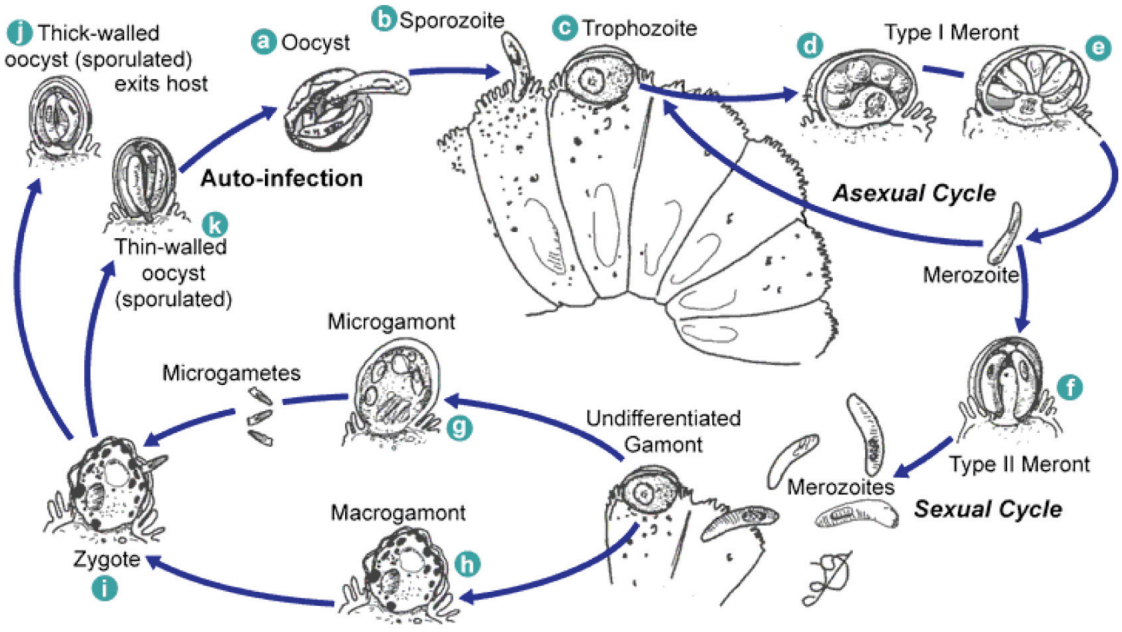
Species or genotype	Former name	Major hosts	Reference
Intestinal species			
C. hominis	human genotype	man	(Morgan-Ryan *et al*., 2002)
C. pestis (C. parvum)	bovine genotype	livestock, man	(Slapeta, 2006)
C. parvum	mouse genotype	mice	(Tyzzer, 1912)
rabbit genotype		lagomorphs	
monkey genotype		monkeys	
ferret genotype		ferrets	
horse genotype		horses	
C. bovis	genotype Bovine B	cattle	(Fayer *et al*., 2005)
C. suis	pig genotype I	pigs	(Ryan *et al*., 2004)
C. canis		dogs	(Fayer *et al*., 2001)
C. felis		cats	(Iseki, 1979)
C. wrairi	guinea pig genotype	guinea pigs	(Vetterling *et al*., 1971)
C. meleagridis		birds	(Slavin, 1955)
C. bayleyi		poultry	(Current *et al*., 1986)
C. saurophilum		lizards	(Koudela and Modry, 1998)
(*C. nasorum*; *nomen nudum*?)		fish	
C. molnari		fish	(Alvarez-Pellitero and Sitja-Bobadilla, 2002)
Skunk genotype		skunks	
Opposum genotype I and II		opposums	
Marsupial genotype I and II		marsupials	
Deer / cervid genotype		Deer, sheep	
Squirrel genotype		squirrels	
Goose genotype I and II		geese	
Duck genotype		ducks	
Snake genotype		snakes	
Bear genotype		bears	
Fox genotype		foxes	
Coyote genotype		coyotes	
Deer mouse genotype		deer mice	
Muskrat genotype I and II		muskrats	
Pig genotype II		pigs	
Gastric species			
C. andersoni	*C. muris*	cattle	(Lindsay *et al*., 2000a)
C. muris		rodents	(Tyzzer, 1907)
C. galli		chickens	(Ryan *et al*., 2003)
C. serpentis		reptiles	(Levine, 1980)
Lizard genotype		lizards	
Tortoise genotype		turtles	
Woodcock genotype		woodcocks	

Panmixia therefore seems to occur, however not frequently among natural isolates, probably due to the limited opportunity for the union of dissimilar subgenotypes (Feng *et al.*, 2000). In contrast, limited polymorphism has been observed in *C. hominis* and subsequent predominance of a small number of subgenotypes, which might reflect the epidemic nature of *C. hominis* infections: originating from one or few individuals the infection spreads rapidely in a susceptible population, with limited opportunity for genetic recombination with other genotypes.

1.2.2 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 host small intestine. Until recently *Cryptosporidium* was considered as an obligate intracellular parasite, but *in vitro* studies revealed that the entire life cycle can be completed without the need for host cells, although not readily accomplished. The amplification *in vivo* occurs in the host cell, which might suggest that *Cryptosporidium* only recently adapted to its intracellular habitat (Hijjawi *et al.*, 2004; Girouard *et al.*, 2006).

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 December 5th 2006: http://www.dpd.cdc.gov/dpdx/HTML)*



The life cycle (Figure 1.1) initiates with the ingestion of the infective oocyst (a) and the excystation of 4 sporozoites (b), which is mainly triggered by temperature and pH (Hijjawi *et al.*, 2002; Smith *et al.*, 2005a). The respiratory tract is occasionally infected after inhalation of infective oocysts (Mascaro *et al.*, 1994; Okhuysen *et al.*, 1999). During cell invasion, the developmental stages are not located in the host cytoplasm, but are surrounded by host microvillous membranes that rise up and link over the top of the parasite to form the parasitoforous membrane. Hence, the parasite lies protected between the cell membrane and the cell cytoplasm in the so-called parasitophorous vacuole. This intracellular yet extracytoplasmatic location is unique for *Cryptosporidium* and enables the parasite to evade both the extracellular and intracellular host defense mechanisms (Hijjawi *et al.*, 2004).

After cell invasion the sporozoite forms a circular stage known as the trophozoite (c). The first part of the cycle consists of an asexual development with two successive generations of merogony, resulting in the formation of meronts (d). Although the number varies *in vitro*, type I meronts (e) usualy contain 8 small merozoites which penetrate new host cells to form type II meronts (f) with 4 large merozoites. Type I merozoites are also believed to have the ability to recycle contineously and to be responsible for auto-infection, especially in immunocompromised human patients. The type II 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. After 2 asexual divisions (sporogony) the oocyst contains 4 sporozoites. Approximately 80% of the oocysts have a thick wall (j) 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).

Most extracellular stages in the life cycle of *Cryptosporidium* are very succeptible to the protective mechanisms of the small intestine. They therefore remain under the protective mucus layer upon release from the host cell and infect adjacant intestinal cells within a few seconds, to limit contact with the hostile gut environment (Meloni and Thompson, 1996). However, two new extracellular stages, commonly refered to as the extracellular trophozoite/gamont-like stages, have been described in the life

cycle of *C. parvum* and *C. andersoni*. These new stages seem to be adapted to extended extracellular existence and have been described in *in vitro* cultures, but also *in vivo* in infected mice and in an infected steer. Similar extracellular stages have also been reported in gregarines, suggesting close taxonomic affinity. Whether these stages can be fertilised and encyst (syzygy), as in gregarines, is unclear (Hijjawi *et al.*, 2002).

1.2.3 Epidemiology and prevalence

Epidemiology

Calves are infected by oral ingestion of infectious oocysts. The oro-faecal transmission may be direct through contact with infected animals, or indirect through a contaminated environment or through the ingestion of contaminated feed and water. Mechanical transmission through vectors such as tools, care-takers, or even arthropods and birds is possible (Graczyk *et al.*, 1999; Fayer *et al.*, 2000). Especially young calves and to a lesser extent the dam should be considered as direct sources for calfhood infection. Calves start excreting oocysts as soon as 3-5 days after parturition but the peak excretion, with up to 10^{10} oocysts per gram of faeces (OPG), occurs around the age of 12 days. The oocyst excretion lasts for 6-14 days (O'Handley *et al.*, 1999; Nydam *et al.*, 2001; Castro-Hermida *et al.*, 2002b). High oocyst excretion is most frequently observed in calves younger than one month and from the age of 5 weeks onwards the OPG decreases (Fayer *et al.*, 1998a; Maldonado-Camargo *et al.*, 1998; O'Handley *et al.*, 1999; Becher *et al.*, 2004; Kvac *et al.*, 2006). Although the excretion of oocysts by adult cattle is generally low, dams have been advocated as a potential source of infection, based on a presumed periparturient rise, which has been reported in ewes (Xiao *et al.*, 1994), and also in cattle for *C. andersoni* (Ralston *et al.*, 2003b). Others did not confirm the periparturient rise of *C. parvum* in cattle (Scott *et al.*, 1995; Atwill *et al.*, 1998; Atwill and Pereira, 2003), or did not differentiate the oocysts (Faubert and Litvinsky, 2000). Due to the low OPG (Atwill *et al.*, 2003) and the predominance of *C. andersoni* in adult cattle (Fayer *et al.*, 2006b), dams are probably of minor importance, especially on dairy farms (Naciri *et al.*, 1999; Sturdee *et al.*, 2003).

As indirect sources for infection the housing should be contemplated, such as the maternity pen and the calf facilities (Sturdee *et al.*, 2003). The role of the maternity

pen in the epidemiology largely depends on the time of stay before transfer to the calf facilities and on the number of cows in the maternity pen (Garber *et al.*, 1994). When calves are transposed shortly after birth, the calf housing facilities are more relevant. In group housing direct contact between calves favours transmission (Castro-Hermida *et al.*, 2002a), but also in individual pens transmission is frequent, either by a vector, such as the care-taker, or by effluent of faecal material. Furthermore, transmission to subsequent calves in one particular pen is possible (O'Handley *et al.*, 1999). In general, calves housed in indoor calf pens are at greater risk of infection than calves housed outside in hutches (Quigley *et al.*, 1994).

As for *Giardia*, several parasite characteristics contribute to the succesfull transmission of *Cryptosporidium* infections in calves (Table 1.3). The high number of infective oocysts excreted by calves with patent cryptosporidiosis is in contrast with the low number of oocysts needed for infection. Furthermore, oocysts can survive for more than 12 weeks in soil and up to 24 weeks in water at temperatures between -4°C and 20°C. Due to faecal micro-organisms the degradation of oocysts is precipitated in bovine faeces, but still oocysts remain infective for at least 70 days in cattle manure (Fayer *et al.*, 1998c; Olson *et al.*, 1999; Jenkins *et al.*, 2002). Freezing is lethal for oocysts, although a small proportion of the oocysts can remain viable at freezing temperatures for up to 2 months. Whether these viable oocysts are still infective is uncertain (Robertson *et al.*, 1992; Walker *et al.*, 2001). Desiccation is 100% lethal within 4 hours. Oocyst infectivity decreases after heating at temperatures above 55°C, with shorter exposure time as temperature increases (Fayer, 1994; Fujino *et al.*, 2002).

Several risk factors for infection have been identified, including management factors such as herd size, the number of young calves, the stocking rate or the length of the calving season. Higher stocking densities increase the likelihood of transmission between infected and succeptible animals by increasing the contact rate, especially between young animals (Garber *et al.*, 1994). A long calving season or year round calvings result in a prolonged recruitement of susceptible calves (Atwill *et al.*, 1999b; Hoar *et al.*, 2001). Seasonal fluctuations in prevalence and oocyst shedding have been identified in human and wildlife populations (Atwill *et al.*, 2004; Roy *et al.*, 2004), but not in cattle (Atwill *et al.*, 1999b; Wade *et al.*, 2000b; Becher *et al.*, 2004), except when coinciding with the calving season and the resulting higher stocking densities (Huetink *et al.*, 2001; Sturdee *et al.*, 2003; Lorino *et al.*, 2005;). An important animal feature seems to be the age of the calf: on *Cryptosporidium* positive farms there is a

cumulative incidence of 100% (O'Handley *et al.*, 1999), with a maximum risk for infection between 9 and 12 days of age. On these positive farms 50% of the calves is infected before the age of 10 days. Furthermore, the younger the calf acquiring infection, the longer the period of oocyst shedding (Maldonado-Camargo *et al.*, 1998; Castro-Hermida *et al.*, 2002b). Occasionally calves older than one month get a primary infection with *C. parvum*, mostly due to the absence of previous infection (Huetink *et al.*, 2001).

Prevalence

Out of the 16 *Cryptosporidium* species, several have been described in cattle. Next to the ubiquitous *C. parvum* the ruminant specific species *C. bovis* and *C. andersoni*, and the cervine genotype have been described on a regular basis in cattle. Occasionally reported are *C. suis* (Fayer *et al.*, 2006b), *C. felis* (Bornay-Llinares *et al.*, 1999) and *C. hominis* (Smith *et al.*, 2005b). The relevance of the latter species to the development of clinical symptoms in cattle or to the zoonotic reservoir function of cattle is probably minimal.

A wide range of calf (0-59%) and farm (0-100%) prevalence estimates has been reported in calves (Table 1.2). Although part of this variation is certainly due to substantial differences in prevalence between farms, the variation is also due to differences in study design, such as the number of animals or farms included, the age and breed of the animals. The choice of diagnostic technique might also influence the prevalence estimate, since some techniques are more sensitive than others. However, there is no gold standard reference test for the diagnosis of *Cryptosporidium*, which troubles the comparison of studies using different diagnostic techniques.

Breed might be important, since in beef calves the *Cryptosporidium* prevalence is lower compared to dairy calves of similar age (Table 1.2), although the lower prevalence can be largely attributed to a combination of risk factors. In a cow-calf system with seasonal calving periods applied on many beef farms the calves remain with the dam, resulting in a more efficient transfer of colostrum and lower calf stocking densities. Furthermore, the cow-calf herds tend to stay on pasture, where the *Cryptosporidium* oocysts are more exposed to dessication and direct sunlight, especially in arid areas such as California (Atwill *et al.*, 1999a) or Zambia (Geurden *et al.*, 2006). Cow-calf herds are therefore considered a limited risk for environmental contamination (Gow and Waldner, 2006).

Table 1.2: Overview of prevalence studies of *Cryptosporidium* in cattle in different countries, with the diagnostic technique (D); the farming type (Farm: d=dairy and b=beef); the number of animals ($\#_C$) and farms ($\#_F$), the age of the animals, the calf (P_C) and farm (P_F) prevalence in percentage (%)

Country	D	Farm	$\#_C$	$\#_F$	Age	P_C (%)	P_F (%)	Reference
Calves < 6months								
Canada	M	d	500	51	7-21d	41	76	(Trotz-Williams *et al.*, 2005a)
France	ELISA	d+b	2,068	196	<21d	23	-	(Lefay *et al.*, 2000)
Mexico	M	d	512	31	<28d	25	93	(Maldonado-Camargo *et al.*, 1998)
USA	IFA	d	393	14	<2m	41	100	(Santin *et al.*, 2004)
Czech Republic	M	d	2,056	11	<2m	27	100	(Kvac *et al.*, 2006)
		b	367	11	<2m	1	27	
Tanzania	M	d	116	5	<3m	16	100	(Mtambo *et al.*, 1997)
Zambia	ELISA	d	250	37	<3m	43	76	(Geurden *et al.*, 2006)
		b	238	25	<3m	8	44	
USA	IFA	d	7,369	1,103	<4m	22	59	(Garber *et al.*, 1994)
USA	M	d	2,943	109	<6m	2	13	(Wade *et al.*, 2000b)
Trinidad	M	-	298		<6m	9	-	(Kaminjolo *et al.*, 1993)
Norway	IFA	d	1,386	136	<6m	12	53	(Hamnes *et al.*, 2006a)
Canada	M	d	-	505	<6m	-	89	(Ruest *et al.*, 1998)
Canada	IFA	d	386	20	<6m	59	80	(Olson *et al.*, 1997a)
Canada	IFA	b	605	100	<6m	3	15	(Olson *et al.*, 1997a)
Calves > 6months								
USA	IFA	d	447	14	3-11m	26	100	(Santin *et al.*, 2004)
Denmark	IFA	d	895	50	<12m	40	96	(Maddox-Hyttel *et al.*, 2006)
USA	PCR	d	571	14	12-24m	12	93	(Fayer *et al.*, 2006b)
Spain	M	d+b	329	30	<24m	21	64	(Quilez *et al.*, 1996b)
Czech Republic	M	d	2,217	11	>3m	0	0	(Kvac *et al.*, 2006)
		b	2,381	11	>3m	0	0	
Canada	IFA	b	560	59	>24m	1	5	(Olson *et al.*, 1997a)

Overall, the highest prevalence is observed in animals under the age of 5 weeks (Quilez *et al.*, 1996b). An age-related resistance to infection has been observed both in calves and in lambs (Harp *et al.*, 1990; Ortega-Mora and Wright, 1994). In post-weaned calves and adult animals the *Cryptosporidium* prevalence is generally low, although high but undifferentiated *Cryptosporidium* prevalences have been reported (Villacorta *et al.*, 1991a; Scott *et al.*, 1995). In the majority of these prevalence studies, molecular or morphological identification is not performed. Recent data however confirm that *C. parvum* is most prevalent in calves up to 3 months, although

in extensive rearing systems the host specific *C. bovis* seems most prevalent (Feng *et al.*, 2006; Geurden *et al.*, 2006). In older calves *C. bovis* and the *Cryptosporidum* deer-like genotype predominate, next to *C. andersoni*. In adult cattle *C. andersoni* and *C. bovis* are most frequently reported (Santin *et al.*, 2004; Fayer *et al.*, 2006b).

1.2.4 Zoonotic aspects

In industrialised countries, the prevalence of *Cryptosporidium* in immunocompetent hosts varies from 0.2% in asymptomatic persons to 2.2% in patients with diarrhea (Guerrant, 1997). Especially children less than 5 years of age are at risk and, to a lesser extent, their parents, since changing diapers has been associated with infection (Hunter *et al.*, 2004). Human cryptosporidiosis can be due to infections with either the human specific *C. hominis* or the zoonotic *C. parvum*, and in a limited number of cases with *C. meleagridis* (Pedraza-Diaz *et al.*, 2001b). Other *Cryptosporidium* species, such as *C. canis*, *C. suis*, *C. muris* and *C. felis* have occasionally been reported, especially in immunodeficient patients (Pedraza-Diaz *et al.*, 2001a; Caccio *et al.*, 2005). For most *Cryptosporidium* species zoonotic transmission is contradicted by the host-parasite co-evolution theory, despite occasional identification in mostly immunocompromised human patients. For *C. parvum* and *C. meleagridis*, the host expansion hypothesis however supports the possibility of zoonotic infections (Xiao *et al.*, 2002), although the identification of *C. parvum* in human stool samples does not conclusively implicate zoonotic transmission. Subgenotype analysis at the 60kDa glycoprotein locus (GP60) (Peng *et al.*, 2001; Alves *et al.*, 2003; Sulaiman *et al.*, 2005; Trotz-Williams *et al.*, 2006) and multilocus microsatellite genotyping (Caccio *et al.*, 2000; Mallon *et al.*, 2003b), revealed both human specific and zoonotic subgenotypes within *C. parvum*. Moreover, in most waterborne outbreaks, including the large Milwaukee outbreak in 1993, human sources of infection are indeed identified (Peng *et al.*, 1997), and in only 1 waterborne outbreak in Canada livestock was implicated (Fayer *et al.*, 2000). This might indicate that next to a zoonotic cycle a human specific cycle of *C. parvum* exists, and that human infections are largely due to human sources of infection. However, molecular information is mainly obtained from outbreaks, whereas most human cryptosporidial infections are not outbreak related (Feltus *et al.*, 2006). Although in sporadic cases the source of infection is mostly unknown, direct contact with infected farm animals has repeatedly been associated

with infection (Hunter *et al.*, 2004; Hunter and Thompson, 2005). Other risk factors are traveling abroad, contact with a diarrhetic patient or swimming (Robertson *et al.*, 2002; Roy *et al.*, 2004).

It is clear that *C. parvum* can be maintained in a variety of independent transmission cycles that do not require interaction. The frequency and the circumstances of interaction mainly depend on the opportunity for contact between infected and susceptible hosts. Hence the proportion of *C. parvum* infections is much higher in rural areas, in the proximity of animal reservoirs, than in urban areas (Learmonth *et al.*, 2004). In Europe, the contact between livestock industry and drinking water resources or habitation and recreational areas, might account for the high prevalence of *C. parvum* in human patients, while in other parts of the world most human infections are due to *C. hominis*. Similarly, regulations imposed during the foot and mouth disease outbreak in 2001, including limited acces to the countryside and massive slaughter of cattle, resulted in a decline of human cryptosporidial infections in Scotland (Strachan *et al.*, 2003). Therefore, cattle and in particular young calves should be considered as a reservoir for *C. parvum* (Heitman *et al.*, 2002). Furthermore, mixed *C. parvum* and *C. hominis* infections (Tanriverdi *et al.*, 2003) and single *C. hominis* infections (Smith *et al.*, 2005b) have occasionally been reported in cattle. Sheep and other livestock seem to be of lesser importance (Ryan *et al.*, 2005) and companion animals are probably negligible as reservoir for human infection (Hunter and Thompson, 2005). Although wildlife, such as deer, elk and bison, can be infected with the same species as cattle and man (Olson *et al.*, 2004), the relevance of wildlife in the epidemiology of bovine or human cryptosporidiosis in Northwestern Europe is uncertain. Cattle seem therefore to be the most important reservoir for zoonotic transmission (Hunter and Thompson, 2005).

Although direct contact with calves caused infections in farm households (Miron *et al.*, 1991; Lengerich *et al.*, 1993) and farm visitors (Evans and Gardner, 1996; Sayers *et al.*, 1996) or veterinarians (Preiser *et al.*, 2003), the majority of the zoonotic infections are due to indirect transmission through water or food (Sulaiman *et al.*, 1999; Stantic-Pavlinic *et al.*, 2003; Hunter *et al.*, 2004). The oocysts are transported to surface water by the drainage from manure storage areas, run-off from manure-laden soil or direct contamination of water by cows (Fayer *et al.*, 1998b; Graczyk *et al.*, 2000; Sischo *et al.*, 2000; Jellison *et al.*, 2002). *Cryptosporidium* oocysts are prevalent in agricultural soil (Barwick *et al.*, 2003) and able to survive for several

months (Jenkins *et al.*, 2002). Rainfall and run-off are major contributers to the total *Cryptosporidium* load in surface waters and drinking water reservoirs (Kistemann *et al.*, 2002) and highest oocyst levels in agricultural surface waters coincide with the calving season (Bodley-Tickell *et al.*, 2002). Since most commonly used disinfection procedures in water treatment plants do not completely remove oocysts, water should be considered as an important vehicle for zoonotic infections.

1.2.5 Pathogenesis and clinical symptoms

The intestinal *Cryptosporidium* species, *C. bovis* and *C. parvum*, complete their life cycle in the ileum, and occasionally in the colon, caecum and duodenum (Sanford and Josephson, 1982). The pathogenicity of *C. bovis* is believed to be limited, probably due to immunity in older calves (Harp *et al.*, 1990; Santin *et al.*, 2004). In young calves *C. parvum* predominates and is considered as highly pathogenic. The invasion and colonisation of the epithelial surface by the different parasite stages result in loss of epithelial cells and microvillus brush border. Furthermore, the epithelial tight junctions are disrupted leading to an increased epithelial permeability. As a consequence there is a decreased intestinal surface area, a loss of membrane-bound digestive enzymes, such as lactase, and an impaired nutrient and electrolyte transport. Furthermore, neuropeptides lead to chloride secretion. In histo-pathological preparations villus shortening and fusion, as well as crypt hyperplasia and an increase in intra-epithelial lymphocytes can be observed (Sanford and Josephson, 1982; Tzipori *et al.*, 1983; Fayer *et al.*, 1998a; Robinson *et al.*, 2003).

Clinical symptoms are most frequently observed in calves between the age of 5 days and 1 month and include a malabsorptive and secretory diarrhea which is usually self-limiting within 2 weeks (O'Handley *et al.*, 1999). The diarrhea can be mild to severe with pale yellowish watery or mucoid faeces. Calves can be dehydrated, depressed and anorectic. The severity and duration of the clinical symptoms is highly variable, depending on concurrent viral, bacterial or parasitic infections, but also on host factors (Fayer *et al.*, 1998a). 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.*, 1999b). Calves with severe cryptosporidiosis can take several weeks to fully recover, and there is certainly an initial negative impact on production due to weight loss or impaired weight gain, and due to treatment expenses. Whether

cryptosporidial infections early in life have long-term detrimental effects is uncertain (Ralston *et al.*, 2003a). Calves that recover from a *Cryptosporidium*-associated diarrhea usually do not have recurrent clinical infections. The oocyst shedding also appears to be minimal after infection.

Infection with *C. andersoni* has been reported world-wide in post-weaned cattle (Enemark *et al.*, 2002; Olson *et al.*, 2004). *C. andersoni* invades the peptic and pyloric glands of the abomasum causing glandular dilatation and hypertrophy of the gastric mucosa and thinning of the epithelial lining. Although occasionally reported (Daniel *et al.*, 2005), infection with *C. andersoni* does usually not result in a pronounced diarrhea, but mainly causes maldigestion by inhibition of protein digestion due to increased gastric pH and subsequent decreased gastric proteolytic activity. This results in a moderate to severe impairment of weight gain, decreased feed efficiency and reduced milk production (Anderson, 1987; Esteban and Anderson, 1995; Ralston *et al.*, 2003a).

1.2.6 Diagnosis

The diagnosis of cryptosporidiosis in calves is traditionally performed by microscopical examination of a faecal smear, although immunological assays and PCR are increasingly applied in human and veterinary medicine. Most clinically affected calves excrete a high number of oocysts (Mohammed *et al.*, 1999; Uga *et al.*, 2000; Nydam *et al.*, 2001), facilitating clinical diagnosis. In epidemiological studies however, the intermittent excretion of oocysts needs to be taken into account, although multiple samplings do not necessarily yield an increased sensitivity (Hanscheid and Valadas, 2006).

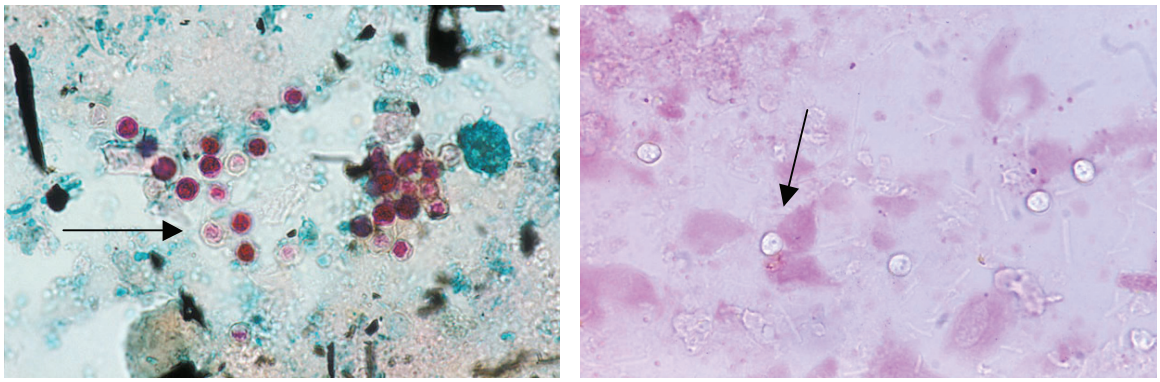
Microscopical examination

The most widely used technique for the diagnosis of *Cryptosporidium* is the detection of oocysts in a faecal smear, either native or after staining. The most commonly used staining methods are the modified Ziehl-Neelsen staining (Henricksen and Pohlenz, 1981) and the carbolfuchsine staining (Heine, 1982) (Figure 1.2). The oocysts of *Cryptosporidium* parasites from the intestinal group, such as *C. parvum*, *C. hominis* and many other *Cryptosporidium* species and genotypes are morphologically indistinguisable. They are spherical to elipsoidal with a mean size of 5.0 by 4.5µm

(range 4.5-5.4 by 4.2-5.0) (Upton and Current, 1985; Fayer *et al.*, 2005). The oocysts of the gastric *C. andersoni*, are larger and measure 7.4 by 5.5µm (range 6.0-8.1 by 5.0-6.5µm) (Lindsay *et al.*, 2000a). In addition, other faecal components ('ghosts') of similar size and shape, such as yeasts or *Cyclospora*, are difficult to distinguish from *Cryptosporidium* oocysts (Henriksen and Pohlenz, 1981; Clarke and McIntyre, 2001), which troubles interpretation and can lead to large variability between laboratories depending on the experience of the microscopist. Overall, microscopy has a low sensitivity compared to immunological techniques and PCR, especially for the diagnosis of mild infections. Even after concentration many of the infections with low oocyst excretion are not diagnosed by microscopy (Fayer *et al.*, 2000).

Figure 1.2: *Cryptosporidium* oocysts as diagnosed by microscopical examination (1000X) using a modified Ziehl-Neelsen staining (a) or the carbolfuchsine (b) staining

(a) (b)

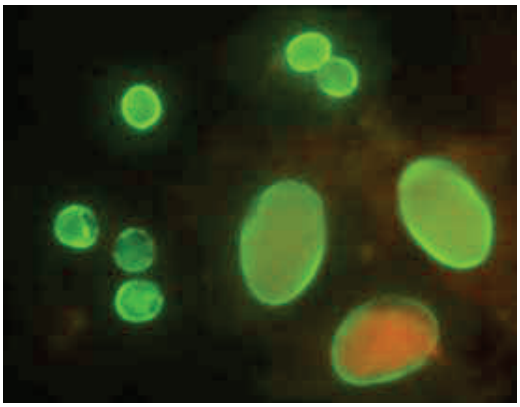


Immunological assays

Immunological assays commonly used in veterinary medicine comprise immunofluorescence assay (IFA), antigen enzym-linked immunosorbent assays (ELISA) and solid-phase qualitative immunochromatographic assays (dip sticks). Immunoserological detection of specific antibodies is unsuitable for clinical diagnosis, as the increase in specific antibody titer can not be detected until after the clinical phase of infection (O'Donoghue, 1995). Although developed and evaluated for use in human stool samples, both IFA and ELISA are applied on a regular basis for the diagnosis of clinical cryptosporidiosis in calves. IFA (Figure 1.3) can be used as a qualitative and a quantitative assay and the detection limit is believed to be as low as 1,000 oocysts per gram of faeces (Xiao and Herd, 1993), although in human stool samples a 100% recovery rate was not reached until seeded with 10,000 OPG (Weber

et al., 1991). The major advantage is the high sensitivity and specificity compared to microscopy, both for IFA (Quilez *et al.*, 1996a) and for ELISA (McCluskey *et al.*, 1995). A major disadvantage is the need for appropriate equipment and qualified personel. In contrast, dip sticks do not need laboratory settings and can be used on site by the veterinarian. In human stool samples, the dip sticks seem to be both sensitive and specific (Depierreux *et al.*, 2001; Llorente *et al.*, 2002), and also in calves they have been shown to be a valid alternative for laboratory diagnosis (Trotz-Williams *et al.*, 2005b).

Figure 1.3: *Cryptosporidium* oocysts (small and round) and *Giardia* cysts (large and ovoid) visualised using a mixture of fluorescein labelled monoclonal antibodies (http://www.dpd.cdc.gov/dpdx/HTML/Cryptosporidiosis.asp?body=Frames).



Polymerase chain reaction (PCR)

PCR is an alternative for microscopy and immunology, especially for the diagnosis of chronic or asymptomatic cryptosporidiosis in immunocompromised human patients (McLauchlin *et al.*, 2003). Several genes are commonly targeted (Caccio *et al*., 2005). Both in human and in animal (Webster *et al.*, 1996; Scorza *et al.*, 2003) faecal samples, PCR has a superior sensitivity compared to conventional methods with a detection limit close to 1 oocyst (Widmer, 1998). Furthermore, several PCR protocols are able to distinguish between different *Cryptosporidium* species and genotypes, which might be important from an epidemiological perspective. Despite the multiple advantages PCR has several limitations, next to the requirement of equipment and trained staff. False positive amplification results from the detection of naked nucleic acids, non-viable microorganisms or laboratory contamination. False negative results can occur through DNA denaturation or the presence of inhibitors in DNA extracted from faecal samples (Fayer *et al.*, 2000; Higgins *et al.*, 2001). Given the high

excretion of oocysts during patent infections, the use of PCR as a routine technique for the diagnosis of clinical cryptosporidiosis in calves is probably not required, although it might be useful as a diagnostic tool for tracking outbreak sources.

1.2.7 Treatment and control

Several chemotherapeutic agents have been tested for the treatment of bovine cryptosporidiosis, but none resulted in a 100% reduction of oocyst excretion.

In Belgium and other European countries, only halofuginone lactate is registered for treatment in calves, at a dose rate of 100µg/kg bodyweight (BW) per day during seven consecutive days. Toxicity can be observed at a dose rate of 500 µg/kg. For preventive treatment, halofuginone should be administered within 48h after parturition, and for curative treatment, within 24h after the onset of the clinical symptoms. Treatment with halofuginone lactate reduces the occurence of diarrhea and postpones the oocyst excretion. Overall, results were better in experimental settings than under natural conditions with a high environmental infection pressure (Villacorta *et al.*, 1991b; Naciri *et al.*, 1993; Lefay *et al.*, 2001; Joachim *et al.*, 2003; Jarvie *et al.*, 2005). Since infection is not completely eliminated, treatment with halofuginone lactate allows the development of a specific immunity (Peeters *et al.*, 1993).

Although not registered for the treatment of cryptosporidiosis, prophylaxis with paromomycin sulphate is effective in preventing oocyst excretion and clinical signs and mortality in calves and goat kids, at a dose rate of 100mg/kg BW per day during 10 or 11 consecutive days. Similar to halofuginone, efficacy was higher under experimental (Fayer and Ellis, 1993b; Mancassola *et al.*, 1995) compared to natural conditions (Chartier *et al.*, 1996; Grinberg *et al.*, 2002; Johnson *et al.*, 2000). In naturally infected lambs curative treatment with paromomycin at a similar dose rate during 2-3 consecutive days resulted in a reduction of oocyst excretion and clinical symptoms (Viu *et al.*, 2000).

Azithromycin is another antibiotic with therapeutic efficacy against *Cryptosporidium* (Elitok *et al.*, 2005), but is not registered for use in calves. Furthermore, the efficacy of several chemotherapeutics, such as sulphamidine (Moon *et al.*, 1982b; Joachim *et al.*, 2003), and coccidiostatics, such as decoquinate (Mancassola *et al.*, 1997; Lindsay *et al.*, 2000b; Moore *et al.*, 2003), have been tested, with no or limited reduction of oocyst excretion. Several new compounds have been shown to decrease severity of

diarrhea and to shorten the duration of oocyst shedding, such as the excipients α- (Castro-Hermida and Ares-Mazas, 2003) and especially β-cyclodextrin (Castro-Hermida *et al.*, 2001 a and b) and in human patients nitazoxanide (Rossignol *et al.*, 2006). Alternative treatment with lactic acid-producing bacteria (Harp *et al.*, 1996b) or with concentrated garlic preparations (Hoflack *et al.*, 2006) were shown to be not effective to prevent oocyst excretion and clinical symptoms.

Since halofuginone only partially reduces oocyst excretion under natural conditions, the control of cryptosporidiosis relies on a combination of animal treatment and appropriate hygienic measures and management. Due to the particular parasite characteristics (Table 1.3), such as the high oocyst excretion by infected calves, the environmental resistance of excreted oocysts and the presence of asymptomatic carriers, cryptosporidiosis should be considered as an endemic problem on infected farms. Hygienic measures must therefore aim to minimize the environmental infection pressure in order to prevent the spread of infection to susceptible calves and to break the transmission cycle. Frequent removal of bedding and thourough cleaning combined with disinfection help to reduce the oocyst load in the environment (Garber *et al.*, 1994; Castro-Hermida *et al.*, 2002a). Unfortunately, *Cryptosporidium* is extremely resistant to commonly used disinfectants (Carpenter *et al.*, 1999). The most effective, but also the most toxic disinfectants against cryptosporidial oocysts are ammonia, methyl bromide, ethylene oxide and ozone (Jenkins *et al.*, 1998; Fayer, 2004). Oocysts are susceptible to extreme temperatures and to dessication (Fujino *et al.*, 2002). Cleaning with hot water followed by drying is therefore an effective way to reduce oocyst infectivity (Harp *et al.*, 1996a).

Good management practices include warm and dry individual calf facilities, preferably outside (Castro-Hermida *et al.*, 2002a; Quigley *et al.*, 1994). High stocking densities are to be avoided (Atwill *et al.*, 1999b). Furthermore, a quarantaine unit should be present at the farm to isolate clinically affected calves, with separate tools, boots and coveralls to prevent spread of infection. It is also important to ensure adequate colostrum intake (Mohammed *et al.*, 1999), since colostral antibodies protect calves from developing severe clinical symptoms by blocking parasite invasion and immobilisation of gut luminal parasitic forms. Micronutrients may also play a significant role in the clinical outcome of cryptosporidiosis as severe clinical signs were observed in calves with selenium deficiency (McAllister *et al.*, 2005; Olson *et al.*, 2004).

Table 1.3: Parasite characteristics of *Cryptosporidium* and *Giardia*, epidemiological consequence and the appropriate preventive measures

Parasite characteristic	Epidemiological consequence	Preventive measure
-high excretion of (oo)cysts in the faeces	-infection pressure can increase in a short period of time	-avoid crowding -isolation of excreting calves -hygiene*
-(oo)cysts are extremely resistant to environmental conditions	-excreted oocysts can survive for several weeks or months in the environment	-hygiene*
-(oo)cysts are extremely resistant to chemical disinfection	-disinfection with common desinfectants (chlorination) is not effective enough	-products based on ammonia, chlorine dioxide, hydrogen dioxide or ozone
-(oo)cysts are only sensitive for heat and desiccation	-infection is mostly seen in the humid environment of stables	-disinfection using steam -avoid crowding -hygiene*

*hygiëne: - frequent removal of faeces
- thourough cleaning, preferably high pressure water cleaning
- if possible, followed by vacancy during several weeks

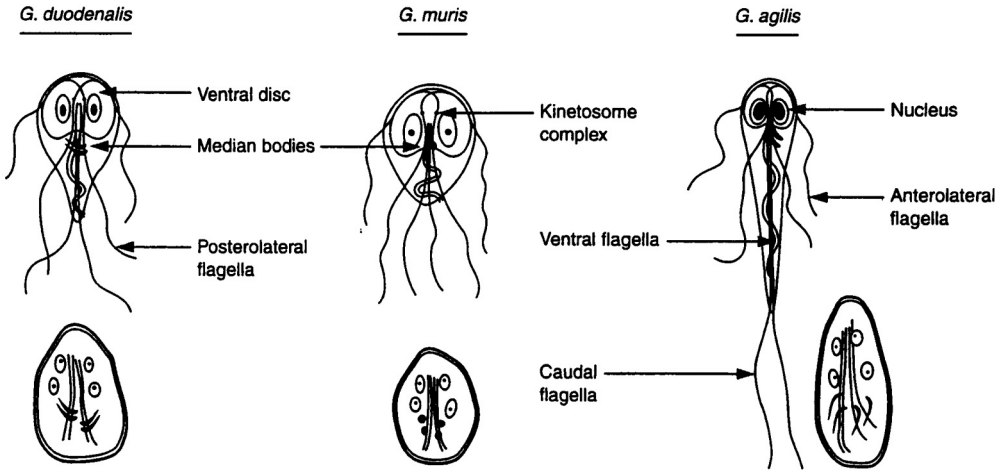
Vaccination could provide a valuable alternative to chemotherapeutic treatment. In experimental settings calves were protected against a single infection dose after active vaccination with gamma-irradiated (Jenkins *et al.*, 2004) or lyophilized oocysts (Harp and Goff, 1995). Due to the occurence of clinical symptoms and oocyst excretion within days after parturition, a single vaccination of the calves is not capable of mediating a significant immune protection prior to infection (Harp *et al.*, 1996b). Furthermore, maternal antibodies have a blocking effect on orally procured vaccinal antigens (de Graaf *et al.*, 1999a). Passive immunisation via colostral antibodies, through immunisation of the dam prior to parturition, has been shown to be effective in calves (Perryman *et al.*, 1999) and goat kids (Sagodira *et al.*, 1999). Although colostral immunisation provides the most promising alternative for chemotherapeutic treatment, the succesful use of vaccines should be supported by hygienic and management measures to minimize the exposure of calves to cryptosporidial oocysts in the initial days of life and to allow the protective immune responses to be generated.

1.3 *Giardia*

1.3.1 Classification

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 (Figure 1.4). The ventral adhesive disc delineates *Giardia* from other Hexamitidae. The genus name *Giardia* was proposed in honour of the Belgian taxonomist Alfred Mathieu Giard (Kunstler, 1882). To commemorate the man who gave the first detailed parasite decription, *Lamblia* (Blanchard, 1888) was also proposed as generic name. Lambl himself had named the parasite *Cercomonas intestinalis* in 1859. In human medical literature the names *Lamblia intestinalis* and *Giardia lamblia* have both consistently been used to designate *Giardia* isolates from human patients. Furthermore, the species names *intestinalis* and *duodenalis* were both frequently used (Thompson *et al.*, 2000). At present *G. duodenalis* is considered the only valid species name for the protozoan parasite as described by Filice (1952).

Figure 1.4: Trophozoite and cyst morphology of *Giardia duodenalis*, *G. muris* and *G. agilis* (redrawn from Thompson *et al.*, 2004)



Over 50 different *Giardia* species have been described, principally based on host specificity. This host-specific taxonomy was later replaced by a taxonomy based on morphological characteristics such as trophozoite shape and lenght and shape of the median bodies (Filice, 1952). Three morphological distinct groups or *Giardia* species were described (Figure 1.4), of which *G. muris* and *G. duodenalis* are able to infect

mammals. The third species, *G. agilis*, is isolated from amphibians. Since the introduction of the new taxonomy, morphological characteristics have been used to describe 3 additional *Giardia* species (Table 1.4): *G. psittaci* and *G. ardeae* originate from birds and have been described based on morphological differences in the trophozoites observed by scanning electron microscopy (Erlandsen and Bemrick, 1987; Erlandsen *et al.*, 1990); *G. microti* has been differentiated based on cyst morphology and is found in a variety of rodents (Erlandsen *et al.*, 1988).

Although morphological characteristics still provide the basis of the current taxonomy, it was only a temporary solution in expectation of more discriminatory criteria. Phenotypic studies (Farbey *et al.*, 1995; Homan and Mank, 2001; Read *et al.*, 2002), along with epidemiological observations (Cribb and Spracklin, 1986), cross-transmission experiments (Kirkpatrick and Green, 1985; Erlandsen *et al.*, 1988; Majewska, 1994; Monis *et al.*, 1999; McDonnell *et al.*, 2003; Monis and Thompson, 2003), electrophoretic studies (Meloni *et al.*, 1995; Monis *et al.*, 2003) and genetic characterisation, have since revealed that *G. duodenalis* should be considered a species complex. At present 7 major assemblages (A to G) have been identified within *G. duodenalis*, some of which have distinct host preferences or a limited host range (Ey *et al.*, 1997; Mayrhofer *et al.*, 1995; Monis *et al.*, 1998, 1999). The first assemblages to be described were assemblage A and B (Monis *et al.*, 1996), named the Polish and Belgian group in Europe (Homan *et al.*, 1992) and groups 1-2 and 3 in Northern America (Nash, 1997), respectively. Next to the assemblages A and B which are both prevalent in human patients, several host-specific assemblages have been identified in animals. Assemblages C and D primarily infect dogs, the hoofed livestock assemblage or assemblage E is identified in artiodactyl species, assemblage F is primarily identified in cats and assemblage G in rats. 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, resulting in 5 new species names (Thompson and Monis, 2004). Including the six species previously recognised in the morphological taxonomy, a total of 11 *Giardia* species have currently been described (Table 1.4). Since these new species names have only recently been introduced and are not yet commonly used, the

'assemblage nomenclature' is preferred throughout the manuscript to designate the different species within the *G. duodenalis* morphological group.

Table 1.4: *Giardia* species and *G. duodenalis* assemblages, with the proposed new species name, and host range

***Giardia* species**	**Host range**	
G. agilis	Amphibians	
G. ardeae	Birds	
G. psitacci	Budgerigar	
G. microti	Rodents	
G. muris	Rodents	
G. duodenalis		**New species name**
assemblage A	Man, dog, cat, livestock,...	*G. duodenalis*
assemblage B	Man, dog, cat, rodents,...	*G. enterica*
assemblage C / D	Dog, coyote, mouse	*G. canis*
assemblage E	Livestock	*G. bovis*
assemblage F	Cat	*G. cati*
assemblage G	Rat	*G. simondi*

1.3.2 Life cycle

Giardia has a simple and direct life cycle consisting of two stages: an infectious cyst 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, cysts are exposed to the acidic environment of the proximal gastro-intestinal tract resulting in the release of the excyzoite in the upper part of the small intestine. The short-living excyzoite has eight flagellae and a metabolism intermediate between a trophozoite and a cyst. The excyzoite has 4 nuclei with 4N nuclear ploidy, following a nuclear DNA replication in the trophozoite and another one in the cyst stage. After release from the differentiated cyst, the excyzoite undergoes cel division, without further DNA replication, generating four disease-causing trophozoites with diploid nuclear ploidy. The cellular amplification might explain the low dose (10 to 100 cysts) needed for infection (Bernander *et al.*, 2001). The trophozoites have a convex dorsal surface and a ventral adhesive disk which is used to attach to the intestinal mucosa (figure 1.4).

For the colonisation of the duodenum and the jejunum, attachment to epithelial cells is essential. Hydrodynamic forces generated beneath the ventral adhesive disc by continuous activity of the ventral flagella, contractile cytoskeletal proteins and lectins have been suggested as possible attachment mechanisms (Inge *et al.*, 1988; Farthing, 1997; Sousa *et al.*, 2001). Unlike *Cryptosporidium*, the trophozoites of *Giardia* are not invasive. They multiply 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 of trophozoites in the jejunum. Cysts are passed in the faeces and are immediately infectious upon excretion allowing completion of the life cycle within 72h (Thompson *et al.*, 1993; Adam, 2001;).

1.3.3 Epidemiology and prevalence

Epidemiology

Calves are infected by oral intake of infectious cysts. As soon as 3 days after infection (Xiao and Herd, 1994) cysts can be recovered from the faeces. The maximum cyst excretion (up to 10^6 cysts per gram of faeces) is observed at the age of 2 to 3 weeks. This coincides with the depletion of colostral immunity within 2 weeks after birth (O'Handley *et al.*, 2003). From the age of 3 to 4 weeks cyst excretion starts to decrease and becomes intermittent. Around 2 to 3 months of age the number of excreted cysts further decreases and in older calves and adult animals cyst excretion is minimal (Xiao *et al.*, 1994; Nydam *et al.*, 2001). Young calves can therefore be considered as the major source of infection for susceptible calves. Dams and older calves can however not be excluded, since a limited number of cysts can be sufficient for infection (Bernander *et al.*, 2001). As in sheep, horses and pigs, a periparturient rise of the cyst excretion has been suggested in cattle, but has not been confirmed (Xiao and Herd, 1994; Wade *et al.*, 2000a).

Similar to *Cryptosporidium*, several parasite characteristics facilitate infection with *Giardia*, such as the high excretion of cysts by infected calves and the low dose needed for infection. Furthermore, *Giardia* cysts are immediately infectious upon excretion and do not need to sporulate in the environment. Cysts are also very resistant and are able to survive for several months in the environment, resulting in a gradual increase in environmental infection pressure (Xiao *et al.*, 1993; Olson *et al.*, 1999; Wade *et al.*, 2000a). Infection can occur either by direct contact with an

infected animal or indirectly through the environment, such as maternity pens or calf housing. Although the maternity pen should be considered, it is probably a minor source of infection, given the colostral immunity and the low number of cysts excreted by the dam. Furthermore, most dairy and to a lesser extent beef calves are removed from the dam shortly after parturition and housed in calf facilities, which are probably the major source of indirect infection. Calves kept indoors are three times more likely to acquire infection than calves reared outside (Ruest *et al.*, 1998). Similar to *Cryptosporidium*, particularly group housing, but also individual housing is considered to favour transmission. Infection can occur via vectors or through the subsequent use of calf pens, due to the environmental resistance of the infectious cyst.

Prevalence

Worldwide cross-sectional studies have reported a *Giardia* prevalence varying from 20% to 73% in calves younger than six months. In calves older than 6 months the prevalence seems somewhat lower. Substantial differences in prevalence partially account for this variability, next to differences in study design, such as the number of animals or farms included in the studies. Since there is no gold standard reference test for the diagnosis of *Giardia* in calves, the sensitivity and specificity of each test is not known. Therefore the use of different diagnostic techniques might thwart comparison between prevalence studies. Furthermore, the age of the animals needs to be considered, since the highest prevalence is reported in calves up to 3 months of age (Ralston *et al.*, 2003b; Becher *et al.*, 2004). In contrast to *Cryptosporidium* (Ralston *et al.*, 2003b; Gow and Waldner, 2006; Kvac *et al.*, 2006), *Giardia* seems to be equally prevalent in dairy and beef calves. The number of farms with *Giardia* positive calves is between 45% and 100% (Table 1.5). On these positive farms, the cumulative incidence is assumed to be 100% (Xiao, 1994; O'Handley *et al.*, 1999), indicating that every calf on that farm will get infected. In cattle the livestock specific assemblage E is most prevalent, although up to 20% zoonotic assemblage A isolates have been reported, either in pre-weaned calves (O'Handley *et al.*, 2000b; Appelbee *et al.*, 2003; Becher *et al.*, 2004; Berrilli *et al.*, 2004; Trout *et al.*, 2004; Itagaki *et al.*, 2005), post-weaned calves (Trout *et al.*, 2005), or adult cattle (Trout *et al.*, 2006; Uehlinger *et al.*, 2006a).

Table 1.5: The calf prevalence (P_C) and farm prevalence (P_F) of *Giardia* in different countries. The type of farm (Farm: b=beef and d=dairy), the number of calves ($\#_C$) and farms ($\#_F$) is presented along with the diagnostic assay (D) used in the study: Immunofluorescence assay (IFA), polymerase chain reaction (PCR) or microscopy (M).

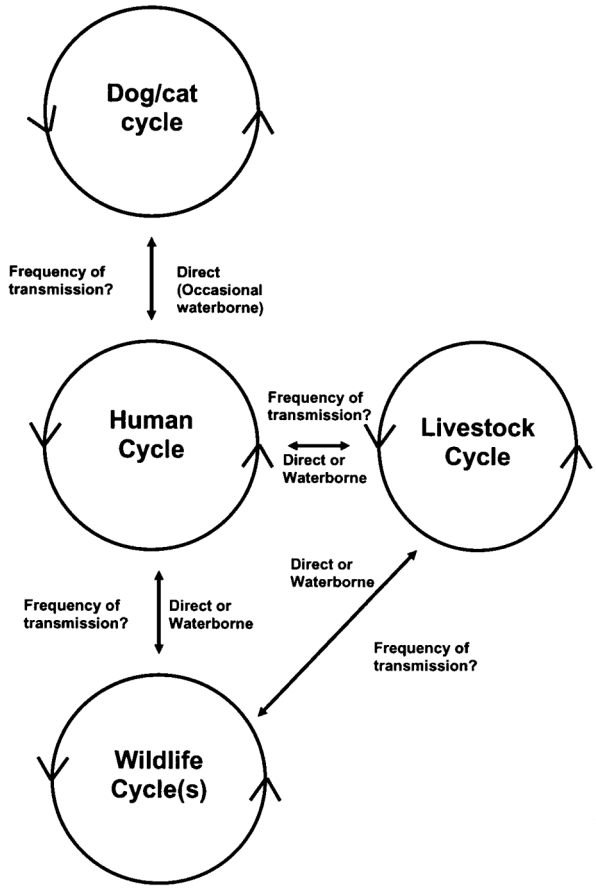
Country	D	Farm	$\#_C$	$\#_F$	age	P_C	P_F	Reference
Calves < 6m								
USA	IFA	d	407	14	<2m	40	100	(Trout *et al.*, 2004)
New Zealand	IFA	d	715	12	<2m	40	100	(Hunt *et al.*, 2000)
Canada	IFA	b	495	9	<3m	34	100	(Appelbee *et al.*, 2003)
Canada	IFA	b	605	100	<6m	23	48	(Gow and Waldner, 2006)
Canada	IFA	d	386	20	<6m	73	100	(Olson *et al.*, 1997a)
Canada	M	d	-	505	<6m	-	45	(Ruest *et al.*, 1998)
Norway	IFA	d	1,386	136	<6m	49	93	(Hamnes *et al.*, 2006b)
USA	M	d	2,943	109	<6m	20	70	(Wade *et al.*, 2000b)
Calves > 6m								
USA	IFA	d	464	14	3-11m	31	100	(Trout *et al.*, 2005)
Denmark	IFA	d	895	50	<12m	50	100	(Maddox-Hyttel *et al.*, 2006)
USA	PCR	d	571	514	12-24m	36	100	(Trout *et al.*, 2006)
Spain	M	d	199	30	<24m	26	53	(Quilez *et al.*, 1996b)
Canada	IFA	b	605	100	>24m	17	69	(Gow and Waldner, 2006)

1.3.4 Zoonotic aspects

Zoonotic transmission either occurs by direct faeco-oral transmission or indirectly, by the ingestion of contaminated water or food. The high prevalence of *Giardia* in a wide range of domestic and wild animals suggests a possible reservoir function for human infection, although little is known about the frequency of zoonotic transmission (Heitman *et al.*, 2002; Hoque *et al.*, 2002; Jakubowski and Graun, 2002; Caccio *et al.*, 2003). In 1979 the WHO recognised the zoonotic potential of *Giardia*. Cross-transmission experiments (Kirkpatrick and Green, 1985; Erlandsen *et al.*, 1988; Majewska, 1994; Monis *et al.*, 1999; McDonnell *et al.*, 2003; Monis and Thompson, 2003) and immunoblotting of sera from both human and animal carriers (Buret *et al.*, 1990a) later confirmed the zoonotic transmission. Several of these cross-transmission experiments however displayed contradictory results, which could only partially be attributed to differences in experimental design (Monis and Thompson, 2003), suggesting that not all *Giardia* isolates have zoonotic potential. The development of

axenic culturing has certainly contributed to the understanding that not all *Giardia* cysts from animal sources contribute to human infections. Since not all isolates of *G. duodenalis* amplify equally in laboratory axenic culture (Andrews *et al.*, 1992), including a large proportion of human isolates, early genetic data are biased by the rather small pool of axenic culture-selected isolates (Monis *et al.*, 2003) and can hardly be considered as representative of the extensive gene pool existing in nature (Thompson, 2004). The introduction of polymerase chain reaction (PCR) has circumvented the need for in-vitro culture, with direct characterisation of parasite DNA from faecal and environmental samples. Based on the notion of genetic difference within *G. duodenalis* and based on geographically dispersed prevalence estimates in different animal species and in humans, several major cycles of transmission have been described (Figure 1.5).

Figure 1.5: Major transmission cycles of *Giardia duodenalis* (redrawn from Thompson *et al.*, 2004)



The human cycle

In industrialised countries *Giardia* is detected in up to 14% of symptomatic patients (Homan and Manck, 2001). The greatest zoonotic risk results from infection with assemblage A isolates and to a lesser extent isolates from assemblage B. Epidemiological evidence however suggests that man should be considered as the main reservoir for human infections (Caccio *et al.*, 2003; Sulaiman *et al.*, 2004). Transmission among human hosts occurs either indirectly through the ingestion of cysts in contaminated water or food, or directly in environments were hygiene levels may be compromised, such as day care centres. In particular nappy handling, traveling and swimming seem to be important risk factors for infection (Hoque *et al.*, 2002, 2003). Although both assemblage A and B are commonly identified in human stool samples, assemblage B seems most prevalent in human infections (Amar *et al.*, 2002; Read *et al.*, 2002; Traub *et al.*, 2004; van der Giessen *et al.*, 2006).

The wildlife cycle

Both terrestrial and marine wildlife have been considered as a possible source for waterborne infections. In Northern America the beaver has been implicated most often in water contamination, to the extent that giardiosis is commonly refered to as 'beaver fever'. Next to beavers, *Giardia* has been reported in muskrats, moose and deer (Rickard *et al.*, 1999; Dunlap and Thies, 2002; Heitman *et al.*, 2002; Hamnes *et al.*, 2006b;). In Uganda, *Giardia* has been reported in gorillas (Graczyk *et al.*, 2002). Similarly, high prevalences of *Giardia* were reported in marine wildlife, such as oysters, mussels (Graczyk and Schwab, 2000), seals and whales (Olson *et al.*, 1997b; Measures and Olson, 1999; Appelbee *et al.*, 2005). Although the zoonotic assemblages A and B of *G. duodenalis* were identified in wildlife (Appelbee *et al.*, 2002, 2003; Trout *et al.*, 2003; Santin *et al.*, 2005;), it is more likely that wildlife is itself infected by cysts originating from human faecal material instead of being the source of human infections. The genetic characterisation of isolates from marsupials in Australia indicated that they are probably not an important source of infection, despite the high prevalence reported in these animals (Thompson, 2004). The importance of wildlife as a source of infection for human infections in more densily populated areas such as North-West Europe, has yet to be determined.

The companion animal cycle

Giardia is considered as one of the most common enteric parasites in dogs and cats. A number of studies indicate that *Giardia* is highly prevalent in cats worldwide (Swan and Thompson, 1986; Collins *et al.*, 1987; Hill *et al.*, 2000; Spain *et al.*, 2001; Barutzki and Schaper, 2003; McGlade *et al.*, 2003). Although the zoonotic assemblages A and B have been reported (van Keulen *et al.*, 2002; Berrilli *et al.*, 2004), most cat isolates are identified as either assemblage D (McGlade *et al.*, 2003) or the cat specific assemblage F (McGlade *et al.*, 2003; Itagaki *et al.*, 2005; Lalle *et al.*, 2005b; Fayer *et al.*, 2006a). Therefore cats should be considered as a possible although not important reservoir for zoonotic transmission. The prevalence of *Giardia* in dogs has been reported with considerable variation, mainly depending on the population under study. Overall prevalence seems to be moderate in well-cared household dogs (Jacobs *et al.*, 2001) and higher in puppies (Hahn *et al.*, 1988) and breeding kennels (Swan and Thompson, 1986; Bugg *et al.*, 1999; Barutzki and Schaper, 2003; Capelli *et al.*, 2003; Papini *et al.*, 2005). Molecular characterisation of dog isolates identified both host-adapted and zoonotic assemblages. High contact frequency between dogs leads to competitive interactions and a domination of the dog specific assemblages, such as in the Aboriginal communities in Australia where dogs tend to stay in packs, favouring dog to dog transfer and resulting in infections with predominantly dog specific assemblages C and D, despite a close contact with human sources of infection, such as human faeces with predominantly assemblage A isolates (Hopkins *et al.*, 1997; Monis *et al.*, 1998). In remote tea growing communities in northeast India dogs are however not allowed to roam free. Hence, dogs are more likely to be exposed to human rather than canine sources of infection and predominantly assemblage A infections were identified in these dogs (Traub *et al.*, 2002, 2004). The Aboriginal and Indian community settings can be considered as high infection pressure environments, facilitating the transmission from one host to another. In domestic households in Western urban settings, dogs seem to be just as likely to harbour the zoonotic assemblage A as the dog specific assemblage D (van Keulen *et al.*, 2002; Abe *et al.*, 2003; Berrilli *et al.*, 2004; Itagaki *et al.*, 2005; Lalle *et al.*, 2005a). Dogs should therefore be considered as an important reservoir of infection.

The livestock cycle

High prevalences of *Giardia* have been reported in farm animals, such as cattle, sheep and goats (Xiao, 1994; Ryan *et al.*, 2005; Bomfim *et al.*, 2005). In horses the prevalence is low (Atwill *et al.*, 2000) and the relevance to human epidemiology is probably minor, although the zoonotic assemblage A has been identified in equine samples (Traub *et al.*, 2005). In sheep the livestock specific assemblage E was mainly identified (Ryan *et al.*, 2005), except in Europe where assemblage A and B infections were reported (Giangaspero *et al.*, 2005; Aloisio *et al.*, 2006). In cattle the livestock specific assemblage E is most prevalent, although up to 20% zoonotic assemblage A isolates have been reported (O'Handley *et al.*, 2000b; Appelbee *et al.*, 2003; Becher *et al.*, 2004; Berrilli *et al.*, 2004; Trout *et al.*, 2004; Itagaki *et al.*, 2005). Occassionally, assemblage B is identified in calves (van Keulen *et al.*, 2002; Lalle *et al.*, 2005b;). However, even a low prevalence of assemblage A or B isolates could pose a significant public health risk, since infected animals tend to excrete a large number of cysts.

Due to the limited prevalence and the probable transient nature of zoonotic assemblage infections in cattle and small ruminants (Thompson, 2004), these studies might suggest that the public health risk from domestic ruminants is minimal, at least in rural regions of Northern America and Australia. Anthropozoonotic transmission seems to occur more frequently when contact between human and livestock sources of infection is more intensive (Graczyk *et al.*, 2002; Uehlinger *et al.*, 2006a). Furthermore, a significant association was found between human giardiosis and both livestock density and manure use on agricultural land (Odoi *et al.*, 2004). Transmission from cattle to humans may therefore be more important in North-Western European regions, with more intensified cattle industry and closer contact between agriculture and human populations, compared to rural areas in Northern America and Australia. Studies from Europe seem indeed to indicate that the zoonotic assemblage A might be equally or more prevalent than assemblage E in cattle, although data are limited (van der Giessen *et al.*, 2002; Lalle *et al.*, 2005b). Furthermore, PCR favours the propagation of the most abundant gene present in a sample. Hence, the occurence of mixed assemblage A/E infections might be underestimated in calves.

1.3.5 Pathogenesis and clinical symptoms

Pathogenesis

Although the clinical significance of *Giardia* is beyond dispute in man and animals (StJean, 1987; Farthing *et al*., 1997), the pathogenesis of giardiosis is not clearly understood. The pathogenesis has been studied in human epithelial cell lines (Buret *et al.*, 1990b), in laboratory animals (Buret *et al.*, 1990c; Buret *et al.*, 2002; Scott *et al.*, 2002), in goat kids (Koudela and Vitovec, 1998) and in calves (Taminelli *et al.*, 1989; Ruest *et al.*, 1997). Giardiosis essentially leads to microvillus alterations. These are not only a direct consequence of the interaction between trophozoites and epithelium, but are also mediated by host factors, since B-and T-cell deficient mice do not exhibit the changes in microvillus height or digestive enzymes in response to a *Giardia* infection (Scott *et al.*, 2000). The pathogenesis of giardiosis can therefore be considered as a multifactorial process, involving both parasitic and host immune factors.

The most significant alteration is an increase in epithelial permeability which appears to result from enterocyte apoptosis (Cevallos *et al.*, 1995; Chin *et al.*, 2002) and from cytoskeletal reorganisation induced by trophozoite toxic products (Buret *et al.*, 2002; Scott *et al.*, 2002). The cytoskeletal alterations lead to local disruption of tight-junctional proteins and to contraction of the circumferential filament bands. Whether *Giardia* secretory-excretory products induce direct proteolytic degradation of tight-junctional proteins is uncertain. The increased epithelial permeability leads to a higher number of intraepithelial lymphocytes (IEL) and to activation of T-lymphocytes. Both CD4+ and CD8+ T-cells are activated, resulting in villus atrophy and crypt hyperplasia (Buret *et al.*, 1992; Ruest *et al.*, 1997; Scott *et al.*, 2002, 2004). In human patients malabsorption has been associated with an increased number of IEL and a decreased villus to crypt ratio (Farthing, 1997; Ruest *et al.*, 1997). Trophozoite toxins and T-cell activation also initiate a diffuse shortening of brush border microvilli and a decreased activity of the small intestinal brush border enzymes, especially lipase, some proteases and the dissacharidases lactase, maltase and sucrase (Buret *et al.*, 1990b; Scott *et al.*, 2000).

Clinical symptoms

Since the pathogenesis of giardiosis is a combination of parasite and host factors, the subsequent clinical symptoms may vary considerably from host to host. The diffuse microvillus shortening leads to a decrease in overall absorptive area in the small intestine and an impaired intake of water and nutritients (Buret *et al.*, 1992; Cevallos *et al.*, 1995). The combined effect of the decreased resorption and the brush border enzyme deficiencies results in malabsorptive diarrhea and lower weight gain, both in murine models (Buret *et al.*, 1990b) and in ruminant experimental models (Ruest *et al.*, 1997; Koudela and Vitovec, 1998). The reduced activity of lipase and the increased production of mucine by goblet cells may explain the steatorrhea and muceous diarrhea which has been described in *Giardia* infected hosts (Zajac, 1992; Moncada *et al.*, 2003). The primary pathogenic effect of natural *Giardia* infections in ruminants was prone to debate (Quilez *et al.*, 1996b; Ruest *et al.*, 1998; Hunt *et al.*, 2000; Huetink *et al.*, 2001), but is at present well recognised (Aloisio *et al.*, 2006; O'Handley *et al.*, 1999). Surprisingly, a mixed infection with *Cryptosporidium* was found to cause less morphological damage in the small intestine of calves than a single *Giardia* infection, which could indicate that these parasites have an antagonistic effect on each other rather than a synergestic effect (Ruest *et al.*, 1997).

Human patients infected with assemblage A are more likely to develop clinical symptoms that infections with assemblage B (Read *et al.*, 2002), although infections with assemblage B seem to result in more persistent diarrhea (Homan and Mank, 2001). Whether a similar difference in clinical outcome occurs between the host specific assemblage E and the zoonotic assemblage A in calves, is not known.

1.3.6 Diagnosis

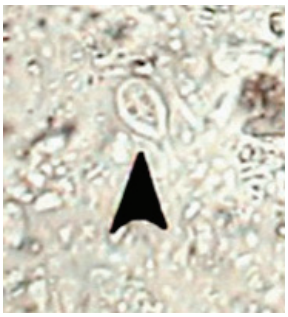
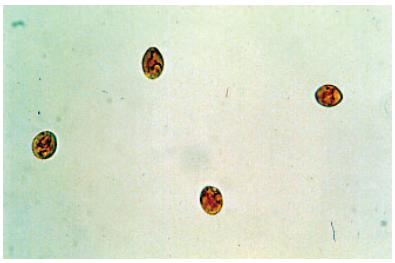
Due to the vague symptoms, the clinical diagnosis of giardiosis in calves is difficult. The most consistent is the excretion of pasty to fluid faeces, often with mucus. Sometimes steatorrhoea is observed (Moncada *et al.*, 2003). The diarrhea does not respond well to antibiotic treatment. Although acute diarrhea can occur, more often a chronic and intermittent diarrhea is observed. Next to diarrhea, an impaired weight gain despite good appetite seems to be a typical clinical symptom of giardiosis in calves (St. Jean, 1987). The diagnosis based on clinical symptoms needs to be confirmed by the detection of the parasite in a faecal sample, either by microscopical

examination, antigen detection or polymerase chain reaction (PCR). Given the intermittent excretion of cysts, especially in the chronic phase of infection, multiple samplings can be necessary, either from the same animal for 3 consecutive days (O'Handley *et al.*, 1999) or from several calves within the same housing. Since the peak excretion is observed in animals around 2-4 weeks of age, preferentially young animals are sampled for diagnosis.

Microscopical examination

Both the trophozoites and the cysts of *Giardia* can be detected by microscopy, either directly or after concentration with sucrose, zinc sulphate or formalin. Steathorrhea, which is often observed in giardiosis, can interfere with sucrose flotation (Xiao and Herd, 1993). Trophozoites can sometimes be detected in faecal samples from calves with diarrhea due to the increased peristalsis. Trophozoites are bilaterally symmetrical, piriform to ellipsoidal in shape with a lenght of 12-15µm and a width of 6-8µm (Figure 1.4 and 1.6). Median bodies, four pairs of flagellae and a ventral disk can be seen (Thompson *et al.*, 1993). Given the characteristic movement of the trophozoites, they are preferably visualized in a native smear using recently obtained faeces.

Figure 1.6: *Giardia* trophozoite in a native smear (a) and *Giardia* cyst after iodine staining (b) using microscopy at 400x magnification

(a)  (b) 

More frequently the detection of cysts in the faeces is prefered for diagnosis. Prior to examination cysts can be stained. Frequently used stains are iodine (Figure 1.6) (Zajac, 1992) and trichrome (Addiss *et al.*, 1991). The major advantage of microscopical examination is the limited cost of consumables. The major

disadvantage is the need for a skilled and experienced microscopist and the alledgedly lower sensitivity compared to immunological assays.

Immunological diagnosis

For the detection of parasite antigen immunofluoresence assays (IFA) (Garcia *et al.*, 1992; Xiao and Herd, 1993; Zimmerman and Needham, 1995), enzyme-linked immunosorbent assays (ELISA) (Zimmerman and Needham, 1995; Mank *et al.*, 1997; Boone *et al.*, 1999) and rapid solid-phase qualitative immunochromatography assays (Garcia *et al.*, 2003) are commercially available. All tests were developed and evaluated for use in human stool samples. IFA (Figure 1.3) and ELISA use monoclonal antibodies against cyst wall proteins. IFA can be used as a quantitative test. In calves the detection limit is estimated to be around 1000 cysts per gram of faeces (CPG) (Xiao and Herd, 1993).

Partially due to the requirement of laboratory settings and trained personnel, the main disadvantage of IFA and ELISA is the high cost compared to microscopical examination, and the time lost by transport to and analysis in the laboratory. This could be circumvented by the use of immunochromatography enabling on-site diagnosis within 15 minutes. Immunochromatography uses monoclonal antibodies directed against specific trophozoite or cyst wall proteins. In human medicine several assays are commercialised, including dip-sticks and rapid membrane assays. In veterinary medicine an immunochromathographic assay is commercialised for use in cats and dogs (Idexx®Snap *Giardia* test, Idexx Laboratories, Schiphol-Rijk, The Netherlands).

Polymerase Chain Reaction (PCR)

PCR has primarily been used for the identification of different species and genotypes of *Giardia* for taxonomical research, although there is potential for diagnostic use. Several genes have been commonly used for genotyping (Caccio *et al.*, 2005). The detection limit of PCR is theoretically 1 cyst (Amar *et al.*, 2002), which improves considerably the diagnostic sensitivity (McGlade *et al.*, 2003). However, several factors can interfere with PCR such as inhibition, which is known to occur frequently in DNA extracted from faecal samples. Furthermore, the extraction of parasite DNA from faeces needs to be standardised for diagnostic use. At present PCR is considered as too expensive for use in veterinary diagnostics (da Silva *et al.*, 1999). In human

medicine and in public health services however, PCR is considered as a promising diagnostic tool.

1.3.7 Treatment and control

Nitroimidazoles (NZs), quinacrine or furazolidine are frequently used to treat giardiosis in human patients. Although therapy with these compounds is effective, considerable side-effects have been demonstrated. Metronidazole is even considered to be carcinogenic (Morgan *et al.*, 1993; Harris *et al.*, 2001). Furthermore, resistance to treatment has been described both for metronidazole and furazolidine (Upcroft *et al.*, 1990). In veterinary medicine metronidazole (St. Jean, 1987; Xiao *et al.*, 1993) and dimetridazole (St. Jean, 1987) have been used in companion animals and in calves, achieving symptomatic improvement. Data on reduction of cyst excretion are however not available. Furthermore, the NZs are no longer approved for use in livestock. Recently, nitazoxanide has been shown *in vitro* to be a promising new drug against *Giardia* (Cedillo-Rivera *et al.*, 2002), but no data on *in vivo* activity in calves are available.

Since most treatments against *Giardia* do not have a persistent efficacy, vaccination could provide a valid and long term alternative, especially for the prevention of disease. In the United States a *Giardia* vaccine is now commercially available for use in cats and dogs (Fel-O-Vax *Giardia* or *Giardia* Vax, Fort Dodge Animal Health), but the efficacy of a preventive (Olson *et al.*, 1996) or curative (Olson *et al.*, 2001; Payne *et al.*, 2002; Stein *et al.*, 2003; Anderson *et al.*, 2004) vaccination seems to be variable. In calves vaccination against *Giardia* did not result in a protective immune respons (Uehlinger *et al.*, 2006b).

Benzimidazoles

An alternative for treatment of giardiosis in cattle are the benzimidazole compounds (BZs) which have been well known anthelmintics over the last 30 years with a broad spectrum against gastrointestinal nematodes. Because BZs are not absorbed from the host intestine, they are believed to have a high safety margin and a selective toxicity (Muser and Paul, 1984; Xiao *et al.*, 1996). *In vitro* studies indicated that BZs are more efficacious against *Giardia* than either metronidazole or tinidazole (Edlind *et al.*, 1990; Meloni *et al.*, 1990; Morgan *et al.*, 1993) and that this anti-*Giardia* effect is

irreversible (Morgan *et al.*, 1993). The BZs interfere with the polymerisation of tubulin and are believed to induce mutations in the tubulin encoding gene. Tubulin is a major component of the *Giardia* trophozoite cytoskeletal structures. Therefore all functional activities of tubulin dependent structures such as the median body and the ventral disk, are inhibited. BZ seem not to affect flagellar tubulin, which has a different tubulin subunit structure (Clark and Holberton, 1988). As a result BZs interfere with trophozoite attachment to the intestinal mucosa and prevent intestinal colonisation. The BZ mode of action might also include binding to giardins, which are *Giardia* specific proteins restricted to the ventral disk (Edlind et al, 1990; Meloni *et al.*, 1990).

There have been several studies to evaluate the *in vivo* efficacy of oxfendazole (Villeneuve *et al.*, 2000), albendazole (Barr *et al.*, 1993) and fenbendazole (Barr *et al.*, 1994) in dogs. In calves data on reduction in cyst excretion are only available for treatment with fenbendazole (Xiao *et al.*, 1996; O'Handley *et al.*, 1997) and albendazole (Xiao *et al.*, 1996). Both have been shown to significantly reduce the peak and the duration of cyst excretion and to result in a clinical benefit (O'Handley *et al.*, 2000a), although the dosage of both BZs needed for *Giardia* treatment (5 to 20 mg per kg bodyweight per day during three consecutive days) is higher compared to helminth treatment. Furthermore, the cyst suppressing effect of BZ treatment was either not complete or short-lasting in field conditions, despite the high *in vitro* efficacy of both drugs. This might be either due to a high environmental infection pressure which counters the effect of treatment or to the lack of persistent efficacy of BZs against *Giardia* in calves, resulting in a rapid reinfection shortly after the end of the treatment.

To prevent re-infection from the environment and to improve long term production parameters, it has been hypothezised that calves should be treated with a continuous low dosage of BZs (O'Handley *et al.*, 2000a), since the treatment duration seems to be more important than treatment dosage (O'Handley *et al.*, 1997). However, there are no data on the safety of a long term treatment with BZs and there might be a potential for developing BZs resistant *Giardia* field strains. It seems therefore more advisable to combine animal treatment with environmental measures to reduce infection pressure. The environmental measures are similar to those used for the treatment of cryptosporidiosis (Table 1.3).

Paromomycin

Paromomycin or aminosidin is a broad-spectrum amino-glycoside antibiotic, with well-known efficacy against several protozoan parasites like *Cryptosporidium* in calves (Fayer and Ellis, 1993a; Mancassola *et al.*, 1995; Chartier *et al.*, 1996; Viu *et al.*, 2000; Grinberg *et al.*, 2002), *Histomonas meleagridis* in chickens (Hu and McDougald, 2004) and *Giardia* in rats (Awadalla *et al.*, 1995) and humans (Wright *et al.*, 2003). Paromomycin binds to the small subunit rRNA and inhibits protein synthesis, which has either a direct effect on *Giardia* or an indirect effect of nutrient withdrawal caused by the inhibition of bacterial protein synthesis and destruction of the bacterial flora (Edlind, 1989; Harder *et al.*, 2001; Harris *et al.*, 2001). Paromomycin is poorly absorbed from the gastrointestinal tract and is therefore well tolerated by calves (Grinberg *et al.*, 2002). No data are available on the efficacy of paromoycin against *Giardia* in calves.

1.4 Conclusions

Many studies indicate that *Cryptosporidium* and *Giardia* occur in cattle worldwide, although the prevalence reports vary markedly. Besides substantial differences between farms, regions or countries, the variation in prevalence can also be ascribed to differences in study design (cross-sectional versus longitudinal), in number of farms or animals, or to differences in age and breed of the animals under study. Another reason for variation is the technique used for parasite diagnosis. Both for *Cryptosporidium* and *Giardia* there is no gold standard reference technique. Moreover, the most commonly used diagnostic techniques have not been evaluated for use in cattle. Both in human and veterinary medicine most often a frequentist approach using contingency tables have been applied for the evaluation of diagnostic assays. Contingency tables might however result in an over-or underestimation of the test characteristics, depending on the choice of gold standard reference test. Although able to thwart the estimation of parasite prevalence and the comprehension of the epidemiology, these considerations are rarely taken into acount.

Cattle have always been considered as a potential zoonotic reservoir for human *Cryptosporidium* and *Giardia* infections. More recently, molecular epidemiological studies and subgenotype analysis have provided a more detailed insight in the zoonotic potential of ruminant *Cryptosporidium* and *Giardia* isolates, suggesting a more limited contribution of cattle to human infections. However, most studies were conducted in Northern America and in Australia, while a number of reports in Europe seem to contradict the limited reservoir function of cattle, especially for *Giardia*. Due to the higher population density and a more intensified contact between the human population and agricultural activity in North Western Europe, frequency of transmission might be higher compared to rural areas in Northern America and Australia, resulting in a higher frequency of zoonotic species and genotypes both in human patients and in cattle. Furthermore, in molecular epidemiological studies amplification of parasite DNA is performed by standard PCR which favours the detection of the most abundant species or genotype in a sample, underestimating the possible occurence of mixed infections with both non-zoonotic and zoonotic genotypes in cattle.

In Belgium, halofuginone is registered for the preventive and curative treatment of *Cryptosporidium* in calves, and was shown to be efficacious both in experimental and in natural conditions. For the treatment of *Giardia* in calves, there is however no drug registration, although several benzimidazole compounds were shown to be efficacious in experimental and to a lesser extent in natural conditions. Moreover, the efficacy of animal treatment in combination with hygienic measures in natural conditions has never been evaluated in cattle. A promising alternative for benzimidazole treatment seems to be paromomycin, which has however never been evaluated for the treatment of bovine giardiosis.

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Objectives of the study

The main objective of the thesis is to obtain better insight in the molecular epidemiology of *Cryptosporidium* and *Giardia* in calves in Belgium, and to focus on the treatment and control of *Giardia* in calves. The three specific sub-objectives are:

1- To estimate the prevalence of *Cryptosporidium* and *Giardia* in dairy and beef calves in the province of East Flanders (Belgium) based on a reliable estimate of the test characteristics of the applied diagnostic assays, using a Bayesian approach (chapter 2)

2- To evaluate the zoonotic potential of bovine *Cryptosporidium* and *Giardia* isolates by molecular identification, with emphasis on subgenotype analysis for *Cryptosporidium* (chapter 3.1) and on mixed assemblage A/E infections for *Giardia* (chapter 3.2)

3- To evaluate the efficacy of different dosages of paromomycin against an experimental *Giardia* infection in calves (chapter 4.1), and to determine the efficacy of a combination of fenbendazole treatment and hygienic measures for the control of a natural *Giardia* infection in calves (chapter 4.2)

Chapter 2

Prevalence and

diagnostic test evaluation

Chapter 2.1

Estimation of diagnostic test characteristics and prevalence of *Giardia* in calves in Belgium, using a Bayesian approach

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Based on:

Geurden, T., Claerebout, E., Vercruysse, J., Berkvens, D., 2004, Estimation of diagnostic test characteristics and prevalence of *Giardia duodenalis* in dairy calves in Belgium using a Bayesian approach. Int J Parasitol 34, 1121-1127.

2.1.1 Introduction

Worldwide a *Giardia* prevalence between 20% (Wade *et al.*, 2000) and 73% (Olson *et al.*, 1997a) has been reported in calves. The number of farms with positive calves in these studies varies from 45 (Ruest *et al.*, 1998) to 100% (Olson *et al.*, 1997 a, b). The wide variation in both calf and farm prevalence is not only due to differences in management practice or climate (Xiao *et al.*, 1994), but also to differences in study design, such as the number of farms or animals under study or the sampling strategy (Xiao *et al.*, 1993; Fayer *et al.*, 2000). Another important factor is the age of the animals under study, since both the prevalence and the cyst excretion of *Giardia* peak among young animals. The prevalence is highest in the age group under six months, although the parasite can be found in all age categories (Xiao, 1994; Quilez *et al.*, 1996; Olson *et al.*, 1997b; Fayer *et al.*, 2000; Wade *et al.*, 2000). The number of cysts in the faeces peaks around two weeks of age, continuing to be high until six weeks of age. In older animals the cyst excretion is lower (Xiao, 1994; Nydam *et al.*, 2001), which can thwart diagnosis, especially when using diagnostic techniques with a low sensitivity.

In general, different diagnostic techniques can result in a substantial variation in prevalence estimates, due to differences in sensitivity and specificity. In cattle, for example, the highest *Giardia* prevalences were found by IFA (Olson *et al.*, 1997a; Hunt *et al.*, 2000; Trout *et al*., 2004; Maddox-Hyttel *et al*., 2006). Next to IFA, several other diagnostic assays, such as ME or ELISA, have been used to estimate the prevalence (Xiao and Herd, 1993; Xiao, 1994; Quilez *et al.*, 1996; Olson *et al.*, 1997 a, b; Ruest *et al.*, 1998; Fayer *et al.*, 2000; Hunt *et al.*, 2000; Wade *et al.*,2000). Neither the sensitivity nor the specificity of these tests is known for a bovine population, yet reliable estimates of these parameters are needed to adjust prevalence estimates. Using a frequentist approach, both ELISA and IFA have been compared with ME and with each other for use in human faecal samples, and were considered to be more sensitive and specific than ME. The differences in test characteristics between IFA and ELISA seem to be minimal (Addiss *et al.*, 1991; Scheffler and Van Etta, 1994; Alles *et al.*, 1995; Zimmerman and Needham, 1995; Garcia and Shimizu, 1997; Mank *et al.*, 1997). Similar results have been described in cats (McGlade *et al.*, 2003). However, the estimates of the test characteristics vary considerably among these validation studies. Furthermore, general scepticism is appropriate when

extrapolating test parameters validated on human samples to bovine samples, because these test characteristics would not apply in populations with a different prevalence and epidemiology (Greiner and Gardner, 2000). Since there is no gold standard for the diagnosis of *Giardia* in calves, the infection status of the population under study is uncertain and the accuracy assessment of a new test can be biased by the use of an imperfect reference test as gold standard. In the frequentist approach test evaluation relies on the comparison with a gold standard reference test in a contingency table. Because a gold standard reference test is not as infallible as inherently assumed in the frequentist approach, the evaluation of the new test might result in an underestimation of the test characteristics of the assay to be evaluated. A novel approach to circumvent the gold standard problem is Bayesian analysis, which has proven its potential in validating diagnostic techniques and providing a reliable estimate of the disease prevalence, when at least three independent diagnostic test results are available (Pouedet *et al.*, 2002; Basanez *et al.*, 2004; Dorny *et al.*, 2004; Berkvens *et al.*, 2006).

2.1.2 Materials and methods

Study design

A cross-sectional epidemiological study was conducted. One hundred dairy farms and 50 beef farms in the province of East-Flanders, Belgium, were randomly selected and visited on a single occasion between September 2001 and August 2005. All dairy calves were Holstein or local breed mixed with Holstein. All beef calves were Belgian blue and white (BBW) or local breed mixed with BBW. Faecal specimens were collected from all calves aged from newborn to 10 weeks, present on the farm at the time of the visit and transported to the laboratory. A subset of the dairy samples (the first 235 samples collected on the first 50 farms) was examined using three different diagnostic assays. These data were used in a Bayesian analysis in order to estimate both the sensitivity and the specificity of these three tests in a bovine population and to estimate the prevalence of *Giardia* in dairy calves. The 235 samples were examined within 48 hours after collection with ME and IFA. Afterwards, all samples were preserved at –20°C and examined with ELISA. The faeces collected on the remaining dairy farms and on the beef farms were examined using IFA within 48 hours after collection. The complete dataset of the 100 dairy and of the 50 beef farms was used in order to estimate the overall farm and calf prevalence, the intensity of cyst excretion

and the average prevalence in each age category. The difference in age between dairy and beef calves was compared with a Mann-Withney U test. Probability (P) values <0.05 were considered to indicate a significant difference. The farm prevalence was calculated as the number of farms with at least one positive calf, compared to the total number of farms in the study. The apparent prevalence was calculated as the number of *Giardia* positive calves, as diagnosed by the respective technique, compared to the total number of animals.

Detection of Giardia *cysts*

Microscopical examination (ME)

A sucrose flotation technique was used to demonstrate the presence of *Giardia* cysts in calf faeces. One gram of faeces was suspended in distilled water and strained through a layer of surgical gauze to withhold the large debris. After sedimentation for 1h, the supernatant was decanted and the sediment was collected into a centrifuge tube. After centrifugation at 3000*g* for 5min, the sediment was resuspended in a sucrose solution (1.27 sp gr.) and centrifuged at 1500*g* for 5min. Sucrose solution was added up to the rim of the tube and a cover glass was put on top of the tube. After one min, the cover glass was removed from the tube, placed on a microscope slide and stained with iodine. The entire slide was examined at 400x magnification. An animal was considered positive if a cyst with the correct morphology (12-14μm in length with an axostyle and 2 or 4 nuclei) was observed.

Direct Immunofluorescence Assay (IFA)

The quantitative IFA was developed by using the commercial Merifluor *Cryptosporidium*/*Giardia* kit (Meridian Diagnostics Inc., Cincinnati, Ohio) and by modifying the method of Xiao and Herd (1993). Again 1g of the sample was suspended in distilled water and strained through a layer of surgical gauze. After sedimentation for 1h and centrifugation at 3000*g* for 5min, the sediment was resuspended in distilled water up to a volume of 1ml. After thorough vortexing, an aliquot of 10μl was pippeted 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 cysts per gram faeces (CPG) was obtained by multiplying the total number of cysts on the smear by 100.

Enzyme-linked immunosorbent assay (ELISA)

The TechLab *Giardia* test (Techlab Inc., Blacksburg, Va.) was used to demonstrate the prevalence of cysts in frozen samples. The monoclonal antibody based ELISA was used as instructed by the manufacturer. This assay demonstrates Cyst Wall Protein 1 which is found in the wall of mature cysts and is secreted in large amounts by the encysting trophozoites. Therefore, the antigen can be detected in the early stage of excretion (Boone *et al.*, 1999).

Statistical analysis

A one test approach (Appendix A) was used to estimate the true prevalence, based on the number of negative and positive samples found in the 100 dairy farms and in the 50 beef farms, and on the sensitivity and specificity of the IFA. To estimate the sensitivity and specificity of three assays used for the diagnosis of *Giardia,* a three test Bayesian approach (Pouedet *et al.*, 2002) was used (Appendix B) based on a subset of the data, obtained from the first 50 dairy farms. Different models were constructed and run in WinBugs 1.4 (Spiegelhalter *et al.*, 2003). Typically, a burn-in phase of 5000 iterations was used and the models were run for another 10,000 iterations to obtain estimates. The Brooks, Gelman & Rubin convergence statistic was used to assess model convergence and only properly converged models were further considered. Model selection proceeded on grounds of identifiability of the model and through minimisation of the deviance information criterion (DIC) while ensuring a positive number of parameters (p_D) estimated in the model (Spiegelhalter *et al.*, 2002) and, on the other hand, on a Bayesian p-value (Gelman *et al.*, 2004). The latter is a measure for the goodness-of-fit of the model (a comparison of the data to the posterior predictive distribution) and essentially represents the proportion of positive differences at consecutive iterations between the deviance for 'cell' counts, sampled from the model, and the deviance calculated for the data. A model, based on a multinomial distribution and including all possible interactions between the three individual tests requires 15 parameters to be estimated. These are the prevalence, the sensitivity and specificity of the first test, two conditional sensitivities and two conditional specificities for the second test, and finally four conditional sensitivities and four conditional specificities for the third test (Figure 2.1).

Figure 2.1: Conditional probabilities for Bayesian models up to six tests

[1] Prevalence $Pr\left(D^{+}\right)$

[2] Se_1 $Pr\left(T_1^{+} \mid D^{+}\right)$

[3] Sp_1 $Pr\left(T_1^{-} \mid D^{-}\right)$

[4] $Pr\left(T_2^{+} \mid D^{+} \cap T_1^{+}\right)$

[5] $Pr\left(T_2^{+} \mid D^{+} \cap T_1^{-}\right)$

[6] $Pr\left(T_2^{-} \mid D^{-} \cap T_1^{-}\right)$

[7] $Pr\left(T_2^{-} \mid D^{-} \cap T_1^{+}\right)$

[8] $Pr\left(T_3^{+} \mid D^{+} \cap T_1^{+} \cap T_2^{+}\right)$

[9] $Pr\left(T_3^{+} \mid D^{+} \cap T_1^{+} \cap T_2^{-}\right)$

[10] $Pr\left(T_3^{+} \mid D^{+} \cap T_1^{-} \cap T_2^{+}\right)$

[11] $Pr\left(T_3^{+} \mid D^{+} \cap T_1^{-} \cap T_2^{-}\right)$

[12] $Pr\left(T_3^{-} \mid D^{-} \cap T_1^{-} \cap T_2^{-}\right)$

[13] $Pr\left(T_3^{-} \mid D^{-} \cap T_1^{-} \cap T_2^{+}\right)$

[14] $Pr\left(T_3^{-} \mid D^{-} \cap T_1^{+} \cap T_2^{-}\right)$

[15] $Pr\left(T_3^{-} \mid D^{-} \cap T_1^{+} \cap T_2^{+}\right)$

[16] $Pr\left(T_4^{+} \mid D^{+} \cap T_1^{+} \cap T_2^{+} \cap T_3^{+}\right)$

...

[23] $Pr\left(T_4^{+} \mid D^{+} \cap T_1^{-} \cap T_2^{-} \cap T_3^{-}\right)$

[24] $Pr\left(T_4^{-} \mid D^{-} \cap T_1^{-} \cap T_2^{-} \cap T_3^{-}\right)$

[25] $Pr\left(T_4^{-} \mid D^{-} \cap T_1^{-} \cap T_2^{-} \cap T_3^{+}\right)$

...

[31] $Pr\left(T_4^{-} \mid D^{-} \cap T_1^{+} \cap T_2^{+} \cap T_3^{+}\right)$

[32] $\Pr\left(T_5^{+} \mid D^{+} \cap T_1^{+} \cap T_2^{+} \cap T_3^{+} \cap T_4^{+}\right)$

...

[50] $\Pr\left(T_5^{-} \mid D^{-} \cap T_1^{-} \cap T_2^{-} \cap T_3^{+} \cap T_4^{-}\right)$

[51] $\Pr\left(T_5^{-} \mid D^{-} \cap T_1^{-} \cap T_2^{-} \cap T_3^{+} \cap T_4^{+}\right)$

...

[63] $\Pr\left(T_5^{-} \mid D^{-} \cap T_1^{+} \cap T_2^{+} \cap T_3^{+} \cap T_4^{+}\right)$

[64] $\Pr\left(T_6^{+} \mid D^{+} \cap T_1^{+} \cap T_2^{+} \cap T_3^{+} \cap T_4^{+} \cap T_5^{+}\right)$

...

[103] $\Pr\left(T_6^{-} \mid D^{-} \cap T_1^{-} \cap T_2^{-} \cap T_3^{+} \cap T_4^{+} \cap T_5^{+}\right)$

[104] $\Pr\left(T_6^{-} \mid D^{-} \cap T_1^{-} \cap T_2^{+} \cap T_3^{-} \cap T_4^{-} \cap T_5^{-}\right)$

...

[127] $\Pr\left(T_6^{-} \mid D^{-} \cap T_1^{+} \cap T_2^{+} \cap T_3^{+} \cap T_4^{+} \cap T_5^{+}\right)$

This model is in fact inestimable since the data only allow 7 parameters to be estimated. Therefore, the model building strategy consists of incorporating extraneous prior information in an attempt to reduce the number of parameters to be estimated. This can be done by stating, for example, that the specificity of one of the tests is 1, leading to the exclusion of all parameters pertaining to false positives for this test: e.g. in Appendix A, equating the specificity of test 1 to 1 ([3] = 1) implicates that [7] must not be estimated, since $\Pr\left(D^- \cap T_1^+\right) = 0$. Probabilistic prior information can also be applied to reduce the possible range of values for a specific parameter. This reduction may affect the possible range of values for other parameters as well. Because of the complexity of the model, it is impossible to predict or calculate what level of reduction in the number of parameters to be estimated will result from this prior information. Therefore DIC is used to evaluate the model fit. DIC is an information criterion and consists of two components: first an equivalent of a likelihood measure (transformed in such a way that a lower value means a better fit) and secondly a penalty for the complexity of the model (the lower the value, the simpler the model). This DIC is to be minimised during the model building process, attempting to find the best fit with the simplest possible model. The p_D that was effectively estimated in the model also represents the complexity of the model and is an indication of the final reduction in the number of parameters that had to be estimated. This approach is described in more detail by Berkvens *et al*. (2006).

2.1.3 Results

In total 499 dairy calves and 333 beef calves were examined for the presence of *Giardia*. The mean age of the dairy calves was 26.43 ±0.93 (mean±standard error) days ranging from 1 to 70 days, with 59% younger than 4 weeks. Seventy-four percent of the dairy calves were female and 26% were male. There was an average number of 5 calves on these dairy farms, ranging from 1 to 19. The mean age of the beef calves was 32.7 ±1.1 days ranging from 1 to 70 days, with 47% younger than 4 weeks. Fifty-two percent of the animals were female and 48% were male. There was an average number of 7 calves on the farms, ranging from 1 to 17. The mean age of the calves on beef farms was significantly ($P<0.001$) higher than on dairy farms. The cross-classified test results obtained by the three individual tests for the detection of *Giardia* are presented in table 2.1.

Table 2.1: The cross-classified test results obtained by the three different tests for the detection of *Giardia* in dairy calves.

ME	IFA	ELISA	# samples
1	1	1	18
1	1	0	2
1	0	0	22
1	0	1	7
0	1	1	13
0	0	1	20
0	1	0	8
0	0	0	145

ME = Microscopical examination; IFA = Immunofluorescence Assay; ELISA = Enzyme-linked immunosorbent assay; # samples= number of test results

Of the 235 dairy calves examined by the three different diagnostic assays, 49 were positive for ME, 41 for IFA and 58 for ELISA (table 2.2). In 163 (69%) calves there was full agreement among test results, either positive or negative. A full conditional model was run, but did not reach convergence. Prior knowledge on the prevalence (constraint between 0 and 0.50) had to be included into the model. Adding information on specificity of both IFA (0.9-1) and ELISA (0.9-1) did not improve the model parameters Bayes-p, DIC and p_D. The prevalence, the sensitivity and specificity of each test was estimated based on the parameters obtained by the first model. The estimated prevalence was 19% (95% probability interval (PI): 11-28%). The sensitivity and specificity of each test is presented in table 2.2.

Table 2.2: The apparent prevalence (AP: in percentage) of *Giardia*, and the test properties of microscopical examination (ME), imunofluorescence assay (IFA) and ELISA as estimated by the three-test Bayesian analysis. (Se=sensitivity; Sp= specificity, with 95% Probability Interval)

	AP	Se	Sp
ME	22	56 (39-73)	87 (81-91)
IFA	17	77 (53-97)	95 (91-99)
ELISA	25	89 (70-99)	90 (83-97)

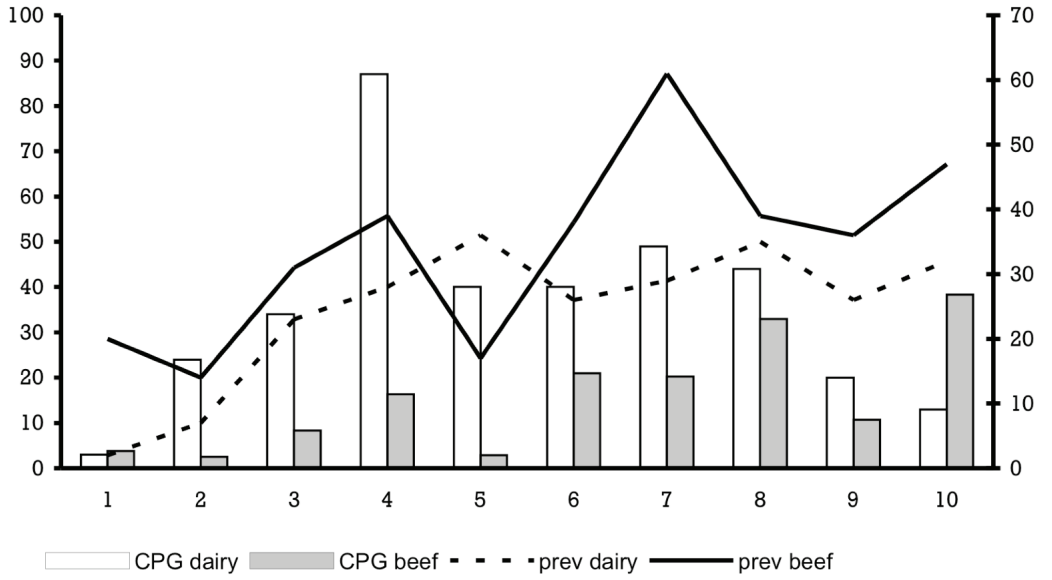
Based on the sensitivity and specificity estimates of IFA, as presented in table 2.1, and on the IFA results of the samples collected on 100 dairy and 50 beef farms, the true prevalence of *Giardia* was estimated (table 2.3).

Table 2.3: The true *Giardia* prevalence (in percentage), with the 95% Probability Interval, for different age categories (w=weeks) in dairy and beef farms, and the farm prevalence (Farm)

	<4w	4-10w	<10w	Farm
Dairy	9 (2-19)	41 (25-61)	22 (12-34)	48
Beef	34 (19-53)	55 (36-80)	45 (30-64)	64

On dairy farms the cyst excretion ranged from 100 to 1,040,000 CPG, with a geometric mean of 3516 CPG. As shown in figure 2.2, the highest geometric mean cyst excretion was observed among calves 4 weeks old and remained high among calves up to the age of 8 weeks, but decreased afterwards. In calves before the age of 2 weeks the geometric mean CPG was low, although the highest individual count was detected in a 12 days old calf. The calf prevalence was highest in dairy calves aged 5 weeks and stayed high among older animals up to 10 weeks, but was low among calves before the age of 2 weeks. On beef farms the cyst excretion ranged from 50 to 246,000 CPG, with a geometric mean of 1841 CPG. The geometric mean cyst excretion was highest among calves between 4 and 10 weeks (figure 2.2). The highest calf prevalence was observed in 7 week old calves, and prevalence was generally high in calves older than 4 weeks. Similar to dairy farms the geometric mean CPG before the age of 2 weeks was low compared to older calves.

Figure 2.2: Prevalence and geometric mean cyst excretion (CPG) of *Giardia* in different age categories (1-10 weeks) on dairy and beef farms, as diagnosed by immunofluorescence assay. The primary Y-axis indicates the geometric CPG (value x100). The secondary Y-axis indicates the prevalence in percentage.



2.1.4. Discussion

This is the first study to use a Bayesian approach to evaluate three different test procedures for the detection of *Giardia* in calves. The lack of diagnostic test evaluations for *Giardia* in dairy calves prior to this study, would have rendered the estimation of the prevalence based on the results of a single diagnostic technique unreliable (Greiner and Gardner, 2000). The apparent prevalence ranged from 17 to 25%, depending on the technique of choice. It has been demonstrated that a Bayesian approach can be used to obtain better estimates of both the prevalence and the test characteristics when the test results of three or four independent assays are available (Pouedet *et al.*, 2002; Basanez *et al.*, 2004; Dorny *et al.*, 2004). In the present study, three diagnostic techniques were performed on 235 samples coming from 50 dairy farms to obtain a more reliable estimate of the test characteristics. ELISA was evaluated as the most sensitive (89%) and IFA as the most specific (95%). The higher sensitivity of the ELISA compared to the IFA may be attributed to the detection of Cyst Wall Protein 1 by ELISA, which is secreted in large amounts by encysting trophozoites, whereas the IFA probably only detects cysts and not trophozoites

(Boone *et al.*, 1999). Despite a lower sensitivity compared to ELISA, the highly specific IFA was preferred as diagnostic assay in the present study because IFA can be used quantitatively. The ME has a low sensitivity compared to ELISA and IFA. Xiao and Herd (1993) also described low recovery rates using the sucrose gradient flotation technique.

The prevalence estimated in the initial 235 dairy samples using the three test Bayesian approach was later confirmed by the 1 test approach with the results of the additional screening of 264 dairy samples using only IFA as diagnostic technique. The prevalence estimate found in the dairy calves is comparable to other studies based on a large number of farms (Wade *et al.*, 2000). In studies with a smaller number of farms (Quilez *et al.*, 1996; Olson *et al.*, 1997 a, b; Hunt *et al.*, 2000), higher prevalence estimates were reported. The prevalence found in the beef farms is comparable to previous prevalence estimates reported in beef farms (Appelbee *et al.*, 2003; Gow and Waldner, 2006). Both the individual calf prevalence and the farm prevalence was high on beef farms compared to dairy farms, possibly due to differences in farm management such as the more frequent use of group housing and the higher stocking rate in beef farms. Next to substantial differences in farm management, the higher average age of beef calves in the present study might account for the higher prevalence, since *Giardia* tends to be more prevalent in calves older than one month. Both on dairy and beef farms the highest proportion of positive calves was found between 4 and 10 weeks. In calves before the age of two weeks the prevalence was low. A similar age distribution was described by Xiao *et al.* (1994) and Quilez *et al.* (1996), while others reported that giardiasis is more prevalent in calves older than 10 weeks (O'Handley *et al.*, 1999; Huetinck *et al.*, 2001), although these results could either be attributed to particular management practices on that farm (Huetinck *et al.*, 2001) or to a slow immune response to a *Giardia* infection (O'Handley *et al.*, 2003) in that calf population.

In accordance with results published by Xiao *et al.* (1994) and Nydam *et al.* (2001), the intensity of cyst excretion exceeded 10^6 cysts per gram of faeces in some calves. The highest cyst excretion in both dairy and beef calves was recorded among calves aged 3 to 10 weeks. In dairy calves excretion peaked at the age of one month, but not in beef calves. The low prevalence and intensity of cyst excretion before the age of two weeks may be due to maternal immunity, as bovine colostrum contains a high level of anti-*Giardia* antibodies. After the maternal antibodies become depleted,

calves become more susceptible to infection with *Giardia*, as they fail to develop a strong and fast specific humoral immunity against infection (O'Handley *et al.*, 2003). The present study indicates that *Giardia* is highly prevalent in calves from the age of three weeks onwards, and that the prevalence is higher in beef compared to dairy calves. IFA and ELISA were both sensitive and specific diagnostic techniques, whereas ME was less sensitive for use in epidemiological studies.

Chapter 2.2

A Bayesian approach for the evaluation of six diagnostic assays and the estimation of *Cryptosporidium* prevalence in calves in Belgium

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Based on:

Geurden, T., Berkvens, D., Geldhof, P., Vercruysse, J., Claerebout, E., 2006, A Bayesian approach for the evaluation of six diagnostic assays and the estimation of *Cryptosporidium* prevalence in dairy calves. Vet Res 37, 1-12.

2.2.1. Introduction

Cryptosporidium has been associated with diarrhoea in cattle, especially in young calves (de Graaf *et al.*, 1999). The prevalence of *Cryptosporidium* in calves aged less than 6 months varies from 1% (Kvac *et al.*, 2006) to 59% (Olson *et al.*, 1997a). Similar to *Giardia,* this variation is not only due to substantial differences in climatological or management conditions, but also to differences in study design, such as the diagnostic technique used in the study. Although there is no gold standard reference test, several conventional diagnostic assays have been widely used for the diagnosis of *Cryptosporidium* in calves, such as ME of a faecal smear after Kinyoun acid fast, modified Ziehl-Neelsen staining or carbolfuchsine staining, and immunological assays, such as IFA and ELISA. More recently, PCR has been shown to be a very sensitive and specific alternative (Morgan *et al.*, 1998). Accuracy assessment of diagnostic assays is traditionally performed by the frequentist approach using contingency tables with the test results of one assay or a combination of assays as the gold standard reference. However, due to a lack of knowledge of the true disease status of the tested animals, diagnostic test evaluation is frequently rendered ineffective leading to an over-or underestimation of the performance of the new test. The test evaluation for the diagnosis of *Cryptosporidium* is illustrative for the problem. In some studies ME was used as gold standard, overrating the ME sensitivity and specificity values compared to ELISA or IFA (Newman *et al.*, 1993; Ignatius *et al.*, 1997) whereas in other studies IFA (Quilez *et al.*, 1996) or PCR (Morgan *et al.*, 1998) was used as gold standard, underestimating the ME test characteristics. Similar reflections can be made for the evaluation of ELISA using IFA as gold standard (Rosenblatt and Sloan, 1993; Garcia and Shimizu, 1997).

The gold standard problem can be circumvented by using spiked samples to evaluate the sensitivity and specificity of diagnostic test methods. A higher recovery rate for IFA compared to ME was described (Weber *et al.*, 1991; Webster *et al.*, 1996), although recovery of *Cryptosporidium* oocysts from spiked specimens is known to be low (Weber *et al.*, 1991). Furthermore, this procedure can only be used in a controlled evaluation protocol and not for evaluation of diagnostic tests in an epidemiological study where the true disease status of the subject is unknown. Recently, Bayesian analysis has proven its value in circumventing the gold standard problem. In the present study, different Bayesian approaches are presented for the evaluation of the

Cryptosporidium prevalence in calves in the province of East Flanders, Belgium, and for the evaluation of diagnostic tests.

2.2.2 Material and methods

Study design

The study design is the same as for the prevalence estimation of *Giardia* (chapter 2.1.2). The samples from the first fifty dairy farms were used for the Bayesian test evaluation. These samples were examined within 48 hours after collection with ME and IFA, preserved at –20°C and later examined with two ELISAs and 2 PCR assays. The ME, IFA and the 2 ELISAs are from hereon referred to as the conventional assays. The samples from the remaining fifty dairy farms and from the fifty beef farms were examined only with IFA. The complete dataset of the 100 dairy and 50 beef farms was used in order to estimate farm and calf prevalence, the intensity of oocyst excretion and the average prevalence in each age category. The farm prevalence was calculated as the number of farms with at least one positive calf, compared to the total number of farms in the study. The apparent prevalence was calculated as the number of *Cryptosporidium* positive calves, as diagnosed by the respective technique, compared to the total number of animals.

Detection of Cryptosporidium *oocysts*

Carbolfuchsine smear method followed by microscopical examination (ME)

A direct faecal smear followed by carbolfuchsine staining (Heine, 1982) was used to demonstrate *Cryptosporidium* oocysts in fresh faecal material. Approximately 2cm^2 of each slide was examined at a 1000x magnification. A sample was considered positive as soon as an oocyst with the correct morphology (4.6-5.6µm x 4-4.8µm) was identified.

Direct Immunofluorescence Assay (IFA)

The commercial Merifluor *Cryptosporidium*/*Giardia* immunofluorescence assay (Meridian Diagnostics, Inc., Cincinnati, Ohio) was used for the quantitative detection to obtain the number of *Cryptosporidium* oocysts per gram of faeces (OPG), following the same procedure as described for *Giardia.*

Enzyme-linked immunosorbent assay

The TechLab (Techlab) *Cryptosporidium* test (Techlab, Inc., Blacksburg, Va.) and the Tetrakit (Tetra) or Bio-X Digestive ELISA Kit (Bio-X diagnostics, Marche-en-Famenne, Belgium) were used to demonstrate the prevalence of *Cryptosporidium* antigen in frozen samples. Both assays were used as instructed by the manufacturer.

PCR diagnosis

DNA was extracted from faecal samples using the QIAamp® Stool Mini Kit (Qiagen). The first PCR (C-PCR) was performed as previously described using the oligonucleotide primers CP-CR and O21F (Morgan *et al.*, 1997) for the amplification of a 312 bp product from the rDNA gene. The second PCR (COWP-PCR) was performed as previously described using the oligonucleotide primers cry-15 and cry-9 (Spano *et al.*, 1997). They were designed to amplify a 550 bp product from the gene encoding for the *Cryptosporidium* oocyst wall protein (COWP). Amplification products were subsequently vizualized on 1.5 % agarose gels with ethidium bromide. A positive and negative control sample was included in each PCR reaction. The positive control consisted of a plasmid originally cloned from a *Cryptosporidium* DNA sample isolated from a calf (kindly provided by Simone Caccio). The negative control was purified water. Amplification products of both PCR assays were randomly selected and sequenced throughout the study to confirm the specificity of both PCR assays.

Statistical analysis

Similar to *Giardia*, a one-test approach (Appendix A) was used to estimate the true prevalence, based on the number of negative and positive samples found in the 100 dairy and 50 beef farms, and on the sensitivity and specificity of the IFA, estimated in the six-test approach. DIC and p_D were used for model evaluation (Spiegelhalter *et al.*, 2002; Berkvens *et al.*, 2006).

A Bayesian analysis framework with only full conditional models was used to draw inferences about the sensitivity and specificity of different assays for the detection of of *Cryptosporidium*. Two different approaches were used to estimate the test characteristics. In a previously described four-test approach (Dorny *et al.*, 2004), a model (Appendix C) based on a multinomial distribution and including all possible interactions between the ME, IFA, Techlab and Tetra was used, requiring 31

parameters to be estimated (figure 2.1). Furthermore, a new six-test approach (Appendix D) was developed, requiring 127 parameters (figure 2.1) to be estimated: the prevalence, the sensitivity and specificity of the first test, two conditional sensitivities and two conditional specificities for the second test, four conditional sensitivities and four conditional specificities for the third test, eight conditional sensitivities and eight conditional specificities for the fourth test, sixteen conditional sensitivities and sixteen conditional specificities for the fifth test and finally thirty-two conditional sensitivities and thirty-two conditional specificities for the sixth test. Since the data only allow 63 parameters to be estimated (64 'classes' of test results with probabilities summing to unity), this model is in fact not identifiable. Therefore, the model building strategy consists of incorporating extraneous prior information, such as expert opinion (Suess *et al.*, 2002), in an attempt to reduce the number of parameters to be estimated. This prior information can be either deterministic or probabilistic. Probabilistic prior information is applied to reduce the possible range of values for a specific parameter (Dunson, 2001; Dorny *et al.*, 2004; Berkvens *et al.*, 2006), which may affect the possible range of values for other parameters as well. Model evaluation was performed as previously described (Spiegelhalter *et al.*, 2003; Gelman *et al.*, 2004). For some parameters no objective prior information can be formulated. Therefore it is necessary to leave prior information on these parameters non-informative, if one wants to maintain a minimum degree of honesty (Gelman *et al.*, 2004; Berkvens *et al.*, 2006).

2.2.3 Results

A total of 499 dairy and 333 beef calves were examined by IFA for the presence of *Cryptosporidium*. Mean age and average number of calves on the farms were described in chapter 2.1.3. A subset of 234 dairy calves were examined for the presence of *Cryptosporidium* using six different assays. The apparent calf prevalence as estimated by the different diagnostic assays, is presented in table 2.5 and ranged from 14% in the Tetra up to 46% in the C-PCR.

Four-test approach

The cross-classified test results obtained by the four tests for the detection of *Cryptosporidium* are presented in table 2.4. In the four-test approach, 150 (64%) calves were in full agreement, either positive or negative.

Table 2.4: The cross-classified test results obtained by the four tests for the detection of *Cryptosporidium* in dairy calves.

ME	IFA	Techlab	Tetra	# samples
1	1	1	1	18
1	1	1	0	5
1	1	0	1	1
1	1	0	0	3
1	0	1	1	1
1	0	1	0	6
1	0	0	1	2
1	0	0	0	34
0	1	1	1	1
0	1	1	0	4
0	1	0	1	0
0	1	0	0	6
0	0	1	1	2
0	0	1	0	13
0	0	0	1	7
0	0	0	0	132

ME = Microscopical examination; IFA=Immunofluorescence; Techlab=Techlab Elisa; Tetra= Tetrakit Elisa; # samples= number of test results

A conditionally dependent test model was used. Prior information on prevalence (prevalence constrained between 0.1-0.8) and on the specificity of the IFA, Techlab and Tetra (0.7-1) had to be included into the model to reach convergence. Adding information on the sensitivity of the IFA (0.4-1) further improved the model but additional constraints did not. Therefore, this model was used to estimate prevalence and test characteristics of the 4 assays. The estimated prevalence was 17% (95% Probability Interval (PI): 10-28%). The estimated sensitivity and specificity of each test are presented in table 2.5.

Table 2.5: The apparent prevalence (AP) of *Cryptosporidium* and the test properties of the diagnostic tests (95% PI) as estimated by the four test approach and the six test approach

		4 test approach		6 test approach	
	AP	**Se (%)**	**Sp (%)**	**Se (%)**	**Sp (%)**
C-PCR	46	ND	ND	79 (69-87)	100
COWP PCR	35	ND	ND	59 (50-67)	100
ME	30	78 (56-95)	79 (72-87)	40 (31-49)	84 (75-97)
IFA	16	78 (54-95)	95 (91-99)	26 (19-34)	94 (88-99)
Techlab	21	76 (54-92)	89 (84-94)	37 (28-46)	84 (76-92)
Tetra	14	59 (39-77)	93 (89-96)	30 (21-42)	88 (81-94)

Se= sensitivity; Sp= specificity; ND= not done

Six-test approach

In the six-test approach the results for 83 (35%) calf samples were in full agreement, either positive or negative (data not shown). Prior information on the specificity of both PCR assays (sp=1) was included in the model, based on previously published specificity estimates (Spano *et al.*, 1997; Morgan *et al.*, 1998). Furthermore, the specificity of both PCR assays was confirmed by the sequencing of amplification products of randomly chosen samples throughout the study (n=25) and all sequences proved to be *Cryptosporidium parvum*. The specificity constraints made the model converge, whereas widening the constraints on the specificity of both PCR assays to a range between 0.8 and 1 prohibited model convergence. Therefore the model with the constraints on PCR specificity (sp=1) was used to estimate the test characteristics of all diagnostic assays (table 2.5). The animal prevalence estimated using this six-test model was 58% (95% PI: 50-66%).

One-test approach

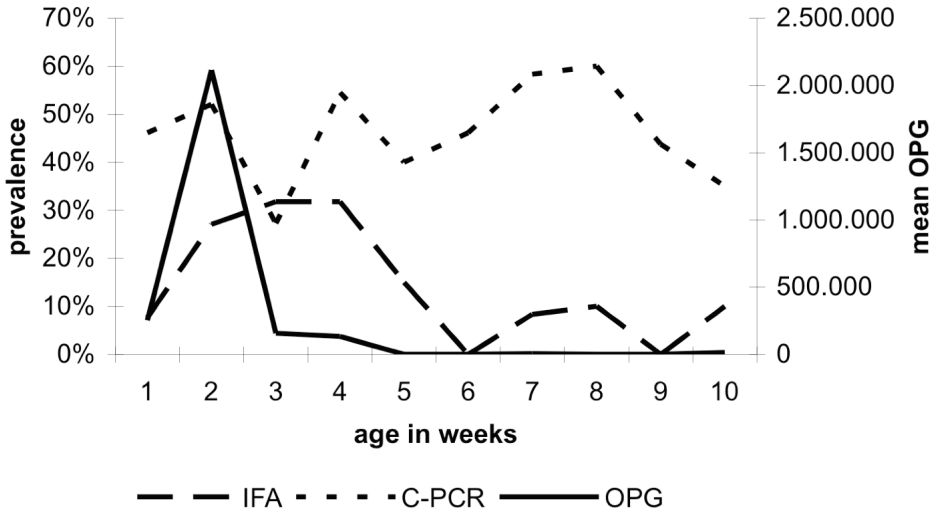
Based on the sensitivity and specificity estimates of IFA obtained in the six-test approach, as presented in table 2.5, and on the IFA results of the samples collected on 100 dairy and 50 beef farms, the true prevalence of *Cryptosporidium* was estimated using the one-test model (table 2.6).

Table 2.6: The true *Cryptosporidium* prevalence (in percentage), with the 95% Probability Interval, for different age categories (w=weeks) in dairy and beef farms, and the farm prevalence (Farm)

	<4w	4-10w	<10w	Farm
Dairy	68 (17-97)	10 (1-27)	37 (7-70)	32
Beef	17 (1-44)	11 (1-30)	12 (1-30)	24

Within the first 234 dairy samples, a high number of positive calves was found between the age of 1 and 4 weeks by all six tests performed. However, in calves older than one month the number of positive calves decreased when using the conventional diagnostic techniques. With the PCR assays a high number of positive test results was not only demonstrated until the age of 4 weeks, but also in calves up to the age of 10 weeks: the apparent prevalence in calves younger and older than one month as estimated by the C-PCR was 45% and 49% respectively. For the COWP the prevalence was 34% and 41% respectively for calves younger and older than one month. In figure 2.3, the proportion of *Cryptosporodium* positive calves, as diagnosed by IFA and C-PCR in the 234 dairy calves are presented along with the oocyst excretion. Since the prevalence for the different age categories estimated by ME, IFA and ELISA were similar, only the data obtained with IFA are presented, in comparison with the data of the C-PCR assay, which were similar to those of the COWP. The number of excreted oocysts by these 234 calves was determined using IFA and ranged from 200 to 24,000,000 OPG. The OPG was highest in calves between the age of 1 to 3 weeks. The highest individual OPG was observed in a 12 days old calf. The data obtained by IFA on the remaining 50 dairy farms confirmed the findings on age dependent prevalence and mean oocyst excretion. The overall geometric mean oocyst excretion in the 499 dairy calves was 29,425 OPG, ranging from 100 to 71,376,000 OPG. On the beef farms the number of positive samples was too low to calculate prevalence and mean oocyst excretion for each age category. The overall geometric mean oocyst excretion in beef calves was 780 OPG, ranging from 50 to 32,400 OPG.

Figure 2.3: Prevalence and mean oocyst excretion (oocysts per gram faeces: OPG) of *Cryptosporidium* in different age categories (1-10 weeks), as diagnosed by IFA and the C-PCR assay.



2.2.4 Discussion

The present study was the first to use a Bayesian approach to evaluate diagnostic assays for the detection of *Cryptosporidium* in calves, and to estimate parasite prevalence. Since there is no gold standard for the diagnosis of *Cryptosporidium* infection, a prevalence estimate based on the results of a single diagnostic assay would have been unreliable: in this study the apparent calf prevalence ranged from 13% to 46 % depending on the technique of choice. Previously a Bayesian approach has proven its potential to circumvent this gold standard problem when 3 (Pouedet *et al.*, 2002) or 4 (Dorny *et al.*, 2004) diagnostic tests are applied. The four-test approach estimated a *Cryptosporidium* calf prevalence of 17%. When the test results of 2 additional PCR assays were combined with the results of the 4 conventional assays and used to develop the six-test Bayesian approach, the prevalence estimate however increased to 58%. The overall true prevalence was estimated by the one-test model based on the IFA results in the 100 dairy farms and the 50 beef farms, taking the six-test model sensitivity and specificity estimates of the IFA into account. The true prevalence was comparable to the six-test model prevalence estimate and is considerably higher than most other prevalence reports. The higher prevalence is largely due to the detection by both PCR assays of positive calves older than one month which the conventional techniques failed to identify. The additional detection

by PCR is probably due to the combined effect of the lower oocyst excretion in older calves and the higher sensitivity of PCR compared to the conventional techniques (Webster *et al.*, 1996). Similar to previous reports (Xiao and Herd, 1994; O'Handley *et al.*, 1999; Nydam *et al.*, 2001), the peak in oocyst excretion was observed in calves between 1 to 4 weeks of age. Although the oocyst excretion decreases in calves older than one month, the prevalence estimated by both PCR assays remained high in calves older than one month, contrary to other studies with a decrease in prevalence found in calves in the age category of 4 to 10 weeks (Quilez *et al.*, 1996; Olson *et al.*, 1997). Calves between the age of 4 to 10 weeks should therefore be considered as an possible source of infection for susceptible animals, although there appears to be a shift in *Cryptosporidium* species with age in calves (Santin *et al.*, 2004). The *Cryptosporidium* prevalence in dairy calves was higher than in beef calves, which is partially due to several risk factors, such as seasonal calvings and farm management (Atwill *et al.*, 1999; Geurden *et al.*, 2006; Gow and Waldner, 2006; Kvac *et al.*, 2006). Furthermore, the dairy calves were younger than the beef calves, and age of the calf is an important risk factor for infection (O'Handley *et al.*, 1999), with a maximum risk for infection between 9 and 12 days of age (Maldonado-Camargo *et al.*, 1998; Castro-Hermida *et al.*, 2002).

The evaluation of the conventional techniques in the four-test approach yielded comparable sensitivity estimates for the ME and two out of the three immunological techniques, confirming previous reports that immunological detection methods are not significantly more sensitive than ME (Rodriguez-Hernandez *et al.*, 1994). The specificity of the immunological techniques however was higher than the specificity of ME. The higher sensitivity of the PCR assays reported by Morgan *et al.* (1998) and Spano *et al.* (1997) was confirmed by the six-test approach. The difference in test characteristics estimates of the conventional assays determined in the four-test and the six-test approach, is due to the inclusion of two diagnostic techniques with a high sensitivity: both PCR assays not only confirmed most samples positive by the four conventional assays, but also detected additional positive samples, resulting in a decrease in sensitivity estimates for the conventional techniques, whereas the specificity estimates for the 4 conventional assays were comparable in both approaches. The results of the six-test approach demonstrate that PCR assays can provide additional epidemiological information compared to the conventional diagnostic tools.

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2.4 Appendices

2.4.1 Appendix A: One test Bayesian model

```
model
{
r[1:2] ~ dmulti(p[1:2], n)

p[1] <- prev*(1-se)+(1-prev)*sp
p[2] <- prev*se + (1-prev)*(1-sp)

prev  ~ dbeta(1,1)
se ~ dbeta(1,1)
sp ~ dbeta(1,1)

for (i in 1:2)
{
    d[i] <- r[i]*log(max(r[i],1)/(p[i]*n))
}
G0 <- sum(d[])
r2[1:2] ~ dmulti(p[1:2], n)

for (i in 1:2)
{
    d2[i] <- r2[i]*log(max(r2[i],1)/(p[i]*n))
}
Gt <- sum(d2[])
bayesp <- step(G0 - Gt)

}

list(r=c(#neg,#pos), n = 000)
```

2.4.2 Appendix B: Three test Bayesian model

```
model
{

r[1:8] ~ dmulti(pr[1:8], n)

pr[1]<-p[1]*p[2]*p[4]*p[8]+(1-p[1])*(1-p[3])*(1-p[7])*(1-p[15])
pr[2]<-p[1]*p[2]*p[4]*(1-p[8])+(1-p[1])*(1-p[3])*(1-p[7])*p[15]
pr[3]<-p[1]*p[2]*(1-p[4])*p[9]+(1-p[1])*(1-p[3])*p[7]*(1-p[14])
pr[4]<-p[1]*p[2]*(1-p[4])*(1-p[9])+(1-p[1])*(1-p[3])*p[7]*p[14]
pr[5]<-p[1]*(1-p[2])*p[5]*p[10]+(1-p[1])*p[3]*(1-p[6])*(1-p[13])
pr[6]<-p[1]*(1-p[2])*p[5]*(1-p[10])+(1-p[1])*p[3]*(1-p[6])*p[13]
pr[7]<-p[1]*(1-p[2])*(1-p[5])*p[11]+(1-p[1])*p[3]*p[6]*(1-p[12])
pr[8]<-p[1]*(1-p[2])*(1-p[5])*(1-p[11])+(1-p[1])*p[3]*p[6]*p[12]

for (i in 1:8)
{
        d[i] <- r[i]*log(max(r[i],1)/(pr[i]*n))
}
G0 <- 2* sum(d[])

r2[1:8] ~ dmulti(pr[1:8],n)
for (i in 1:8)
{
        d2[i] <- r2[i]*log(max(r2[i],1)/(pr[i]*n))
}
Gt <- 2* sum(d2[])

bayesp <- step(G0 - Gt)

p[1]  ~ dbeta(1,1)
p[2]  ~ dbeta(1,1)
p[3]  ~ dbeta(1,1)
p[4]  ~ dbeta(1,1)
p[5]  ~ dbeta(1,1)
p[6]  ~ dbeta(1,1)
p[7]  ~ dbeta(1,1)
p[8]  ~ dbeta(1,1)
p[9]  ~ dbeta(1,1)
p[10]  ~ dbeta(1,1)
p[11]  ~ dbeta(1,1)
p[12]  ~ dbeta(1,1)
p[13]  ~ dbeta(1,1)
p[14]  ~ dbeta(1,1)
p[15]  ~ dbeta(1,1)

se[1] <- p[2]
sp[1] <- p[3]
se[2] <- p[2]*p[4]+(1-p[2])*p[5]
sp[2] <- p[3]*p[6]+(1-p[3])*p[7]
se[3] <- p[2]*(p[4]*p[8]+(1-p[4])*p[9])+(1-p[2])*(p[5]*p[10]+(1-p[5])*p[11])
sp[3] <- p[3]*(p[6]*p[12]+(1-p[6])*p[13])+(1-p[3])*(p[7]*p[14]+(1-p[7])*p[15])

}

list(r= c(#neg,#pos), n = 000)
```

2.4.3 Appendix C: Four test Bayesian model

```
model
{
r[1:16] ~ dmulti( pr[1:16], n )

pr[1] <- p[1]*(1-p[2])*(1-p[5])*(1-p[11])*(1-p[23])+(1-p[1])*p[3]*p[6]*p[12]*p[24]
pr[2] <- p[1]*(1-p[2])*(1-p[5])*(1-p[11])*p[23]+(1-p[1])*p[3]*p[6]*p[12]*(1-p[24])
pr[3] <- p[1]*(1-p[2])*(1-p[5])*p[11]*(1-p[22])+(1-p[1])*p[3]*p[6]*(1-p[12])*p[25]
pr[4] <- p[1]*(1-p[2])*(1-p[5])*p[11]*p[22]+(1-p[1])*p[3]*p[6]*(1-p[12])*(1-p[25])
pr[5] <- p[1]*(1-p[2])*p[5]*(1-p[10])*(1-p[21])+(1-p[1])*p[3]*(1-p[6])*p[13]*p[26]
pr[6] <- p[1]*(1-p[2])*p[5]*(1-p[10])*p[21]+(1-p[1])*p[3]*(1-p[6])*p[13]*(1-p[26])
pr[7] <- p[1]*(1-p[2])*p[5]*p[10]*(1-p[20])+(1-p[1])*p[3]*(1-p[6])*(1-p[13])*p[27]
pr[8] <- p[1]*(1-p[2])*p[5]*p[10]*p[20]+(1-p[1])*p[3]*(1-p[6])*(1-p[13])*(1-p[27])
pr[9] <- p[1]*p[2]*(1-p[4])*(1-p[9])*(1-p[19])+(1-p[1])*(1-p[3])*p[7]*p[14]*p[28]
pr[10]<- p[1]*p[2]*(1-p[4])*(1-p[9])*p[19]+(1-p[1])*(1-p[3])*p[7]*p[14]*(1-p[28])
pr[11]<- p[1]*p[2]*(1-p[4])*p[9]*(1-p[18])+(1-p[1])*(1-p[3])*p[7]*(1-p[14])*p[29]
pr[12]<- p[1]*p[2]*(1-p[4])*p[9]*p[18]+(1-p[1])*(1-p[3])*p[7]*(1-p[14])*(1-p[29])
pr[13]<- p[1]*p[2]*p[4]*(1-p[8])*(1-p[17])+(1-p[1])*(1-p[3])*(1-p[7])*p[15]*p[30]
pr[14]<- p[1]*p[2]*p[4]*(1-p[8])*p[17]+(1-p[1])*(1-p[3])*(1-p[7])*p[15]*(1-p[30])
pr[15]<- p[1]*p[2]*p[4]*p[8]*(1-p[16])+(1-p[1])*(1-p[3])*(1-p[7])*(1-p[15])*p[31]
pr[16]<- p[1]*p[2]*p[4]*p[8]*p[16]+(1-p[1])*(1-p[3])*(1-p[7])*(1-p[15])*(1-p[31])

for( i in 1:16) {
d[i] <- r[i]*log(max(r[i],1)/(pr[i]*n))
                    }
G0 <- 2* sum(d[])

r2[1:16] ~ dmulti(pr[1:16], n)

for( i in 1:16) {
d2[i] <- r2[i]*log(max(r2[i],1)/(pr[i]*n))
                    }
Gt <- 2* sum(d2[])
bayesp <- step(Gt - G0)

p[1]      ~ dbeta(1,1)I(0,0.5)
p[2]      ~ dbeta(1,1)
p[3]      ~ dbeta(1,1)
p[4]      ~ dbeta(1,1)
p[5]      ~ dbeta(1,1)
p[6]      ~ dbeta(1,1)
p[7]      ~ dbeta(1,1)
p[8]      ~ dbeta(1,1)
p[9]      ~ dbeta(1,1)
p[10]     ~ dbeta(1,1)
p[11]     ~ dbeta(1,1)
p[12]     ~ dbeta(1,1)
p[13]     ~ dbeta(1,1)
p[14]     ~ dbeta(1,1)
p[15]     ~ dbeta(1,1)
p[16]     ~ dbeta(1,1)
p[17]     ~ dbeta(1,1)
p[18]     ~ dbeta(1,1)
p[19]     ~ dbeta(1,1)
p[20]     ~ dbeta(1,1)
p[21]     ~ dbeta(1,1)
p[22]     ~ dbeta(1,1)
p[23]     ~ dbeta(1,1)
p[24]     ~ dbeta(1,1)
```

```
p[25]     ~ dbeta(1,1)
p[26]      ~ dbeta(1,1)
p[27]      ~ dbeta(1,1)
p[28]      ~ dbeta(1,1)
p[29]      ~ dbeta(1,1)
p[30]      ~ dbeta(1,1)
p[31]      ~ dbeta(1,1)

}
          list(r=c(#neg,#pos), n = 000)
```

2.4.4 Appendix D: Six test Bayesian model

```
model
{
r[1:64] ~ dmulti(Pr[1:64], n)

Pr[1] <-p[1]*p[2]*p[4]*p[8]*p[16]*p[32]*p[64]+(1-p[1])*(1-p[3])*(1-p[7])*(1-p[15])*(1-p[31])*(1-p[63])*(1-p[127])
Pr[2] <-p[1]*p[2]*p[4]*p[8]*p[16]*p[32]*(1-p[64])+(1-p[1])*(1-p[3])*(1-p[7])*(1-p[15])*(1-p[31])*(1-p[63])*p[127]
Pr[3] <-p[1]*p[2]*p[4]*p[8]*p[16]*(1-p[32])*p[65]+(1-p[1])*(1-p[3])*(1-p[7])*(1-p[15])*(1-p[31])*p[63]*(1-p[126])
Pr[4] <-p[1]*p[2]*p[4]*p[8]*p[16]*(1-p[32])*(1-p[65])+(1-p[1])*(1-p[3])*(1-p[7])*(1-p[15])*(1-p[31])*p[63]*p[126]
Pr[5] <-p[1]*p[2]*p[4]*p[8]*(1-p[16])*p[33]*p[66]+(1-p[1])*(1-p[3])*(1-p[7])*(1-p[15])*p[31]*(1-p[62])*(1-p[125])
Pr[6] <-p[1]*p[2]*p[4]*p[8]*(1-p[16])*p[33]*(1-p[66])+(1-p[1])*(1-p[3])*(1-p[7])*(1-p[15])*p[31]*(1-p[62])*p[125]
Pr[7] <-p[1]*p[2]*p[4]*p[8]*(1-p[16])*(1-p[33])*p[67]+(1-p[1])*(1-p[3])*(1-p[7])*(1-p[15])*p[31]*p[62]*(1-p[124])
Pr[8] <-p[1]*p[2]*p[4]*p[8]*(1-p[16])*(1-p[33])*(1-p[67])+(1-p[1])*(1-p[3])*(1-p[7])*(1-p[15])*p[31]*p[62]*p[124]
Pr[9] <-p[1]*p[2]*p[4]*(1-p[8])*p[17]*p[35]*p[68]+(1-p[1])*(1-p[3])*(1-p[7])*p[15]*(1-p[30])*(1-p[61])*(1-p[123])
Pr[10]<-p[1]*p[2]*p[4]*(1-p[8])*p[17]*p[35]*(1-p[68])+(1-p[1])*(1-p[3])*(1-p[7])*p[15]*(1-p[30])*(1-p[61])*p[123]
Pr[11]<-p[1]*p[2]*p[4]*(1-p[8])*p[17]*(1-p[35])*p[69]+(1-p[1])*(1-p[3])*(1-p[7])*p[15]*(1-p[30])*p[61]*(1-p[122])
Pr[12]<-p[1]*p[2]*p[4]*(1-p[8])*p[17]*(1-p[35])*(1-p[69])+(1-p[1])*(1-p[3])*(1-p[7])*p[15]*(1-p[30])*p[61]*p[122]
Pr[13]<-p[1]*p[2]*p[4]*(1-p[8])*(1-p[17])*p[36]*p[70]+(1-p[1])*(1-p[3])*(1-p[7])*p[15]*p[30]*(1-p[60])*(1-p[121])
Pr[14]<-p[1]*p[2]*p[4]*(1-p[8])*(1-p[17])*p[36]*(1-p[70])+(1-p[1])*(1-p[3])*(1-p[7])*p[15]*p[30]*(1-p[60])*p[121]
Pr[15]<-p[1]*p[2]*p[4]*(1-p[8])*(1-p[17])*(1-p[36])*p[71]+(1-p[1])*(1-p[3])*(1-p[7])*p[15]*p[30]*p[60]*(1-p[120])
Pr[16]<-p[1]*p[2]*p[4]*(1-p[8])*(1-p[17])*(1-p[36])*(1-p[71])+(1-p[1])*(1-p[3])*(1-p[7])*p[15]*p[30]*p[60]*p[120]
Pr[17]<-p[1]*p[2]*(1-p[4])*p[9]*p[18]*p[36]*p[72]+(1-p[1])*(1-p[3])*p[7]*(1-p[14])*(1-p[29])*(1-p[59])*(1-p[119])
Pr[18]<-p[1]*p[2]*(1-p[4])*p[9]*p[18]*p[36]*(1-p[72])+(1-p[1])*(1-p[3])*p[7]*(1-p[14])*(1-p[29])*(1-p[59])*p[119]
Pr[19]<-p[1]*p[2]*(1-p[4])*p[9]*p[18]*(1-p[36])*p[73]+(1-p[1])*(1-p[3])*p[7]*(1-p[14])*(1-p[29])*p[59]*(1-p[118])
Pr[20]<-p[1]*p[2]*(1-p[4])*p[9]*p[18]*(1-p[36])*(1-p[73])+(1-p[1])*(1-p[3])*p[7]*(1-p[14])*(1-p[29])*p[59]*p[118]
Pr[21]<-p[1]*p[2]*(1-p[4])*p[9]*(1-p[18])*p[37]*p[74]+(1-p[1])*(1-p[3])*p[7]*(1-p[14])*p[29]*(1-p[58])*(1-p[117])
Pr[22]<-p[1]*p[2]*(1-p[4])*p[9]*(1-p[18])*p[37]*(1-p[74])+(1-p[1])*(1-p[3])*p[7]*(1-p[14])*p[29]*(1-p[58])*p[117]
Pr[23]<-p[1]*p[2]*(1-p[4])*p[9]*(1-p[18])*(1-p[37])*p[75]+(1-p[1])*(1-p[3])*p[7]*(1-p[14])*p[29]*p[58]*(1-p[116])
Pr[24]<-p[1]*p[2]*(1-p[4])*p[9]*(1-p[18])*(1-p[37])*(1-p[75])+(1-p[1])*(1-p[3])*p[7]*(1-p[14])*p[29]*p[58]*p[116]
Pr[25]<-p[1]*p[2]*(1-p[4])*(1-p[9])*p[19]*p[38]*p[76]+(1-p[1])*(1-p[3])*p[7]*p[14]*(1-p[28])*(1-p[57])*(1-p[115])
Pr[26]<-p[1]*p[2]*(1-p[4])*(1-p[9])*p[19]*p[38]*(1-p[76])+(1-p[1])*(1-p[3])*p[7]*p[14]*(1-p[28])*(1-p[57])*p[115]
Pr[27]<-p[1]*p[2]*(1-p[4])*(1-p[9])*p[19]*(1-p[38])*p[77]+(1-p[1])*(1-p[3])*p[7]*p[14]*(1-p[28])*p[57]*(1-p[114])
Pr[28]<-p[1]*p[2]*(1-p[4])*(1-p[9])*p[19]*(1-p[38])*(1-p[77])+(1-p[1])*(1-p[3])*p[7]*p[14]*(1-p[28])*p[57]*p[114]
Pr[29]<-p[1]*p[2]*(1-p[4])*(1-p[9])*(1-p[19])*p[39]*p[78]+(1-p[1])*(1-p[3])*p[7]*p[14]*p[28]*(1-p[56])*(1-p[113])
Pr[30]<-p[1]*p[2]*(1-p[4])*(1-p[9])*(1-p[19])*p[39]*(1-p[78])+(1-p[1])*(1-p[3])*p[7]*p[14]*p[28]*(1-p[56])*p[113]
Pr[31]<-p[1]*p[2]*(1-p[4])*(1-p[9])*(1-p[19])*(1-p[39])*p[79]+(1-p[1])*(1-p[3])*p[7]*p[14]*p[28]*p[56]*(1-p[112])
Pr[32]<-p[1]*p[2]*(1-p[4])*(1-p[9])*(1-p[19])*(1-p[39])*(1-p[79])+(1-p[1])*(1-p[3])*p[7]*p[14]*p[28]*p[56]*p[112]
Pr[33]<-p[1]*(1-p[2])*p[5]*p[10]*p[20]*p[40]*p[80]+(1-p[1])*p[3]*(1-p[6])*(1-p[13])*(1-p[27])*(1-p[55])*(1-p[111])
Pr[34]<-p[1]*(1-p[2])*p[5]*p[10]*p[20]*p[40]*(1-p[80])+(1-p[1])*p[3]*(1-p[6])*(1-p[13])*(1-p[27])*(1-p[55])*p[111]
Pr[35]<-p[1]*(1-p[2])*p[5]*p[10]*p[20]*(1-p[40])*p[81]+(1-p[1])*p[3]*(1-p[6])*(1-p[13])*(1-p[27])*p[55]*(1-p[110])
Pr[36]<-p[1]*(1-p[2])*p[5]*p[10]*p[20]*(1-p[40])*(1-p[81])+(1-p[1])*p[3]*(1-p[6])*(1-p[13])*(1-p[27])*p[55]*p[110]
Pr[37]<-p[1]*(1-p[2])*p[5]*p[10]*(1-p[20])*p[41]*p[82]+(1-p[1])*p[3]*(1-p[6])*(1-p[13])*p[27]*(1-p[54])*(1-p[109])
Pr[38]<-p[1]*(1-p[2])*p[5]*p[10]*(1-p[20])*p[41]*(1-p[82])+(1-p[1])*p[3]*(1-p[6])*(1-p[13])*p[27]*(1-p[54])*p[109]
Pr[39]<-p[1]*(1-p[2])*p[5]*p[10]*(1-p[20])*(1-p[41])*p[83]+(1-p[1])*p[3]*(1-p[6])*(1-p[13])*p[27]*p[54]*(1-p[108])
Pr[40]<-p[1]*(1-p[2])*p[5]*p[10]*(1-p[20])*(1-p[41])*(1-p[83])+(1-p[1])*p[3]*(1-p[6])*(1-p[13])*p[27]*p[54]*p[108]
Pr[41]<-p[1]*(1-p[2])*p[5]*(1-p[10])*p[21]*p[42]*p[84]+(1-p[1])*p[3]*(1-p[6])*p[13]*(1-p[26])*(1-p[53])*(1-p[107])
Pr[42]<-p[1]*(1-p[2])*p[5]*(1-p[10])*p[21]*p[42]*(1-p[84])+(1-p[1])*p[3]*(1-p[6])*p[13]*(1-p[26])*(1-p[53])*p[107]
Pr[43]<-p[1]*(1-p[2])*p[5]*(1-p[10])*p[21]*(1-p[42])*p[85]+(1-p[1])*p[3]*(1-p[6])*p[13]*(1-p[26])*p[53]*(1-p[106])
Pr[44]<-p[1]*(1-p[2])*p[5]*(1-p[10])*p[21]*(1-p[42])*(1-p[85])+(1-p[1])*p[3]*(1-p[6])*p[13]*(1-p[26])*p[53]*p[106]
Pr[45]<-p[1]*(1-p[2])*p[5]*(1-p[10])*(1-p[21])*p[43]*p[86]+(1-p[1])*p[3]*(1-p[6])*p[13]*p[26]*(1-p[52])*(1-p[105])
Pr[46]<-p[1]*(1-p[2])*p[5]*(1-p[10])*(1-p[21])*p[43]*(1-p[86])+(1-p[1])*p[3]*(1-p[6])*p[13]*p[26]*(1-p[52])*p[105]
Pr[47]<-p[1]*(1-p[2])*p[5]*(1-p[10])*(1-p[21])*(1-p[43])*p[87]+(1-p[1])*p[3]*(1-p[6])*p[13]*p[26]*p[52]*(1-p[104])
Pr[48]<-p[1]*(1-p[2])*p[5]*(1-p[10])*(1-p[21])*(1-p[43])*(1-p[87])+(1-p[1])*p[3]*(1-p[6])*p[13]*p[26]*p[52]*p[104]
Pr[49]<-p[1]*(1-p[2])*(1-p[5])*p[11]*p[22]*p[44]*p[88]+(1-p[1])*p[3]*p[6]*(1-p[12])*(1-p[25])*(1-p[51])*(1-p[103])
Pr[50]<-p[1]*(1-p[2])*(1-p[5])*p[11]*p[22]*p[44]*(1-p[88])+(1-p[1])*p[3]*p[6]*(1-p[12])*(1-p[25])*(1-p[51])*p[103]
Pr[51]<-p[1]*(1-p[2])*(1-p[5])*p[11]*p[22]*(1-p[44])*p[89]+(1-p[1])*p[3]*p[6]*(1-p[12])*(1-p[25])*p[51]*(1-p[102])
Pr[52]<-p[1]*(1-p[2])*(1-p[5])*p[11]*p[22]*(1-p[44])*(1-p[89])+(1-p[1])*p[3]*p[6]*(1-p[12])*(1-p[25])*p[51]*p[102]
Pr[53]<-p[1]*(1-p[2])*(1-p[5])*p[11]*(1-p[22])*p[45]*p[90]+(1-p[1])*p[3]*p[6]*(1-p[12])*p[25]*(1-p[50])*(1-p[101])
Pr[54]<-p[1]*(1-p[2])*(1-p[5])*p[11]*(1-p[22])*p[45]*(1-p[90])+(1-p[1])*p[3]*p[6]*(1-p[12])*p[25]*(1-p[50])*p[101]
Pr[55]<-p[1]*(1-p[2])*(1-p[5])*p[11]*(1-p[22])*(1-p[45])*p[91]+(1-p[1])*p[3]*p[6]*(1-p[12])*p[25]*p[50]*(1-p[100])
Pr[56]<-p[1]*(1-p[2])*(1-p[5])*p[11]*(1-p[22])*(1-p[45])*(1-p[91])+(1-p[1])*p[3]*p[6]*(1-p[12])*p[25]*p[50]*p[100]
Pr[57]<-p[1]*(1-p[2])*(1-p[5])*(1-p[11])*p[23]*p[46]*p[92]+(1-p[1])*p[3]*p[6]*p[12]*(1-p[24])*(1-p[49])*(1-p[99])
Pr[58]<-p[1]*(1-p[2])*(1-p[5])*(1-p[11])*p[23]*p[46]*(1-p[92])+(1-p[1])*p[3]*p[6]*p[12]*(1-p[24])*(1-p[49])*p[99]
Pr[59]<-p[1]*(1-p[2])*(1-p[5])*(1-p[11])*p[23]*(1-p[46])*p[93]+(1-p[1])*p[3]*p[6]*p[12]*(1-p[24])*p[49]*(1-p[98])
Pr[60]<-p[1]*(1-p[2])*(1-p[5])*(1-p[11])*p[23]*(1-p[46])*(1-p[93])+(1-p[1])*p[3]*p[6]*p[12]*(1-p[24])*p[49]*p[98]
```

```
Pr[61]<-p[1]*(1-p[2])*(1-p[5])*(1-p[11])*(1-p[23])*p[47]*p[94]+(1-p[1])*p[3]*p[6]*p[12]*p[24]*(1-p[48])*(1-p[97])
Pr[62]<-p[1]*(1-p[2])*(1-p[5])*(1-p[11])*(1-p[23])*p[47]*(1-p[94])+(1-p[1])*p[3]*p[6]*p[12]*p[24]*(1-p[48])*p[97]
Pr[63]<-p[1]*(1-p[2])*(1-p[5])*(1-p[11])*(1-p[23])*(1-p[47])*p[95]+(1-p[1])*p[3]*p[6]*p[12]*p[24]*p[48]*(1-p[96])
Pr[64]<-p[1]*(1-p[2])*(1-p[5])*(1-p[11])*(1-p[23])*(1-p[47])*(1-p[95])+(1-p[1])*p[3]*p[6]*p[12]*p[24]*p[48]*p[96]

for (i in 1:64)
{
        d[i] <- r[i]*log(max(r[i],1)/(Pr[i]*n))
}
G0 <- 2* sum(d[])

r2[1:64] ~ dmulti(Pr[1:64],n)
for (i in 1:64)
{
        d2[i] <- r2[i]*log(max(r2[i],1)/(Pr[i]*n))
}
Gt <- 2* sum(d2[])
bayesp <- step(G0 - Gt)

p[1] ~ dbeta(1,1)
...
p[127] ~ dbeta(1,1)

se[1] <- p[2]
sp[1] <- p[3]

se[2] <- p[2]*p[4]+(1-p[2])*p[5]
sp[2] <- p[3]*p[6]+(1-p[3])*p[7]

se[3] <- p[2]*(p[4]*p[8]+(1-p[4])*p[9])+(1-p[2])*(p[5]*p[10]+(1-p[5])*p[11])
sp[3] <- p[3]*(p[6]*p[12]+(1-p[6])*p[13])+(1-p[3])*(p[7]*p[14]+(1-p[7])*p[15])

se[4] <- p[2]*(p[4]*(p[8]*p[16]+(1-p[8])*p[17])+(1-p[4])*(p[9]*p[18]+(1-p[9])*p[19]))
        +(1-p[2])*(p[5]*(p[10]*p[20]+(1-p[10])*p[21])+(1-p[5])*(p[11]*p[22]+(1-p[11])*p[23]))
sp[4] <- p[3]*(p[6]*(p[12]*p[24]+(1-p[12])*p[25])+(1-p[6])*(p[13]*p[26]+(1-p[13])*p[27]))
        +(1-p[3])*(p[7]*(p[14]*p[28]+(1-p[14])*p[29])+(1-p[7])*(p[15]*p[30]+(1-p[15])*p[31]))

se[5] <- p[2]*(p[4]*(p[8]*(p[16]*p[32]+(1-p[16])*p[33])+(1-p[8])*(p[17]*p[34]+(1-p[17])*p[35]))
        +(1-p[4])*(p[9]*(p[18]*p[36]+(1-p[18])*p[37])+(1-p[9])*(p[19]*p[38]+(1-p[19])*p[39])))
        +(1-p[2])*(p[5]*(p[10]*(p[20]*p[40]+(1-p[20])*p[41])+(1-p[10])*(p[21]*p[42]+(1-p[21])*p[43]))
        +(1-p[5])*(p[11]*(p[22]*p[44]+(1-p[22])*p[45])+(1-p[11])*(p[23]*p[46]+(1-p[23])*p[47])))

sp[5] <- p[3]*(p[6]*(p[12]*(p[24]*p[48]+(1-p[24])*p[49])+(1-p[12])*(p[25]*p[50]+(1-p[25])*p[51]))
        +(1-p[6])*(p[13]*(p[26]*p[52]+(1-p[26])*p[53])+(1-p[13])*(p[27]*p[54]+(1-p[27])*p[55])))
        +(1-p[3])*(p[7]*(p[14]*(p[28]*p[56]+(1-p[28])*p[57])+(1-p[14])*(p[29]*p[58]+(1-p[29])*p[59]))
        +(1-p[7])*(p[15]*(p[30]*p[60]+(1-p[30])*p[61])+(1-p[15])*(p[31]*p[62]+(1-p[31])*p[63])))


se[6]<- p[2]*(p[4]*(p[8]*(p[16]*(p[32]*p[64]+(1-p[32])*p[65])+(1-p[16])*(p[33]*p[66]+(1-p[33])*p[67]))
        +(1-p[8])*(p[17]*(p[34]*p[68]+(1-p[34])*p[69])+(1-p[17])*(p[35]*p[70]+(1-p[35])*p[71])))
        +(1-p[4])*(p[9]*(p[18]*(p[36]*p[72]+(1-p[36])*p[73])+(1-p[18])*(p[37]*p[74]+(1-p[37])*p[75]))
        +(1-p[9])*(p[19]*(p[38]*p[76]+(1-p[38])*p[77])+(1-p[19])*(p[39]*p[78]+(1-p[39])*p[79]))))
        +(1-p[2])* (p[5]*(p[10]*(p[20]*(p[40]*p[80]+(1-p[40])*p[81])+(1-p[20])*(p[41]*p[82]+
        (1-p[41])*p[83]))+(1-p[10])*(p[21]*(p[42]*p[84]+(1-p[42])*p[85])+(1-p[21])*(p[43]*p[86]+(1-
p[43])*p[87])))
        +(1-p[5])*(p[11]*(p[22]*(p[44]*p[88]+(1-p[44])*p[89])+(1-p[22])*(p[45]*p[90]+(1-p[45])*p[91]))
        +(1-p[11])*(p[23]*(p[46]*p[92]+(1-p[46])*p[93])+(1-p[23])*(p[47]*p[94]+(1-p[47])*p[95]))))


sp[6] <- p[3]*(p[6]*(p[12]*(p[24]*(p[48]*p[96]+(1-p[48])*p[97])+(1-p[24])*(p[49]*p[98]+(1-p[49])*p[99]))
        +(1-p[12])*(p[25]*(p[50]*p[100]+(1-p[50])*p[101]) +(1-p[25])*(p[51]*p[102]+(1-p[51])*p[102])))
        +(1-p[6])*(p[13]*(p[26]*(p[52]*p[104]+(1-p[52])*p[105])+(1-p[26])*(p[53]*p[106]+(1-p[53])*p[107]))
        +(1-p[13])*(p[27]*(p[54]*p[108]+(1-p[54])*p[109])+(1-p[27])*(p[55]*p[110]+(1-p[55])*p[111])))
        +(1-p[3])*(p[7]*(p[14]*(p[28]*(p[56]*p[112]+(1-p[56])*p[113])+(1-p[28])*(p[57]*p[114]+(1-
p[57])*p[115]))
        +(1-p[14])*(p[29]*(p[58]*p[116]+(1-p[58])*p[117])+(1-p[29])*(p[59]*p[118]+(1-p[59])*p[119])))
        +(1-p[7])*(p[15]*(p[30]*(p[60]*p[120]+(1-p[60])*p[121])+(1-p[30])*(p[61]*p[122]+(1-p[61])*p[123]))
```

```
        +(1-p[15])*(p[31]*(p[62]*p[124]+(1-p[62])*p[125])+(1-p[31])*(p[63]*p[126]+(1-p[63])*p[127])))
}
list(r= c(#neg,#pos), n = 000)
```

Chapter 3

Molecular characterisation of *Cryptosporidium* and *Giardia* in calves in Belgium

Chapter 3.1

Molecular characterisation of *Cryptosporidium* in calves in Belgium

3.1.1 Introduction

Several *Cryptosporidium* species and genotypes have been described in cattle: apart from the ubiquitous and zoonotic *C. parvum*, the ruminant specific species *C. bovis* and *C. andersoni*, and the *Cryptosporidium* cervine genotype are reported on a regular basis in cattle (Caccio *et al.*, 2005; Fayer *et al.*, 2006a). Occasionally *C. suis* (Fayer *et al.*, 2006b), *C. felis* (Bornay-Llinares *et al.*, 1999), *C. canis* (Fayer *et al*., 2001) and *C. hominis* (Smith *et al.*, 2005) are diagnosed, but the contribution of the latter species to the development of clinical symptoms in cattle is probably limited. Since *C. parvum* is most prevalent and clinical cryptosporidiosis mainly occurs in calves younger than 1 month, *C. parvum* is believed to be the most pathogenic species in calves, although comparative studies with related species have not yet been conducted. Human cryptosporidiosis can be due to infections with either the human specific *C. hominis* or the zoonotic *C. parvum*, and in a more limited number of cases with *C. meleagridis*, which is also zoonotic (Pedraza-Diaz *et al.*, 2001). Probably due to the more intensified contact between human activity and livestock industry, *C. parvum* is the main cause (57% on average) of human cryptosporidiosis in Europe, while in America, Africa and Australia *C. hominis* is most prevalent (62% on average) (Caccio *et al*., 2005). At least part of the human *C. parvum* infections can be ascribed to contact with livestock, either direct or indirectly through contaminated water or food. However, the identification of *C. parvum* in human stool samples does not conclusively implicate farm animals as a source of infection. Subgenotype analysis at the 60kDa glycoprotein locus (gp60) (Peng *et al.*, 2001; Alves *et al.*, 2003; Sulaiman *et al.*, 2005; Trotz-Williams *et al.*, 2006) and multilocus microsatellite genotyping (Caccio *et al.*, 2000; Mallon *et al.*, 2003), revealed both human specific and zoonotic subgenotypes within *C. parvum*. In most waterborne outbreaks, including the large Milwaukee outbreak in 1993, human sources of infection were identified (Peng *et al.*, 1997). Only in one Canadian waterborne outbreak could livestock be conclusively identified as the source of infection (Fayer *et al.*, 2000). Most of the isolates included in molecular characterisation studies are obtained from outbreaks. In sporadic cases of cryptosporidiosis the source of infection is often hard to trace, yet direct contact with infected livestock has repeatedly been associated with infection (Hunter and Thompson, 2005). Since the majority of human cryptosporidial infections are sporadic

cases (Feltus *et al*., 2006), the relevance of zoonotic transmission might be underestimated based on the data obtained from outbreaks.
The objective of the present study was to identify the *Cryptosporidium* species in dairy and beef calves in East Flanders, Belgium. Furthermore, samples from clinically affected calves were included to study the association between diarrhea and species or genotype. By subgenotyping of the *C. parvum* positive samples the potential of calves as a zoonotic reservoir for human infection was investigated.

3.1.2 Materials and methods

In order to have a representative view of the different *Cryptosporidium* species and subgenotypes in the calf population of East Flanders, *Cryptosporidium* positive isolates from dairy and beef calves from a cross-sectional epidemiological study (chapter 2) were selected for genotyping. Furthermore, samples from calves younger than 6 weeks with clinical cryptosporidiosis, sent to the diagnostic laboratory by practicing veterinarians, were withheld. DNA was extracted using the QIAamp® Stool Mini Kit (Qiagen) according to the manufacturer's instructions, although an initial step of 3 freeze-thaw cycles (freezing in liquid nitrogen for 5 minutes and heating at 95˚C for 5 minutes) was incorporated in the protocol to maximise oocyst lesion. The eluted DNA was dissolved in 15 µl ultra-pure water.

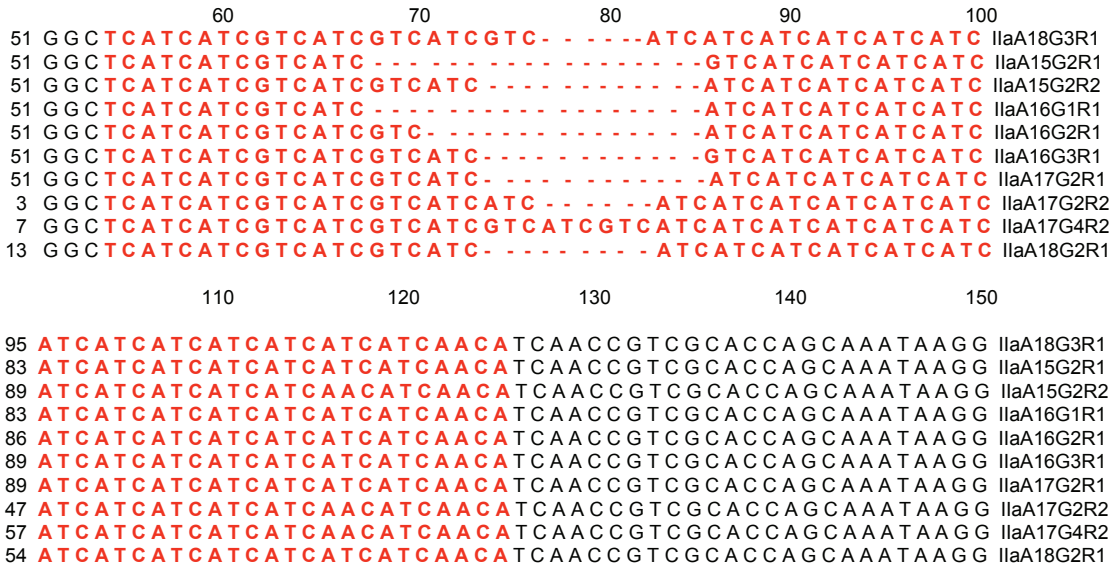
The primers used in the present study are presented in table 3.1. For the amplification of the *Cryptosporidium* 70 kDa heat shock protein (HSP-70) gene the primer pair HSP-F4 and HSP-R4 was used in the primary PCR, and HSP-F3 and HSP-R3 in the nested reaction. The PCR protocol was performed as previously described (Morgan *et al.*, 2001). For the *Cryptosporidium* 18S ribosomal DNA gene (18S rDNA) the primer pair 18SiCF2 and 18SiCR2 was used in the primary reaction, and 18SiCF1 and 18SiCR1 in the nested PCR reaction. Reactions were performed as described by Ryan *et al.* (2003). For the subgenotyping of *C. parvum* positive samples, the 60kDa glycoprotein (gp60) was targeted, using the primers AL 3531 and AL 3534 for the primary and AL 3532 and AL 3533 for the nested PCR. The reaction was performed as described by Peng *et al.* (2001). In all the above-mentioned reactions bovine serum albumin (BSA) was added to a final concentration of 0.1µg BSA/µl reaction mixture.

Table 3.1: Primers used for the molecular characteristation of *Cryptosporidium* isolates.

Forward (5'-3')	**Reverse (5'-3')**
HSP-F4 (GGT GGT GGT ACT TTT GAT GTA TC)	HSP-R4 (GCC TGA ACC TTT GGA ATA CG)
HSP-F3 (GCT GST GAT ACT CAC TTG GGT GG)	HSP-R3 (CTC TTG TCC ATA CCA GCA TCC)
18SiCF2 (GAC ATA TCA TTC AAG TTT CTG ACC)	18SiCR2 (CTG AAG GAG TAA GGA ACA ACC)
18SiCF1 (CCT ATC AGC TTT AGA CGG TAG G)	18SiCR1 (TCT AAG AAT TTC ACC TCT GAC TG)
AL 3531 (ATA GTC TCC GCT GTA TCC)	AL 3534 (GCA GAG GAA CCA GCA TC)
AL 3532 (TCC GCT GTA TTC TCA GCC)	AL 3533 (GAG ATA TAT CTT GGT GCG)

Amplification products were visualized on 1.5 % agarose gels with ethidium bromide. A positive (Plasmid DNA) and negative (PCR water) control sample was included in each PCR reaction. PCR products were purified using the Qiaquick® purification kit (Qiagen) and fully sequenced using 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 Seqman II (DNASTAR, Madison WI, USA).

Figure 3.1: Subgenotyping within *Cryptosporidium parvum* allele IIa based on the number of TCA (A), TCG (G) or rare (R) repeats in the 60kDa glycoprotein sequence



Sequences were compared with known sequences by blasting against the NCBI databank. Furthermore, phylogenetic analysis was performed on more conserved positions of the nucleotide alignment created with Muscle (Edgar, 2004). Editing of the alignment was performed with BioEdit (Hall, 1999). Distance matrices were calculated based on Poisson correction and trees were constructed with the neighbor-joining algorithm using the software packages TREECON (Van de Peer and De Wachter, 1994). Bootstrap analysis with 100 replicates was performed to test the significance of nodes. The nomenclature of the gp60 subgenotyping within allele IIa was based on the number of TCA (A), TCG (G) or rare (R) repeats (figure 3.1) within the amplified region containing the mini or microsatellite motifs (Chalmers *et al*., 2005)

The age of the *C. parvum* and the *C. bovis* positive calves was compared with a two-way Mann-Withney U test. Probability (P) values <0.05 were considered to indicate a significant difference.

3.1.3 Results

The number of samples and the results of the molecular characterisation are presented in Table 3.2. At least one isolate from the 32 *Cryptosporidium* positive dairy farms was included. Isolates from 9 of the 12 positive beef farms were included, since no amplification product could be obtained from isolates of the three remaining farms. Both in beef and in dairy farms *C. parvum* was predominantly identified, next to the cattle specific *C. bovis*. In the samples from the calves with clinical cryptosporidiosis only *C. parvum* was identified. In all *C. parvum* positive samples the subgenotype IIa (IIaA13G2R1 (n=1), IIaA14G2R1 (n=1), IIaA15G2R1 (n=81), IIaA16G2R1 (n=3)) was identified, except in one sample from a clinically affected calf, with the IId subgenotype (IIdA22G1). All but one of the calves infected with *C. bovis* were older than 4 weeks and the average age of the *C. bovis* positive calves was higher (P<0.001) than the age of the *C. parvum* positive calves. In one beef calf, the porcine specific *C. suis* was identified.

Table 3.2: Results of the molecular characterisation for *Cryptosporidium* in the dairy, beef, clinically affected calves and overall, with the age and age range (in days) for each species (n=total number of samples; HSP-70=70kDa Heat shock Protein; 18S=18S ribosomal DNA; gp60=60kDa glycoprotein; NA=No Amplification; nd=not done)

	n		HSP 70	18S	GP60	age (range)
Dairy	73	*C. parvum*	67	67	53 IIa	24.8 (3-68)
		C. bovis	6	6	nd	53.8 (22-69)
		NA	0	0	14	
Beef	11	*C. parvum*	7	6	5 IIa	18.4 (3-46)
		C. bovis	2	3	nd	51.3 (36-70)
		C. suis	1	1	nd	27
		NA	1	1	2	
Clinical	31	*C. parvum*	31	31	28 IIa+ 1 IId	<42
		NA	0	0	2	
Overall	115	*C. parvum*	105	104	86 IIa +1IId	
		C. bovis	8	9	nd	
		C. suis	1	1	nd	
		NA	1	1	18	

3.1.4 Discussion

Although human sources of infection are most often implicated in waterborne *Cryptosporidium* outbreaks, livestock and in particular young calves have to be considered as a potential source of human cryptosporidiosis, in outbreak related (Fayer *et al.,* 2000) and in sporadic cases (Goh *et al*., 2004; Feltus *et al*., 2006). Since not all *Cryptosporidium* species in cattle have zoonotic potential, molecular identification should be included in epidemiological studies to further elucidate the relevance of cattle in the epidemiology of human cryptosporidiosis (Traub *et al*., 2005). However, only a limited number of studies did combine prevalence estimation and molecular identification, both in the United States (Santin *et al*., 2004; Fayer *et al*., 2006a) and in Australia (Becher *et al*., 2004). In other studies molecular characterisation was performed on a limited (Hajdusek *et al*., 2004) or large number (McLauchlin *et al*., 2000; Peng *et al*., 2003) of *Cryptosporidium* isolates from calves, but not within the framework of a cross-sectional epidemiological study, which might lead to a selection bias towards clinical samples. The samples in the present study were collected within a cross-sectional epidemiological framework.

The guidelines proposed by Caccio *et al*. (2005) were taken into account, emphasizing that the genetic characterisation of *Cryptosporidium* isolates should be based on 2

genetic loci, of which at least one is the conserved 18S rDNA gene. For further subgenotyping the gp60 locus was targeted. The genotyping results of the HSP-70 and 18S rDNA gene concurred in all but two samples, in which one of the two genes did not amplify. The majority of the *Cryptosporidium* positive samples from the dairy and beef calves was identified as *C. parvum*. Since the calves included in the epidemiological study were all youger than 70 days, the current results are in accordance with previous findings that *C. parvum* is the most prevalent species in pre-weaned calves (Santin *et al*., 2004). The 70-days age limit also accounts for the limited number of *C. bovis* infections, and for the absence of *C. andersoni* or the *Cryptosporidium* cervine genotype in the present study. All but one of the *C. bovis* infections were identified in calves older than 1 month, confirming that *C. bovis* is more prevalent in older calves compared to neonatal calves (Santin *et al*., 2004). In 1 beef calf *C. suis* was found, indicating a rather erratic parasitism of this *Cryptosporidium* species in cattle (Fayer *et al*., 2006b). In the clinically affected calves which were all under 6 weeks of age, solely *C. parvum* was identified, confirming that neonatal cryptosporidial diarrhea in calves is primarily caused by *C. parvum* (de Graaf *et al.*, 1999; Santin *et al*., 2004).

Unlike previous reports (Tanriverdi *et al.*, 2003; Santin *et al.*, 2004), no mixed infections were found in the present study. The lack of mixed infections could be attributed to the exponential nature of PCR and the tendency of *C. parvum* to outgrow other *Cryptosporidium* species. To detect small *Cryptosporidium* subpopulations, a species-specific multiplex PCR or real-time PCR assay is preferred (Tanriverdi *et al.*, 2003).

Of the *Cryptosporidium* species commonly found in calves, only *C. parvum* is known to infect human patients. Within *C. parvum* anthroponotic and zoonotic subgenotypes have been described. The molecular analysis on the gp60 gene identified all but one of the *C. parvum* positive calf isolates as the zoonotic IIa allele, with predominantly subtype IIaA15G2R1. This allele subtype has previously been reported as most prevalent in calves in Portugal (Alves *et al*., 2006), in the UK (Chalmers *et al*., 2005), and in Northern America (Peng *et al*., 2003; Trotz-Williams *et al*., 2006; Xiao *et al*., 2007). Furthermore, this subtype is known to frequently infect human patients (Chalmers *et al.*, 2005; Sulaiman *et al*., 2005; Feltus *et al*., 2006), indicating the zoonotic reservoir function for these calves. In total, 4 different allele IIa subtypes have been identified in the present study. When the IIaA15G2R1 subtype is not found

other subtypes are more frequently identified (Trotz-Williams *et al*., 2006). The predominant occurrence of the IIaA15G2R1 subtype in humans raises some concerns about the selection of highly infectious strains by intensive animal husbandry systems (Xiao *et al*., 2007). The occurrence of subgenotype IId in calves is rare, but has previously been reported (Alves *et al.*, 2003, 2006). As expected, the anthroponotic *C. parvum* allele IIc was not found in any of the calves.

The present study therefore demonstrates a high prevalence of zoonotic *C. parvum* infections in calves in Belgium, and confirms that calves should be considered as a potential zoonotic reservoir for human infections.

Chapter 3.2

Molecular characterisation of *Giardia* in calves in Belgium

3.2.1 Introduction

The livestock specific assemblage E is the most prevalent *Giardia* species in cattle. However, the zoonotic assemblage A has been reported in up to 20% of either pre-weaned calves (O'Handley *et al.*, 2000; Appelbee *et al.*, 2003; Becher *et al.*, 2004; Berrilli *et al.*, 2004; Trout *et al.*, 2004; Itagaki *et al.*, 2005), post-weaned calves (Trout *et al.*, 2005), or adult cattle (Trout *et al.*, 2006; Uehlinger *et al.*, 2006). Occassionally, assemblage B was identified in calves (van Keulen *et al.*, 2002; Lalle *et al.*, 2005). In human patients both assemblage A and B are commonly identified, with assemblage B as most prevalent (Amar *et al.*, 2002; Read *et al.*, 2002; Traub *et al.*, 2004; van der Giessen *et al.*, 2006). A species-specific pathogenicity has been observed in human patients (Homan and Mank, 2001; Read *et al.*, 2002), but not in calves.

Although little is known about the frequency of transmission between cattle and man, zoonotic infections either occur by direct contact or indirectly, by the ingestion of contaminated water or food (Caccio *et al.*, 2005). Most studies seem to suggest that the public health risk from cattle is minimal, at least in Northern America and in Australia (Sulaiman *et al.*, 2004; Caccio *et al.*, 2005). Similar to *Cryptosporidium*, anthropozoonotic transmission is more likely to occur in endemic locations with closer contact between human and livestock sources of infection (Graczyk *et al.*, 2002; Uehlinger *et al.*, 2006). Transmission from cattle to man may therefore be more frequent in North-Western Europe, given the intensified cattle industry and close contact between agricultural and human activity. Studies from Europe indeed indicate a high prevalence of the zoonotic assemblage A in cattle, although the available data are scarce and were not collected within an epidemiological study (Berrilli *et al.*, 2004; Lalle *et al.*, 2005). Furthermore, the occurrence of mixed assemblage A/E infections in calves might be underestimated, since the exponential nature of PCR favours the amplification of the most abundant gene present in a sample. Due to the high frequency of transmission in calves, the host-specific assemblage E is likely to out-compete underlying assemblage A infections, as is described in dogs (Hopkins *et al.*, 1997).

Therefore, the different *Giardia* assemblages in calf samples collected in an epidemiological study in East Flanders (Belgium), were identified using the β-giardin gene, as described by Caccio *et al.* (2005). A novel assemblage-specific PCR was

developed based on the triose phosphate isomerase (tpi) gene, to study the occurrence of mixed infections in calves.

3.2.2 Materials and methods

In order to have a representative view of the different *Giardia* species or assemblages in the calf population of East Flanders, *Giardia* positive isolates from dairy and beef calves from a cross-sectional epidemiological study (chapter 2) were selected for genotyping. Furthermore, samples from calves younger than 1 year with clinical giardiosis were collected on farms with a *Giardia* outbreak (chapter 4.2), or sent to the laboratory by veterinarians in practice. DNA was extracted using the QIAamp® Stool Mini Kit (Qiagen) according to the manufacturer's instructions, although an initial step of 3 freeze-thaw cycles (freezing in liquid nitrogen for 5 minutes and heating at 95˚C for 5 minutes) was incorporated in the protocol to maximise cyst lesion. The eluted DNA was dissolved in 15 µl ultra-pure water.

Amplification of the β-giardin gene

For the amplification of the *Giardia* β-giardin gene the primer pair G7 and G759 was used in the primary PCR reaction, and G7n and G759n in the nested PCR reaction (Table 3.3). The PCR protocol was performed as previously described (Lalle *et al.*, 2005), with BSA added to a final concentration of 0.1µg BSA/µl reaction mixture. Amplification products were subsequently visualized on 1.5 % agarose gels with ethidium bromide. A positive (Plasmid DNA) and negative (PCR water) control sample was included in each PCR reaction. PCR products were purified using the Qiaquick® purification kit (Qiagen) and fully sequenced using 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 Seqman II (DNASTAR, Madison WI, USA). Sequences were compared to previously published sequences by blasting against the NCBI databank. The subgenotype analysis was based on alignment by ClustalW and comparison to the substitutions described by Lalle *et al*. (2005). Furthermore, phylogenetic analysis was performed on more conserved positions of the nucleotide alignment created with Muscle (Edgar, 2004). Editing of the alignment was performed with BioEdit (Hall, 1999). Distance matrices were calculated based on Poisson correction and trees were constructed with

the neighbor-joining algorithm using the software packages TREECON (Van de Peer and De Wachter, 1994). Bootstrap analysis with 100 replicates was performed to test the significance of nodes.

Table 3.3: Primers used for the molecular characteristation of *Giardia* isolates.

Forward (5'-3')	**Reverse (5'-3')**
G7 (AAG CCC GAC CTC ACC CGC AGT GC)	G759 (GAG GCC GCC CTG GAT CTT CGA GAC GAC)
G7n (GAA CGA ACG AGA TCG AGG TCC G)	G759n (CTC GAC GAG CTT CGT GTT)
AL 3543 (AAA TIA TGC CTG CTC GTC G)	AL 3546 (CAA ACC TTI TCC GCA AAC C)
Af (CGC CGT ACA CCT GTC A)	Ar (AGC AAT GAC AAC CTC CTT CC)
Ef2 (CCC CTT CTG CCG TAC ATT TAT)	Er (GGC TCG TAA GCA ATA ACG ACT T)

Amplification of the triose phosphate isomerase (tpi) gene

For the amplification of the *Giardia* tpi gene the primer pair AL 3543 and AL 3546 was used in the primary PCR. The PCR reaction was performed as described in Sulaiman *et al.* (2003), with BSA added to a final concentration of 0.1µg BSA/µl reaction mixture. In the nested PCR reaction new species-specific primers were used (figure 3.2). Assemblage A specific primers (Af and Ar: table 3.3) were designed based on Genbank sequences (AY368157 to AY368161; GIU57897 and AY655704), to amplify a 332 bp PCR product. Assemblage E specific primers (Ef2 and Er: table 3.3) were designed based on Genbank deposited sequences (AY228645 to AY228647; AY655705 to AY655706), for the amplification a 388 bp PCR product. The reaction mixture for the secondary species-specific tpi PCR consisted of a master mix containing 0.25µl Taq DNA polymerase, 0.5µl dNTP mixture, BSA a to a final concentration of 0.1µg BSA/µl reaction mixture, 2.5 µl standard Taq buffer, 10 pM of each primer and 2,5 µl of template DNA in total volume of 25 µl. Subsequent steps were initial denaturation for 10 min at 94°C, followed by 35 cycles of denaturation for 45s at 94°C, annealing for 45s at 60°C and an extension for 45s at 72°C. Amplification products were subsequently visualized on 1.5 % agarose gels with ethidium bromide. A positive (Plasmid DNA) and negative (PCR water) control sample was included in each PCR reaction.

To verify the specificity of the species-specific primers, a subset of the samples was selected for sequencing, both from the assemblage A and E positive samples. The PCR products were purified using the Qiaquick® purification kit (Qiagen) and fully

sequenced with 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 Seqman II (DNASTAR, Madison WI, USA). Sequences were identified by blasting against the NCBI databank.

Figure 3.2: Selection of the species-specific primers based on the alignment of the triose phosphate isomerase nucleotide sequences (a) forward primers (b) reverse primers

(a)

(b)

The age of the calves positive for the different *Giardia* assemblages was compared with a two-way Mann-Withney U test. Probability (P) values <0.05 were considered to indicate a significant difference.

3.2.3 Results

At least one isolate from 32 out of 48 *Giardia* positive dairy farms was included in the molecular study. For the beef farms, isolates from 21 of the 32 positive farms were included. From the isolates of the remaining farms, there was either not enough faeces from the positive samples to extract DNA or no amplification product could be obtained from the sample(s). The number of samples included and the results of the molecular characterisation on the β-giardin gene are presented in Table 3.4.

Table 3.4: Results of the molecular characterisation from the β-giardin gene for *Giardia* in the dairy, beef, clinically affected calves and overall, with the age and age range in days (n=total number of samples; %= percentage of samples identified as the respective Assemblage)

	n	Ass A	Ass E	age (range)
Dairy	49	29 (59%)	20 (41%)	39 (11-70)
Beef	44	7 (16%)	37 (84%)	39 (6-70)
Clinical	27	7 (26%)	20 (74%)	< 352
Overall	120	43 (36%)	77 (64%)	

Overall, the livestock specific assemblage E (64%) was predominantly identified by the β-giardin gene. In beef calves (84%) and in calves with clinical giardiosis (74%) the livestock specific assemblage E was most prevalent, whereas in dairy calves the zoonotic assemblage A (59%) was identified in the majority of the samples. In 25 of the 32 dairy farms (78%) and in 12 of the 21 beef farms (57%) assemblage A was identified in at least one isolate. Within assemblage A the subgenotypes A2 and A3 were identified, and within assemblage E the subgenotypes E2 and E3. There was no difference in the age of the calves infected with assemblage A or E ($P>0.05$).

The results of the species-specific tpi PCR are presented in table 3.5. For 19 samples amplified with the β-giardin gene no result could be obtained using the tpi PCR protocol, due to the absence of faecal material or lack of amplification. In the majority of the samples the identification of the β-giardin gene was confirmed by the identification of the tpi gene, either as mono-infection or as mixed assemblage A/E infection. In 3 dairy samples, assemblage A was found on the β-giardin gene, and assemblage E on the tpi gene (no mixed infection could be detected in these samples). In total, 69% of the samples identified as assemblage A and 20% of the samples

identified as assemblage E by the β-giardin gene, were diagnosed as a mixed infection by the tpi gene.

Table 3.5: Results of the molecular characterisation from the triose phosphate isomerase gene for *Giardia* in the dairy, beef, clinically affected calves and overall with the age and age range in days (n=total number of samples; %= percentage of samples identified as the respective Assemblage)

	n	Ass A	Ass E	Mixed Ass A/E	age (range)
Dairy	45	11 (24%)	17 (38%)	17 (38%)	39 (11-70)
Beef	31	2 (6%)	22 (71%)	7 (22%)	37 (6-64)
Clinical	25	3 (12%)	15 (60%)	7 (28%)	< 352
Overall	101	16 (16%)	54 (53%)	31 (31%)	

The specificity of the species-specific PCR was confirmed by the sequencing of species-specific PCR products from dairy, beef and clinical samples. Eighteen of the 47 samples (38%) yielding a positive amplification for the assemblage A specific PCR and 33 of the 85 samples (39%) yielding a positive amplification for the assemblage E specific PCR, were selected for sequencing. All assemblage A positive samples were confirmed as assemblage A, and all assemblage E positive samples were confirmed as assemblage E.

3.2.4 Discussion

Since not all *Giardia* species found in cattle are relevant in a public health perspective, molecular identification of calf isolates should be included in epidemiological studies to elucidate the zoonotic potential (Traub *et al*., 2005). In general, cattle are not considered as an important source for human giardiasis (Caccio *et al.*, 2003; Sulaiman *et al.*, 2004), due to the high prevalence of the livestock specific assemblage E in cattle, observed in the United States (Trout *et al*., 2004, 2005 and 2006), Canada (Applebee *et al*., 2003), New Zealand (Hunt *et al*., 2000) and in Australia (O'Handley *et al*., 2000; Becher *et al*., 2004). In Europe, either parasite prevalence was estimated without molecular characterisation (Castro-Hermida *et al.*, 2006; Hamnes *et al*., 2006; Maddox-Hyttel *et al*., 2006), or molecular characterisation

was performed on samples not collected within a cross-sectional epidemiological study design (Berelli *et al*., 2004; Lalle *et al*., 2005). This is the first molecular epidemiological study for *Giardia* in calves in Europe.

Whether the use of a single locus for the identification of *Giardia* is sufficient for epidemiological research, remains uncertain (Caccio *et al*., 2005). Therefore, two different genetic loci were targetted in the present study. The genotyping results of the β–giardin and the tpi gene agreed in all samples, except in 3 dairy calves. Overall, the majority of the *Giardia* positive samples was identified as assemblage E of *G. bovis*, confirming previous reports in the United States (Trout *et al*., 2004, 2005, 2006), Canada (Applebee *et al*., 2003), New Zealand (Hunt *et al*., 2000) and in Australia (O'Handley *et al*., 2000; Becher *et al*., 2004). However, a substantial part of the isolates was identified as the zoonotic assemblage A, especially in the dairy calves, which confirms the scarce European data indicating that assemblage A is prevalent in calves (Berrilli *et al.*, 2004; Lalle *et al.*, 2005). Furthermore, assemblage A was identified in 78% of the dairy farms and 57% of the beef farms included in the genotyping study, indicating a wide distribution of the zoonotic assemblage A. Similar to the present study, a wide distribution of assemblage A among farms was also reported in the United States (Trout *et al*., 2004). Contrary to previous reports (Lalle *et al.*, 2005; van Keulen *et al*., 2002), assemblage B was not found in the present study. Furthermore, mixed assemblage A/E infections were detected with the species-specific tpi PCR. Most (69%) of the assemblage A infections found with the β-giardin gene were found to be a mixed A/E infection with the tpi gene whereas only 20% of the β-giardin diagnosed assemblage E infections were identified as a mixed A/E infection with tpi, confirming that the livestock specific assemblage E is the most prevalent in calves. The absence of mixed infections in previous reports can be attributed to the exponential nature of PCR and the subsequent failure to detect small *Giardia* subpopulations. Hence, this is the first report of mixed *Giardia* assemblage A/E infections in calves.

The high prevalence of the zoonotic *Giardia* assemblage A infections in calves in East Flanders might be due to the proximity of intensified livestock industry to human activity, facilitating interaction between the human and livestock transmission cycle. The higher likelihood of anthropozoonotic transmission in an endemic focus has been described between the companion animal and the human transmission cycle. Dogs in

frequent contact with their owners tend to be infected with the zoonotic assemblage A, while dogs staying in packs are more likely to be infected with the dog specific assemblage D (Hopkins et al., 1997; Traub *et al*., 2004). Similarly, the more frequent occurrence of the zoonotic assemblage A in dairy calves compared to beef calves, both in the present study and in previous reports (O'Handley *et al*., 2000; Appelbee *et al*., 2003; Trout *et al*., 2004, 2005 and 2006), might be due to the seperation of dairy calves from the dam shortly after birth and the more frequent contact with human caretakers, whereas beef calves tend to stay with the dam. Contrary to *Cryptosporidium*, there was no correlation between infection with either assemblage and the age of the calf. Although post-weaned calves and adult cattle were not included in the present study, this seems to confirm previous findings (Trout *et al*., 2004, 2005, 2006).

Once introduced, assemblage A is able to maintain itself in a bovine population. Moreover, the occurrence of assemblage A infections in clinically affected calves seems to suggest that infections with assemblage A are not only transient infections (Caccio *et al*., 2005), but contribute to the development of clinical giardiasis in calves. Although mixed infections were not considered, a difference in clinical outcome has been described in human patients (Homan and Mank, 2001; Read *et al.*, 2002). Whether there is a difference in clinical outcome between assemblage A and assemblage E infections in calves, remains uncertain.

The present study demonstrates a high prevalence and wide distribution among farms of *Giardia* assemblage A infections, and the occurrence of mixed assemblage A/E infections in calves. The results suggest that calves, although primarily infected with the livestock specific assemblage E, are also infected to a large extent with the zoonotic assemblage A, and that calves should therefore be considered as a potential zoonotic reservoir for human infections. The occurrence of mixed infections also illustrates the hazard of evaluating the zoonotic potential of *Giardia* isolates from animal origin using molecular identification based on a single gene.

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Chapter 4

Treatment and control of *Giardia* infections in calves

Chapter 4.1

The efficacy of an oral treatment with paromomycin against an experimental infection with *Giardia* in calves

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Based on:

Geurden, T., Claerebout, E., Dursin, L., Deflandre, A., Bernay, F., Kaltsatos, V. Vercruysse, J., 2006, The efficacy of an oral treatment with paromomycin against an experimental infection with *Giardia* in calves. Vet Parasitol 135, 241-247.

4.1.1 Introduction

Infection with *Giardia* leads to villus atrophy and crypt hyperplasia mainly in the duodenum, resulting in an intermittent and mucous diarrhea (Ruest et al., 1997). Although the detrimental effects of giardiosis have never been experimentally examined in calves, in a lamb model a *Giardia* infection caused a decreased weight gain, impaired feed efficiency and reduced carcass weight (Olson *et al.,* 1995). Furthermore, there is clinical evidence that infection in calves is associated with diarrhea and ill thrift and therefore has a negative impact on growth performance (St. Jean *et al.* 1987; Xiao *et al*., 1993; O'Handley *et al*., 1999). *Giardia* infections in calves might also constitute a possible reservoir for human infection (chapter 3.2), although the non zoonotic assemblage E appears to be more prevalent in calves than the zoonotic assemblage A (Thompson and Monis, 2004; Trout *et al*., 2004).

Several compounds like metronidazole (St. Jean *et al*., 1987; Xiao *et al*., 1993), dimetridazole (St Jean et al., 1987), fenbendazole and albendazole (Xiao *et al*., 1996; O'Handley *et al*., 1997, 2000) have been used in the treatment of giardiasis in calves, achieving symptomatic improvement in all treated animals. Data on reduction in cyst excretion are only available for treatment with fenbendazole and albendazole. Both albendazole (20 mg/kg during three consecutive days) and fenbendazole (5-20 mg/kg during three consecutive days) were effective in significantly reducing the cyst excretion after treatment (Xiao *et al*., 1996; O'Handley *et al*., 1997, 2000).

A promising alternative for treatment is paromomycin, since it is poorly absorbed from the gastrointestinal tract and therefore well tolerated by calves (Grinberg *et al*., 2002). Paromomycin or aminosidin is a broad-spectrum amino-glycoside antibiotic, with well-known efficacy against several protozoan parasites like *Cryptosporidium* in calves (Fayer and Ellis, 1993; Mancassola *et al.*, 1995; Chartier *et al.,* 1996; Viu *et al*., 2000; Grinberg *et al*., 2002), *Histomonas* in chickens (Hu and McDougland, 2004) and *Giardia* in rats (Awadalla *et al*., 1995) and in humans (Wright *et al*., 2003). Paromomycin binds to the small subunit rRNA and inhibits protein synthesis, which has either a direct effect on *Giardia* or an indirect effect of nutrient withdrawal caused by the inhibition of bacterial protein synthesis and destruction of the bacterial flora (Edlind, 1989; Harder *et al*., 2001; Harris *et al*., 2001).

In the present study the efficacy of a five-day treatment with three different dosages of paromomycin against an experimental infection with *Giardia* was evaluated.

4.1.2 Materials and methods

Study design

Twenty-four male Holstein calves aged 3 to 5 weeks were screened upon arrival for the presence of Bovine Viral Diarrhea antigen in blood samples and in faecal samples for the presence of *Giardia* cysts using IFA, and *Eimeria* oocysts using the McMaster technique. Only healthy calves without diarrhea and negative for all pathogens were included in the study. All animals were individually housed in pens with concrete floors and walls, which prevented contact between neighbouring calves. The calves remained in the same pen from the day of arrival up to the end of the study. They received a commercial calf feed and milk. Straw bedding was added to the pens daily. This study was conducted in accordance with the VICH Guidelines for Good Clinical Practice and the E.U. Animal Welfare Directives.

Giardia cysts were purified from the faeces of a calf experimentally infected with a field strain. These cysts were confirmed by PCR (Caccio *et al*., 2002) to be Assemblage E. The cysts in the inoculum were enumerated based on multiple counts using IFA and diluted in water to obtain the same infection dose as described in a previous infection study (O'Handley *et al*., 2000). Eleven days before the start of the treatment (D-11), all calves were orally inoculated twice with a 12h interval, with a total infection dose of 10^5 *Giardia* cysts. From 5 days (D-6) after infection until D-2, faeces were collected daily from all calves to confirm the presence of cysts. Calves were blocked by descending order of D-2 cyst excretion and within each block calves were randomly assigned to one of the treatment groups or the control group (6 calves/group). The calves in the three treatment groups received an oral treatment with paromomycin sulfate (Gabbrovet® CEVA Animal Health) at a dose rate of 25, 50 or 75 mg/kg body weight (BW) respectively during 5 consecutive days (D0-D4). The paromomycin sulfate was diluted in water and given orally before the meal using a syringe. The control group received a placebo (water).

All animals were examined three times a week during three consecutive weeks after the start of the treatment for the presence of *Giardia* cysts in their faeces. Data on faecal consistency and general health were recorded during the entire duration of the study on a daily basis. The general health was described as either normal or abnormal. The faecal consistency was scored as either normal (1), pasty (2) or watery (3) and an average faecal consistency score was calculated on D6, D13 and D20. All calves were

weighed prior to treatment (D-1) and at the end of the study (D20) using a cattle-weighing scale with a calibration of 1kg. Calibrated weights were used to confirm the accuracy of the scale prior and after weighing.

Detection and enumeration of Giardia *cysts*

A quantitative direct IFA (Merifluor *Cryptosporidium*/*Giardia* kit; Meridian Diagnostics Inc., Cincinnati, Ohio) was developed by modifying the method of Xiao and Herd (1993). In short, one gram of the faecal sample was suspended in distilled water and strained through a layer of surgical gauze. After sedimentation for 1 hour and subsequent centrifugation of this sediment at 3000 *g* for 5 minutes, the obtained pellet was resuspended in distilled water up to a volume of 1 ml. After thorough vortexing, an aliquot of 20 µl was pippeted 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 cysts per gram faeces (CPG) was obtained by multiplying the total number of cysts on the smear by 50.

Assessment of efficacy

The treatment efficacy in this study was measured based on the reduction in cyst excretion for each treatment group compared to the control group and on differences in cumulative cyst excretion. The reduction in cyst excretion was calculated using the Henderson-Tilton formula (Henderson and Tilton, 1955):

$$100 \text{ X} \left[1 - \frac{\text{Ta X Cb}}{\text{Tb X Ca}}\right]$$

Tb & Ta being the geometric mean cyst count in the treated group before and after treatment, and Cb & Ca being the geometric mean cyst count in the control animals before and after treatment (Presidente, 1985). The Henderson-Tilton formula is considered to be the most appropriate when counts in the control group tend to decrease. Cumulative cyst counts (cysts per gram of faeces; CPG) were calculated for each animal using the trapezium rule, *i.e*. the average CPG of two sequential sampling days was multiplied by the number of days in between the sampling days. The cumulative CPG from the start of the experiment to each sampling day for each animal was then calculated by adding together the period cyst counts and used to calculate the geometric mean cumulative cyst excretion per group.

Differences in cyst excretion, faecal consistency and weight gain between groups were analysed using a Kruskal-Wallis test, followed by a Mann-Whitney test for pairwise comparison of each treated group with the control group. Probability (P) values <0.05 were considered to indicate significant differences.

4.1.3 Results

During and after treatment no adverse reactions were observed. All calves were negative for *Giardia* before the start of the study. The number of animals excreting cysts in the faeces prior and after treatment is presented in Table 4.1, along with the geometric mean (GM) and the range of the faecal cyst counts.

Table 4.1: The geometric mean (mean) and range of the faecal *Giardia* cyst excretion, and the number of animals (#) excreting cysts on each sampling day before and after treatment with paromomycin

	control			25 mg/kg			50 mg/kg			75 mg/kg		
	mean	**range**	**#**	**mean**	**range**	**#**	**mean**	**range**	**#**	**mean**	**range**	**#**
D-1	1828	100-30,550	6	2049	200-35,000	6	1660	50-29,700	6	1572	50-12,450	6
D2	205	0-43,350	4	11	0-3,000	2	8	0-50	3	0	0	0
D4	1270	100-39,300	6	7	0-1,200	2	3	0-50	1	0	0	0
D6	281	0-56,100	4	17	0-8,700	2	3	0-50	1	0	0	0
D9	530	0-35,000	5	21	0-19,000	2	5	0-5,400	1	0	0	0
D11	326	50-16,600	6	168	0-43,850	4	20	0-2,750	3	4	0-350	1
D13	476	50-8,700	6	353	0-17,200	4	13	0-4,150	2	7	0-250	2
D16	142	0-12,550	5	635	0-50,350	5	440	0-19,950	5	291	0-105,000	5
D18	738	0-2,450	6	648	0-44,200	5	188	0-2,850	5	142	0-24,750	4
D20	91	0-3,300	5	1225	0-27,600	5	15	0-3,200	2	372	0-14,700	5

Prior to treatment all animals were excreting *Giardia* cysts and there was no significant difference in cyst excretion between groups. In the group treated with 75 mg/kg, there was no cyst excretion until D11. In the groups treated with 25 and 50 mg/kg, two and one animal respectively excreted cysts during and shortly after treatment. In the final week of the observation period all animals, except 1 animal in the group treated with 75 mg/kg, were intermittently excreting cysts in the faeces.

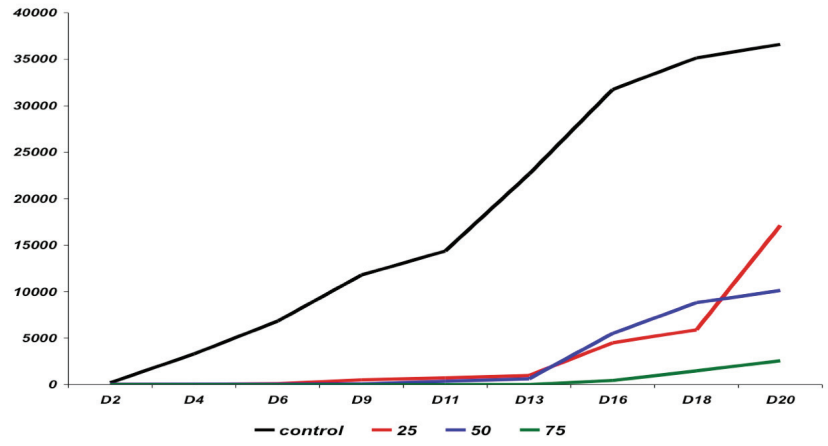
The reduction in cyst excretion calculated with the Henderson-Tilton formula, based on the GM cyst counts, is presented in Table 4.2. For the group treated with 75mg/kg there was a 100% reduction in cyst excretion until D9 and a ≥98% reduction until D13. For the group treated with 50 mg/kg, there was a ≥93% reduction until D13. In the group treated with 25 mg/kg there was a ≥94% until D9. From D16 onwards, the reduction in all treatment groups was low and discontinuous.

Table 4.2: Percentage reduction in geometric mean *Giardia* cyst counts calculated by the Henderson-Tilton formula in all treatment groups after treatment with paromomycin

treatment	D2	D4	D6	D9	D11	D13	D16	D18	D20
25 mg/kg	95.4	99.4	94.6	96.4	53.9	33.8	0.0	0.0	0.0
50 mg/kg	95.6	99.7	98.8	98.9	93.3	96.9	0.0	71.9	82.3
75 mg/kg	100	100	100	100	98.7	98.3	0.0	77.6	0.0

The GM cumulative cyst excretion for each group was calculated based on the individual cumulative cyst excretions and is presented in Figure 4.1. The total cumulative cyst excretion over the three-week period was highest in the control group. In the group treated with 25 mg/kg, the cumulative cyst count increased from D6 onwards. In the groups treated with 50 and 75 mg/kg, the cumulative cyst count increased from D13 onwards. The cumulative cyst excretion until D13 was significantly lower ($P<0.05$) in the groups treated with 50 (GM: 634 CPG; range: 0-23.350 CPG) and 75 mg/kg (GM: 9 CPG; range: 0-850 CPG) compared to the control group (GM: 22.652 CPG; range: 3.550-394.500 CPG). In the group treated with 25 mg/kg (GM: 991 CPG; range: 0-152.900 CPG), the cumulative cyst excretion was not significantly lower than in the control group.

Figure 4.1: The geometric mean cumulative *Giardia* cyst excretion after treatment with paromomycin at 25mg/kg (25), 50 mg/kg (50=), 75 mg/kg (75) and in the control group.



The general health was normal for all animals during the entire extent of the study. The faecal consistency scores were higher in the control group compared to the treated

groups, although there was no significant difference on D6, D13 and D20 (Table 4.3). The arithmetic mean weight gain was 8.2kg (± standard deviation: 3.4kg) for the control group and 8.9kg (±3.4kg), 9.0kg (±2.3kg) and 10.8kg (±2.6kg) for the groups treated with 25, 50 and 75 mg/kg respectively. However, there was no significant difference in weight gain between the treated and the control animals.

Table 4.3: The arithmetic mean (±standard deviation) of the faecal consistency scores on D0, D6, D13 and D20 for all groups (25= 25mg/kg; 50= 50 mg/kg; 75= 75 mg/kg; control= control group)

	DO	D6	D13	D20
control	1.3 (±0.54)	1.27 (±0.29)	1.36 (±0.37)	1.35 (±0.29)
25	1.17 (±0.41)	1.22 (±0.47)	1.24 (±0.37)	1.21 (±0.25)
50	1.5 (±0.51)	1.17 (±0.19)	1.09 (±0.07)	1.19 (±0.16)
75	1.18 (±0.41)	1.06 (±0.15)	1.1 (±0.17)	1.11 (±0.12)

4.1.4 Discussion

All animals in this trial were excreting cysts within 10 days after infection with 10^5 *Giardia* cysts, confirming previous experimental infection results using this infection dose (O'Handley *et al*., 2000). In other studies the efficacy was evaluated based on reduction of the geometric mean cyst counts and on clinical improvement (Xiao *et al*., 1996; O'Handley *et al*., 1997; 2000). However, since there are no official guidelines for efficacy assessment of treatment against protozoal infections in cattle, contrary to nematode infections (Vercruysse *et al*., 2001), we used the Henderson-Tilton formula, which takes the natural course of infection into account, and the reduction in cumulative cyst excretion, as a measure of the reduction in contamination of the environment. Furthermore, faecal consistency and weight gain were monitored as a measure of clinical improvement.

In the highest dosage groups (75 and 50 mg/kg) there was a very high reduction in cyst excretion until D13, which resulted in a significantly ($P<0.05$) lower cumulative cyst excretion in these treatment groups compared to the control group. All animals, except one animal in the group treated with 75 mg/kg, were re-excreting cysts within 2 to 3 weeks after treatment. A similar efficacy and post treatment infection pattern was observed for treatments with fenbendazole and albendazole (Xiao *et al.*, 1996; O'Handley *et al*., 1997; 2000). The re-occurrence of cyst-excretion within two weeks after treatment may be due to reinfection from the contaminated environment shortly

after treatment. *Giardia* has a prepatent period of only 4 to 10 days (Xiao and Herd, 1994; Olson *et al.,* 1995) and, contrary to other studies (Xiao *et al*., 1996; O'Handley *et al*., 1997), housing in this trial was not disinfected. Alternatively some *Giardia* trophozoites may have survived treatment (Xiao *et al*., 1996; O'Handley *et al*., 1997) resulting in a continuous passing of a limited number of cysts in the faeces shortly after treatment. This low cyst excretion might not have been diagnosed in the faeces, since the sensitivity of the IFA used in this study is not 100%.

Due to the high variation within the groups and to the limited number of animals per group, the short term cyst-suppressing effect of treatment did not result in a significantly better faecal consistency score or weight gain, although the animals in all treatment groups had an average weight that was between 0.7 and 2.6kg higher than in the control group. Furthermore, the short term cyst-suppressing effect of both paromomycin and benzimidazole products in a contaminated environment (Xiao *et al.,* 1996; O'Handley *et al.,* 2000) further emphasises the need for an integrated control program combining cleaning and disinfection of the environment with treatment, although even that might not guarantee total elimination of infection (Xiao *et al*., 1996). The higher efficacy at the end of the treatment (D4; >99%) in the groups treated with 25 and 50 mg/kg compared to efficacy on D2 (>95%) suggests that cleaning and disinfection should be performed at the end of the treatment period to further minimise risk of reinfection after treatment, rather than at the start of the treatment.

The present study demonstrates the efficacy and safety of a five-day treatment with paromomycin against an experimental *Giardia* infection in calves. The efficacy of a paromomycin treatment is comparable to the efficacy of both fenbendazole and albendazole treatments, although a dose confirmation study and field efficacy studies need to be conducted to further confirm these results.

Chapter 4.2

Field testing of a fenbendazole treatment combined with hygienic and management measures against a natural *Giardia* infection in calves

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Based on:

Geurden, T., Vercruysse, J., Claerebout, E., 2006, Field testing of a fenbendazole treatment combined with hygienic and management measures against a natural *Giardia* infection in calves. Vet Parasitol 142, 367-371.

4.2.1 Introduction

Several compounds such as fenbendazole and albendazole (Xiao *et al*., 1996; O'Handley *et al*., 1997, 2000), and paromomycin (chapter 4.1) can be used for treatment of giardiasis in calves. Fenbendazole (5-20 mg/kg bodyweight during 3 consecutive days) and paromomycin (50 or 75 mg/kg bodyweight during 5 consecutive days) resulted in a 100% reduction of the cyst excretion after treatment, although calves were re-excreting cysts within 2 to 3 weeks after treatment. Since *Giardia* cysts can survive for 1 week in cattle faeces and up to 7 weeks in soil (Olson *et al*., 1999), the effective treatment period of most treatment protocols (3-5 days) may be too short to prevent reinfection from a contaminated environment shortly after treatment. Furthermore, a high environmental infection pressure can result in an efficacy less than 100% from 1 week after treatment onwards, as in the albendazole trial of Xiao *et al*. (1996). Although in the fenbendazole trials of Xiao *et al*. (1996) and O'Handley *et al*. (1997) calves were housed in a clean and disinfected environment, leading to a 100% efficacy until at least 13 days after treatment, calves were re-excreting cysts after treatment, suggesting failure of disinfection, reinfection through faecal material on limbs or other parts of the calf or survival of *Giardia* trophozoites in the intestine (Xiao *et al*., 1996; O'Handley *et al*., 1997, 2000). The short term cyst-suppressing effect of treatment in a contaminated environment (Xiao *et al.,* 1996; O'Handley *et al.,* 2000) emphasises the need for an integrated control program combining treatment with cleaning and disinfection of the environment at the end of the treatment period to further minimise risk of reinfection after treatment. *Giardia* cysts are known to be resistant to commonly used desinfectants, such as chlorine. Alternative desinfectants, including chlorine dioxide, ozone and ultra violet irradiation have been the focus of research in drinking water treatment processes (Betancourt and Rose, 2004), although there are practical objections against most of these disinfection procedures for use in calf facilities. Alternatively, heat or desiccation (Olson *et al*., 1999) and disinfection with quaternary ammonium (Xiao *et al.,* 1996; O'Handley *et al*., 1997) can be used in calf facilities. In the present study, the efficacy of a combination of animal treatment with fenbendazole and environmental cleaning and disinfection with ammonia 10% or relocation was evaluated on ten commercial farms with a total of 94 animals.

4.2.2 Materials and Methods

Study design

Two studies were performed on 10 commercial farms, with a total of 94 animals. The farms were selected based on a history of chronic diarrhea, dull hair coat, ill thriving and impaired growth in calves between the age of 1 to 6 months (Figure 4.2).

Figure 4.2: Calves clinically affected by *Giardia:* (a) impaired growth and dirty hind quarters due to chronic diarrhea; (b) rough haircoat

a

b

The diagnosis of giardiasis was confirmed through identification of *Giardia* cysts in at least 50% of the animals. Coccidiosis was excluded on all these farms as a possible pathogen. The first study (study 1), which included 5 farms (farms 1 to 5) was performed during the winter of 2004-2005. In the second study (study 2) another 5 farms (farms 6 to 10) were visited during the winter of 2005-2006. On all farms animals were housed together in pens with concrete floor and bedding. Management consisted of adding straw to the bedding on a regular basis (2-3x/week), but the bedding was removed only once, twice or thrice a year, depending on the farm. Disinfection was not performed or with chlorine. Although treatment dosages as low as 5 mg fenbendazole per kg bodyweight are 100% effective in reducing *Giardia* cyst excretion in natural infections (O'Handley *et al*., 1997), a higher fenbendazole dosage (15 mg/kg during 3 consecutive days; Panacur® Intervet, Boxmeer, the Netherlands) was preferred in the present studies because the environmental infection pressure on these farms was considered to be high. One of two possible environmental measure protocols were implemented on the last day of treatment. The choice of the final day of the treatment period for the application of the environmental measures was inspired by observations that the reduction in cyst excretion is maximised at the end of the treatment period (chapter 4.1). The first protocol consisted of transfering the animals to a clean environment which had not been used for animal housing for at least 1 year (transfer protocol). The second protocol was removal of all bedding material, thourough high pressure cleaning with water, followed by complete drying of the housing and disinfection with an ammonia 10% solution (ammonia protocol). A previously described and evaluated immunofluorescence assay (IFA: Merifluor *Cryptosporidium*/*Giardia*; Meridian Diagnostics Inc., Cincinnati, Ohio) was used for detection of *Giardia* cysts (chapter 4.1). The IFA was used qualitatively in the first study and quantitatively in the second study, to calculate the percentage reduction. Clinical symptoms (diarrhea) and general appearance were evaluated at each sampling date and 4 months after the end of the treatment.

Study 1

On farms 2 and 3 animals were moved to a clean environment on the third day of treatment. No calves had been kept in these clean calf facilities at all (farm 2) or for at least one year prior to the treatment (farm 3). On farms 1, 4 and 5 the ammonia protocol was applied on the third day of treatment. Individual faecal samples were

collected from all animals prior to treatment (D-1) and weekly thereafter, until positive animals were identified or until four weeks after treatment (D7, D14, D21 and D28).

Study 2

On farm 6 animals were moved to a clean environment where no calves had been housed for at least one year prior to the treatment. On farms 7, 8 and 9 the ammonia protocol was applied. On farm 10 the treatment was not combined with environmental measures, and the animals remained in the contaminated environment. Based on the results of the first study faecal sampling in study 2 was limited to D21 and D28 after treatment. On the other hand, cyst excretion in study 2 was determined quantitatively instead of qualitatively. Geometric mean (GM) cyst counts were calculated at each sampling date and a percent reduction was calculated as following:

$$\%\text{Reduction} = \frac{GM_{before} - GM_{after}}{GM_{before}} \; x100$$

with GM_{before} being the geometric mean cyst count before treatment and GM_{after} being the geometric mean cyst count after treatment. The geometric mean cyst counts before (D-1) and after treatment (D21 and D28) were compared on each farm, using the Mann-Whitney test. Probability (P) values <0.05 were considered to indicate significant differences.

4.2.3 Results

Study 1

The results of study 1 are presented in table 4.4. Prior to treatment the percentage of positive animals present in the group housing varied between 50% and 85%. No cysts were detected during week 1 or 2 after treatment. Furthermore, no cysts were identified after treatment on farm 2 where animals were moved to a housing which was previously not used for calves. In the remaining farms cysts were detected within 3 weeks after treatment, although not in all animals excreting cysts prior to treatment. There was a noticable improvement in diarrhea towards D28 and at four months after treatment in all farms (data not shown).

Table 4.4: The total number of animals (n) on farms 1 to 10 and the number of animals excreting *Giardia* cysts before treatment (D-1) and on day 7 (D7), day 14 (D14), day 21 (D21) and day 28 (D28) after treatment, along with the treatment and environmental measures ('ammonia' or 'transfer' protocol).

Farm	n	D-1	D7	D14	D21	D28	treatment
1	5	4	0	0	0	2	**15 mg/kg 3d + ammonia**
2	10	5	0	0	0	0	**15mg/kg 3d + transfer**
3	7	4	0	0	3	3	**15 mg/kg 3d + transfer**
4	8	5	0	0	2	3	**15 mg/kg 3d + ammonia**
5	14	12	0	0	3	8	**15 mg/kg 3d + ammonia**
6	11	7	nd	nd	1	1	**15mg/kg 3d + transfer**
7	14	9	nd	nd	0	1	**15 mg/kg 3d + ammonia**
8	8	8	nd	nd	1	0	**15 mg/kg 3d + ammonia**
9	8	7	nd	nd	1	1	**15 mg/kg 3d + ammonia**
10	9	9	nd	nd	7	nd	**15mg/kg 3d**

nd = not done

Study 2

The results of farms 6 to 10 are presented in table 4.4 and 4.5. Since in the first study in none of the animals *Giardia* cysts were identified on D7 or D14 after treatment, it was concluded that D21 and D28 could be considered as adequate sampling days to identify cyst re-appearance after treatment. The percentage of positive animals varied between 64% and 100% prior to treatment (Table 4.4). Furthermore, the intensity of cyst excretion varied between animals of the same farm (Table 4.5).

Table 4.5: The geometric mean and range of the *Giardia* faecal cyst count (cyst per gram of faeces) on D-1 (GM D-1), D21 (GM D21) and D28 (GM D28) and the percent reduction (%) are presented, along with the treatment and environmental measures ('ammonia' or 'transfer' protocol).

Farm	GM D-1	GM D21	GM D28	red D21	red D28	treatment
6	28.4 (0-4,750)	0.4 (0-50)	0.4 (0-50)	98.0%*	98.0%*	**15mg/kg 3d + transfer**
7	109.5 (0-31,000)	0	0.5 (0-300)	100.0%*	99.5%*	**15 mg/kg 3d + ammonia**
8	2988 (150-40,200)	1 (0-300)	0	99.9%*	100.0%*	**15 mg/kg 3d + ammonia**
9	5074 (0-250,000)	0.9 (0-200)	0.9 (0-150)	99.9%*	99.9%*	**15 mg/kg 3d + ammonia**
10	1229 (50-17,050)	1122 (0-66,150)	nd	8.7%	nd	**15mg/kg 3d**

nd = not done; *significant ($P<0.05$) reduction

On farms 6 to 9 *Giardia* cysts were detected within 3 to 4 weeks after treatment in a limited number of animals (Table 4.4). The reduction in geometric mean cyst counts after treatment (D21 and D28) was very high on these farms (≥98.0%; $P<0.05$; Table 4.5). In contrast, on farm 10 a majority of the animals (7 out of 9) was excreting high numbers of cysts 3 weeks after treatment (Table 4.5) and excretion was not significantly lower ($P>0.05$) after treatment. Similar to the first study, diarrhea

improved in all farms towards D28 and at four months after treatment (data not shown), except in farm 10.

4.2.4 Discussion

These results illustrate the clinical importance of a natural *Giardia* infection in calves between the age of 1 to 6 months, since all farms reported problems of chronic and often mucous diarrhea, reduced weight gain and ill thrift. On *Giardia* positive farms most calves are infected when introduced into a contaminated housing (Xiao *et al.*, 1994) and start excreting up to 10^6 cysts per gram of faeces (Xiao *et al.*, 1994), resulting in a rapid increase of the environmental infection pressure. Because only a low number of *Giardia* cysts is needed for infection (Caccio *et al*., 2005) and a specific immune response against *Giardia* infection develops slowly in ruminants (Yanke *et al*., 1998; O'Handley *et al*., 2003), it has been suggested that environmental disinfection in combination with animal treatment is necessary (Xiao *et al*., 1996; O'Handley *et al*., 1997, 2000). This was confirmed by the results of farm 10 where housing was not disinfected after treatment. Calves were re-excreting high numbers of cysts within three weeks after treatment and there was no effect of treatment on clinical symptoms.

Both in the first and in the second study the combination of treatment with 15mg fenbendazole per kg bodyweight and the ammonia protocol on the third day of treatment resulted in a total suppression of cyst excretion during at least 2 weeks. An alternative way of coping with the high environmental infection pressure was to move the calves to a *Giardia* free environment, as suggested in the albendazole trial of Xiao *et al*. (1996) where the high efficacy 6 weeks after treatment was probably due to the transfer of the calves to pasture 2 weeks after treatment. The relocation of the calves to a clean environment resulted in a similar cyst excretion pattern compared to ammonia protocol. Moreover, the relocation of the animals on farm 2 to pens which were never used before to house ruminants, resulted in a 100% reduction of excretion for 4 weeks, indicating that the environment should be considered as the most important source for reinfection. Both environmental protocols resulted in a significant ($P<0.05$) reduction of the cyst excretion during at least 4 weeks after treatment, leading to a low environmental infection pressure and preventing clinical symptoms for at least four months after treatment.

The need for a combination of animal treatment and environmental measures against a natural *Giardia* infection in calves between the age of 1 to 6 months is emphasized by the present results. Treatment with fenbendazole at 15mg/kg during 3 consecutive days combined with the ammonia protocol or relocation of the animals on the third day of treatment did result in a significant ($P<0.05$) reduction of cyst excretion until at least 4 weeks after treatment and in a noticable improvement of clinical symptoms. Although there are no scientific data on the cost-effectiveness of a treatment against *Giardia* in calves, the results of the present study indicate that treatment leads to a noticable improvement of the clinical symptoms caused by a *Giardia* infection, and hence contribute to an improved general health and production parameters.

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Chapter 5

General discussion, future perspectives and conclusions

5.1 Introduction

The main objective of the present thesis was to obtain better insight in the molecular epidemiology of *Cryptosporidium* and *Giardia* in calves in Belgium, and to optimize the treatment of clinical giardiosis in calves. More specifically, the aim in chapter 2 was to obtain a reliable estimate of the parasite prevalence in both dairy and beef calves, based on a Bayesian estimation of test characteristics. In chapter 3, existing and novel tools for molecular characterisation were applied on a selection of parasite positive calf samples to elucidate the distribution of species, genotypes and subgenotypes of *Cryptosporidium* and *Giardia* in the dairy and beef calf population. Finally in chapter 4, the efficacy of a treatment with paromomycin was evaluated in an experimental study design, and of fenbendazole in combination with additional environmental measures under natural conditions.

In the present chapter, the advantages and disadvantages of the applied methodology are discussed. Last but not least, the general conclusions and future perspectives are presented.

5.2 Prevalence estimation

In chapter 2, the prevalence of *Cryptosporidium* and *Giardia* in calves was studied in the province of East Flanders. To estimate the parasite prevalence in a population, a cross-sectional study design is preferred (Greiner and Gardner, 2000), although this methodology can underestimate the parasite prevalence under specific conditions, such as intermittent or seasonal variations in (oo)cyst excretion (O'Handley *et al*., 1999). Prevalence estimates are not only affected by parasite or host factors, but also by the characteristics of the diagnostic tests applied in the study (Greiner and Gardner, 2000; Basanez *et al*., 2004). Although some of the assays used in the present study were previously evaluated for use in human medicine, for clinical diagnosis or in experimental validation studies, general scepticism is appropriate when extrapolating test parameters beyond the limits of the validation study. Furthermore, test evaluation in experimental settings typically results in an overestimation of the test characteristics (Greiner and Gardner, 2000). Therefore, the sensitivity and specificity of the different assays was evaluated for the diagnosis of infection in calf samples collected within an epidemiological study design.

Test are traditionally evaluated by a frequentist approach using contingency tables. In the frequentist approach, test characteristics are estimated based on the 'true disease' status of the animals, as determined by one or a combination of gold standard reference assays. Since even widely accepted gold standards are subject to human or other sources of misclassification errors, the results might be biased in favour of the gold standard reference test. In the present study, a Bayesian approach was preferred over a frequentist approach, since it proved its value in circumventing the gold standard problem (Dorny *et al*., 2004). A further advantage is that full posterior distributions for the parameters of interest (i.e. prevalence and test characteristics) are generated, while a frequentist approach only provides the best estimate of the parameter and a standard error. Further differences between the frequentist and a Bayesian statistical approach are reviewed in more detail by Basanez *et al*. (2004).

In a Bayesian analysis, inferences on prevalence and test characteristics are based on the data, being the cross-classified test results of at least three diagnostic assays, and on prior information, which comprises previously published estimates or expert opinion on model parameters (Suess *et al*., 2002; Branscum *et al.*, 2005). Since the data at hand are established in the experiment and are hence fixed values, the prior information does influence to a large extent the posterior distributions. The incorporation of prior information has therefore been the most controversial element of Bayesian analysis, although there is no alternative to constrain the space on certain parameters, allowing estimation of remaining parameters. A good Bayesian analysis should therefore include a judgement on the importance of the prior information (Dunson, 2001; Basanez *et al*., 2004; Toft *et al*., 2005).

The prior information included in a Bayesian model can be either deterministic or probabilistic. A deterministic constraint sets the parameter at a particular value, whereas a probabilistic constraint specifies a prior distribution for that parameter (Berkvens *et al*., 2006). Some authors advocate the use of sensitivity analysis based on expert opinion to verify the validity of the prior information. A disadvantage of this approach is the bias of the prior information, since the sensitivity analysis is solely based on information previously obtained in contingency studies or on subjective expert opinion (Enoe *et al*., 2000). More recently, an alternative approach was introduced, using the DIC, p_D and the Bayes-p to verify whether the prior information is in conflict with the data, and to quantify the effect of these prior

constraints on the number of effectively estimated parameters (Berkvens *et al.*, 2006). The latter approach was used in the present study.

In chapter 2, the use of wide range constraints (Dunson, 2001) was sufficient to reduce the number of parameters to be estimated, and did not influence the posterior distributions in the 3-test model for *Giardia* and the 4-test model for *Cryptosporidium*. In the *Cryptosporidium* 6-test model however, the use of more stringent constraints was necessary to reduce the high number of parameters. The use of stringent parameters in the six test model directed the Bayesian model and the parameter estimates towards the results of the PCR assays. In the present study, the validity of the previously published prior information on the specificity of both PCR assays (Morgan *et al.*, 1997; Spano *et al.*, 1997) was verified and confirmed by sequencing randomly selected PCR amplification products. Although the posterior distributions in the six-test approach were largely driven by the rigid prior information, these results still illustrate the major advantage of the Bayesian approach over the frequentist approach: the ability to include previously tested assumptions (=prior information) on certain, but not all parameters, while in the frequentist approach, all parameters describing the sensitivity and specificity of the gold standard reference test are inherently set at value 1.

Since the posterior estimates are not rigid parameters, but best-possible estimates based on the observations and the prior information (Dorny *et al.*, 2004), inconsiderate extrapolation of results beyond the limits of the study cannot be made. Since the data at hand resulted from an epidemiological study including both calves with and without clinical symptoms, and depended to a large extent on the choice of diagnostic test, the posterior estimates of the test characteristics must be interpreted taking these limits into account. The low sensitivity estimates of the conventional techniques in the six-test model are mostly due to the low excretion of *Cryptosporidium* oocysts in calves older than one month and to the higher sensitivity of PCR compared to more conventional techniques. It does however not imply that these conventional techniques are not reliable for clinical diagnosis, since clinical symptoms are mostly diagnosed in calves younger than one month and correlate with an increased oocyst excretion. The usefulness of different conventional diagnostic assays was confirmed in a recent study to evaluate test characteristics for the detection of *Cryptosporidium* in faeces of clinically affected calves (Geurden *et al.*,

unpublished results). Sensitivity and specificity should therefore not be considered as values intrinsic to the diagnostic test (i.e. constant and universally applicable), but as variables dependent on parasite prevalence and intensity of excretion (Berkvens *et al*., 2006).

5.3 Molecular characterisation and the zoonotic reservoir function of calves

A substantial part of the *Cryptosporidium* and *Giardia* research in the last decade focussed on taxonomy and on the development of molecular genotyping tools. As reviewed by Caccio *et al*. (2005), molecular tools are now available for both parasites at different levels of specificity, and the application of these tools in epidemiological research has been advocated to obtain better insight into the transmission dynamics (Traub *et al*., 2005). Most molecular studies in calves were however not conducted within the limits of an epidemiological study, possibly biasing the genotyping results towards a clinical perspective. In an epidemiological context and from a public health perspective, a representative sample of the calf population is preferred for genotyping, including both clinically affected and asymptomatic carrier animals, in order to obtain a reliable estimate of the prevalence and the distribution of the respective isolates. Therefore, positive samples from the epidemiological study (chapter 2) were selected to ensure a wide spectrum of farms and isolates. Furthermore, previously described primers and PCR protocols were selected for genotyping, based on their reliability and specificity as genotyping tools (Caccio *et al*., 2005), and on the availability of a sufficient number of reference sequences in the NCBI Genbank. By doing so, the results of the present molecular study could be compared with previous studies in Northern America, Australia and New Zealand, and viewed in a broader perspective. For *Giardia*, 2 new primer pairs were developed for the species-specific amplification of assemblage A and assemblage E isolates. A similar approach has been described for the identification of assemblage A and B in human samples (Bertrand *et al*., 2005).

The molecular identification of *Cryptosporidium* confirmed that calves are a potential zoonotic reservoir for human cryptosporidiosis. Despite the frequent identification of zoonotic species and subgenotypes, calves are considered to be less important than human sources in the epidemiology of human cryptosporidiosis (Caccio *et al*., 2005;

Smith *et al*., 2006). However, several important epidemiological factors have possibly been neglected. Although approximately 90% of the human cases of cryptosporidiosis are not outbreak related (Feltus *et al*., 2006), most molecular information from human isolates originates from outbreaks, in which human sources of infection are known to be most important (Peng *et al*., 1997). The continuous selection of outbreak related isolates might bias the results of molecular characterisation studies towards *C. hominis* or human specific *C. parvum* subgenotypes. Furthermore, in most human outbreaks of cryptosporidiosis, infections are due to a limited number of infection sources, again biasing the results towards an epidemic population structure (Mallon *et al*., 2003). Therefore, the relevance of zoonotic subgenotypes of *C. parvum* in the aetiology of sporadic cases of human cryptosporidiosis is possibly underestimated. In sporadic cases of human cryptosporidiosis, which are often not identified as such (hence the limited number of reports in the medical literature), it is less clear which subgenotypes are prevalent, and to what extent calves contribute to these sporadic infections. Despite the overall bias towards outbreak related samples, *C. parvum* and not *C. hominis* has been shown to be the major cause of human cryptosporidiosis in Europe (Caccio *et al*., 2005). Although it is not clear if zoonotic subgenotypes of *C. parvum* contribute to sporadic cases of human cryptosporidiosis in Belgium, the results of the present study indicate that young calves mainly harbour isolates with a zoonotic potential and are a possible reservoir for human infection.

The high *Giardia* prevalence in calves, the wide distribution among farms of infections with the zoonotic assemblage A, and the occurrence of mixed assemblage A/E infections in calves were not expected, taking previous reports in the United States (Trout *et al*., 2004, 2005, 2006), Canada (Appelbee *et al*., 2003), New Zealand (Hunt *et al*., 2000) and in Australia (O'Handley *et al*., 2000; Becher *et al*., 2004) into account. The results seem therefore to suggest that geographical differences in the prevalence of Assemblage A in calves might occur. Geographical differences were also observed in pre-weaned calves in the United States (Trout *et al*., 2004), although not confirmed in post-weaned (Trout *et al*., 2005) and adult cattle (Trout *et al*., 2006). The current results seem therefore to renew the zoonotic relevance of calves in the epidemiology of giardiosis, which is in contradiction with previous opinions based on data from Northern America and Australia (Olson *et al*., 2004; Caccio *et al*., 2005).

The identification of zoonotic lineages in the majority (for *Cryptosporidium*) or in a substantial part (for *Giardia*) of their parasite population indicates that dairy and beef calves are a potential reservoir for human infections. The relevance of calves in the epidemiology of human infections is however mainly determined by the frequency of transmission to humans. The opportunity for interaction between the livestock and the human transmission cycles is probably less in scarcely populated compared to densely populated agricultural areas with intensified cattle industry, such as East Flanders. Such a densely populated rural region can be considered as a large endemic focus, in which transmission between different reservoirs is likely to occur. Zoonotic transmission of *Cryptosporidium* between different reservoir populations in endemic foci, such as farms, has been described (Miron *et al.*, 1991; Lengerich *et al.*, 1993; Evans and Gardner, 1996; Sayers *et al.*, 1996). Similarly for *Giardia*, transmission of zoonotic assemblages is likely to occur more frequently between dogs and their owners in urban areas than in rural areas (Traub *et al*., 2004). In cattle a high prevalence of the zoonotic assemblage A has been reported in Uganda, due to contact with park rangers (Graczyk *et al*., 2002), and in a bovine teaching herd (Uehlinger *et al.*, 2006a).

Most of these reports consider rather small endemic foci (e.g. farms or dog loving households). However, the same assumptions of facilitated transmission might be valid on a larger scale, on condition that appropriate vehicles for transmission are at hand. In the UK, the spring peak in human cryptosporidiosis coincides with the lambing and calving season and is almost exclusively due to *C. parvum* (McLaughlin *et al*., 2000); in Scotland the foot and mouth disease outbreak in 2001 resulted in a decrease in the number of human cryptosporidiosis cases (Strachan *et al.*, 2003); and in New Zealand the proportion of human *C. parvum* infections in rural areas was found to be higher than in urban areas (Learmonth *et al*., 2004). For *Giardia* a significant association was observed between human giardiosis and both livestock density and manure use on agricultural land (Odoi *et al*., 2004).

The facilitated transmission in a large endemic focus occurs when an appropriate vehicle for infection is present, such as water. The (oo)cysts of both parasites are extremely resistant in water, and are not retained by the commonly used disinfection and filtration procedures (Caccio *et al*., 2005). Furthermore, pasture run-off from livestock is known to be an important source of contamination of drinking water

resources (Sischo *et al*., 2000), especially in the calving season (Bodley-Tickell *et al.*, 2002). Studies attempting to correlate (oo)cyst prevalence in drinking water to the prevalence of disease in man do however not reveal a clear relationship, although the lack of correlation could be masked by several other factors, such as differences in host immunity (Savioli *et al*., 2006).

Next to water, food is an important vector for infection with *Cryptosporidium* or *Giardia,* given the all-year round demand of soft fruit and salad vegetables from around the world, including agricultural production areas with more limited resources for manure management or water treatment. The rapid and cooled transport increases the likelihood of (oo)cyst survival on the food surface, and the subsequent foodborne transmission. In contrast, a negative association between consumption of raw vegetables and infection has been reported and is ascribed to repeated exposure to low infection doses and the subsequent acquisition of immunity (Hunter *et al*., 2004). In community settings with infrequent exposure to infection and hence limited immunity, foodborne transmission through consumption of raw vegetables might however be succesfull, given the limited number of (oo)cysts required for infection.
Fresh-water and marine shellfish are also well known vehicles for transmission, since shellfish concentrate (oo)cysts from contaminated water. Hence, they are good indicators of the infection load in water, but also an important foodborne source for infection (Smith *et al*., 2006). Livestock might contribute to this foodborne and waterborne transmission through spreading of manure onto the fields, and the subsequent direct contamination of crops, or the indirect contamination of surface water through water run-off from manure-laden fields (Olson *et al*., 2004).
Although the potential of calves as zoonotic reservoir for human infection is clear, the frequency and the routes of zoonotic transmission still require further epdidemiological research, preferably in longitudinal studies in well defined endemic foci, using state of the art genotyping and subgenotyping tools (Caccio *et al*., 2005; Smith *et al*., 2006).

5.4 Treatment and prevention of giardiosis in calves

Therapeutic treatment

In Europe, there are no chemotherapeutic compounds registered for the treatment of a *Giardia* infection in calves. From a public health point of view, treatment prevents environmental contamination and possible zoonotic transmission, and from a veterinary perspective treatment can prevent diarrhea and retarded growth. Although the pathogenicity of *Giardia* was reported almost 20 years ago (St. Jean, 1987) and the results of chapter 4 confirm the clinical relevance, *Giardia* is often ignored as a pathogen and as a differential diagnosis for coccidiosis in calves older than 1 month. The study of the treatment with fenbendazole against a natural *Giardia* infection in calves (chapter 4.2), emphasized the need for a combination of animal treatment and environmental measures, but also illustrates the increasing awareness of the pathogenic importance of *Giardia,* since the farms included in the study were visited on demand of the practicing veterinarians.

The experimental study in chapter 4 indicated that treatment with an intermediate or a high dosage of paromomycin is effective against an experimental infection with *Giardia* in calves. The efficacy of paromomycin is comparable to fenbendazole and albendazole under similar experimental conditions (Xiao *et al*., 1996; O'Handley *et al*., 1997, 2000). However, a positive control group treated with BZD was not included in the present dose determination study, and efficacy studies should be compared with caution, since there are no official guidelines for the evaluation of anti-protozoal drugs in cattle, contrary to the evaluation of anthelmintic treatment (Vercruysse *et al*., 2001).

Cyst excretion re-occurred within 13 days in the experimental study (chapter 4.1), and within 4 weeks in the study under natural conditions (chapter 4.2). Although some *Giardia* trophozoites may survive treatment (Xiao *et al*., 1996; O'Handley *et al*., 1997), the excretion after treatment is mainly due to re-infection with cysts from the environment. Since both paromomycin and fenbendazole lack persistent efficacy, calves are re-infected shortly after drug withdrawal. A curative treatment should therefore include hygienic measures to minimize exposure after treatment to infective oocysts from the environment. Since the environmental infection pressure is generally high on farms with a clinical outbreak of giardiosis, re-infection post treatment often

results in the re-occurrence of clinical symptoms. In the study with naturally infected calves, the hygienic measures resulted in a decrease of the environmental infection pressure, to the extent that clinical symptoms were no longer observed after treatment.

In human medicine several chemotherapeutic compounds are commonly used, such as tinidazole, metronidazole, mebendazole and quinacrine. Furthermore, nitazoxanide is proving to be usefull and promising in the treatment of both cryptosporidiosis and giardiosis (Fox and Saravolatz, 2005; Fung and Doan, 2005). However, treatment failures have been reported with all commonly used antigiardial drugs (Whright *et al*., 2003), and none of these compounds have been or are being registered for use in calves. Recently, the antigiardial activity of several plant extracts and spices, such as peppermint and curcumin, was reported both *in vitro* and *in vivo* in mice (Barbosa *et al*., 2006, 2007; Perez-Arriaga *et al*., 2006; Vidal *et al*., 2007). Although they may be promising alternatives for chemoterapeutic treatment, the efficacy of these compounds in calves was however never studied.

Prevention

For the prevention of clinical outbreaks on *Giardia* positive farms, prophylaxis based on treatment is probably not effective. Contrary to the prevention of cryptosporidiosis, the strategic treatment time is unpredictable, due to the prolonged susceptibility of calves to infection with *Giardia* (O'Handley *et al*., 2003). Prevention therefore relies on hygienic and management measures. Since the epidemiology of *Cryptosporidium*, *Giardia* and *Eimeria* spp. is similar, the prevention of giardiosis should fit in a farm based approach to reduce the occurrence of protozoal diarrhea in calves. Despite the lack of well-defined risk factors triggering a clinical outbreak of giardiosis, the general principles of the environmental and management measures are reviewed in table 1.3.

Vaccination could provide an alternative for chemotherapeutic treatment. The efficacy of vaccination is defined by multiple factors, such as the antigen presentation, the ability of the host to mount a protective immune response, the environmental infection pressure, and the route and time of vaccine administration (Olson *et al*., 2000). For *Cryptosporidium*, the efficacy of an active vaccination is limited due to the blocking effect of maternal antibodies, and to the difficulty of mediating a significant

protection against clinical cryptosporidiosis within a month after parturition. Therefore, passive immunisation through transfer of colostral antibodies was found to be more effective. Due to the rapid development of a specific cellular immune response against *Cryptosporidium* in calves, the colostral immunity is sufficient to protect the susceptible calf during the first weeks of life (de Graaf *et al*., 1999).

In contrast, immunity against *Giardia* is mainly humoral (Faubert, 2000) and develops slowly in ruminants (O'Handley *et al*., 2003). Although colostrum contains specific antibodies against *Giardia*, they are depleted within 2 weeks after parturition, whereas clinical symptoms mainly occur in calves older than 1 month. Active immunisation seems therefore more appropriate for the prevention of giardiosis. In the United States a *Giardia* vaccine is commercially available for use in cats and dogs, but the efficacy of a preventive (Olson *et al.*, 1996) or curative (Olson *et al.*, 2001; Payne *et al.*, 2002; Stein *et al.*, 2003; Anderson *et al*., 2004) vaccination was found to be variable. In calves, a preventive vaccination against an experimental infection with *Giardia* did not result in a significant protective immune response (Uehlinger *et al.*, 2006b). This might be due to the antigenic variation in *Giardia,* the high infection dose used in the experiment (10^5 cysts) or the slow immune response in ruminants. Although studies in mice and naturally infected human patients indicate that humoral immunity, mainly IgG and IgA antibodies, contribute to the elimination of *Giardia* trophozoites from the intestine, both calves and lambs did not mount a significant humoral immune response against infection with *Giardia* (Yanke *et al*., 1998; O'Handley *et al*., 2003). Further research into the immune response against *Giardia* in ruminants is therefore required to elucidate the possibility of mediating a protective immune response by vaccination.

5.5 Future perspectives

In chapters 2 and 3 we have clearly demonstrated that *Cryptosporidium* is highly prevalent in dairy and to a lesser extent in beef calves. Most of the isolates found in these calves were identified as zoonotic subgenotypes of *C. parvum*. Similarly, a high *Giardia* prevalence was found both in the dairy and beef calves. Although the majority of the isolates was livestock specific, the zoonotic assemblage A constitutes a considerable proportion of the parasite population in calves, either as a single or as a mixed infection. To further elucidate the transmission between cattle and man, a

longitudinal study in one or more endemic foci is required, based on molecular identification of bovine and human isolates. Since water is a major vehicle for infection, raw and treated surface water, and drinking water should be monitored. The environmental risk factors contributing to the transmission dynamics in an endemic focus can be studied using the Geographic Information System (GIS) technology, which enables to incorporate both geographically referenced information and information on weather patterns into a risk factor analysis (Odoi *et al*., 2004). Furthermore, the relevance of other farm animals and companion animals in the epidemiology of human infections, and the prevalence and distribution of the respective isolates in sporadic cases of human cryptosporidiosis and giardiosis, needs to be determined.

Since chemotherapeutic treatment is not suitable, prevention of clinical giardiosis should rely on management and environmental measures. Several studies have identified multiple, but often contradictory risk factors for infection (Garber *et al*., 1994; Mohammed *et al*., 1999; Castro-Hermida *et al*., 2002), possibly due to the rather small number of farms in relation to the high number of risk factors included in those studies. Therefore, longitudinal studies on infected farms should be conducted to identify these factors contributing to outbreaks of clinical infection. Given the large number of possible risk factors and the interaction between risk factors, a large number of farms should be monitored.

For the prevention of infection, vaccination constitutes a valid alternative. The efficacy of vaccination is, among other factors, defined by the ability of the host to mount a protective immune response. Due to the luminal localization of the parasite, the adaptive immune response against *Giardia* requires the effector molecules to reach the lumen in sufficient quantities to be effective, and is believed to be locally generated by the mucosa-associated lymphoid tissue. The immune response against *Giardia* is mostly studied in *in vitro* studies, in murine animal models and in a small number of ruminant models. In these ruminant models *Giardia* specific serum IgG and IgM antibody titres in infected animals did not significantly differ from control animals (Yanke *et al*., 1998; O'Handley *et al*., 2003), although serum IgM antibodies did rise in the early stage of infection. Similar to murine models an elevated serum IgA response was observed in infected lambs (Yanke *et al*., 1998). The authors

concluded that ruminants did not mount a rapid humoral immune response against *Giardia* infection, ignoring that serum and local IgA responses are not always well correlated. A ruminant-based infection model could therefore help in further elucidating the adaptive immune response against *Giardia,* and to finetune the vaccination strategy.

5.6 General conclusions

The results of the present study indicate that both *Cryptosporidium* and *Giardia* occur frequently in calves, and that calves should be considered as a reservoir for human cryptosporidiosis and giardiosis. For the control of giardiosis in calves several compounds are effective, although in natural conditions control solely based on animal treatment is not sufficient, but should be combined with environmental hygienic measures.

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Summary

Summary

Cryptosporidiosis mainly occurs in calves younger than 1 month, and causes a mild to profuse diarrhea, leading to weight loss and dehydration. Sometimes infection results in mortality, especially in combination with other intestinal pathogens. In contrast, an infection with *Giardia* is most frequently diagnosed in calves older than 1 month and clinical symptoms comprise a chronical and intermittent diarrhea, and ill thriving. Both *Cryptosporidum* and *Giardia* are also frequently diagnosed pathogens in human cases of diarrhea, weight loss and impaired development. Cattle and in particular young calves are considered as a possible reservoir for zoonotic transmission, although the relevance of cattle in the epidemiology of human infections remains unclear.

In **chapter 1**, the most recent findings on *Cryptosporidium* and *Giardia* are reviewed, with emphasis on prevalence, molecular epidemiology, diagnosis and treatment. In the review conclusions, some remaining questions are highlighted, leading to the specific objectives of the present thesis.

Previously published studies indicate that both parasites occur frequently in cattle worldwide, although the reported prevalences vary considerably. The variability can partially be ascribed to differences in study design, such as the technique used for parasite diagnosis. Although prevalence estimation and the comprehension of the parasite epidemiology depend on a reliable diagnosis, both for *Cryptosporidium* and *Giardia* there is no gold standard reference test and most diagnostic techniques have never been evaluated for use in cattle. The first objective of this thesis was therefore to estimate the prevalence of *Cryptosporidium* and *Giardia* in dairy and beef calves in the province of East Flanders (Belgium), based on a reliable Bayesian estimate of the test characteristics (**objective 1**).

The inclusion of molecular identification in epidemiological research has been advocated to assess the role of cattle in the epidemiology of human infections, since not all *Cryptosporidium* and *Giardia* species, genotypes or subgenotypes have zoonotic potential. In Northern America and in Australia, molecular epidemiological studies and subgenotype analysis suggest a limited contribution of cattle to human infections. Similar molecular epidemiological studies have not yet been conducted in Europe, but the higher population density and closer contact between agriculture and

human population might result in a more frequent transmission between cattle and humans. Moreover, in the molecular epidemiological studies the possible occurrence of mixed *Giardia* infections in cattle with both non-zoonotic and zoonotic assemblages has not been taken into account. Therefore, the second objective was to evaluate the zoonotic potential of bovine *Cryptosporidium* and *Giardia* isolates by molecular identification, with emphasis on subgenotype analysis for *Cryptosporidium* and on mixed assemblage A/E infections for *Giardia* (**objective 2**).
For the treatment of *Giardia* in calves, several benzimidazole compounds have shown to be highly efficacious in experimental conditions. In natural conditions the efficacy was less, possibly due to re-infection of the calves from the environment. Additional environmental and management measures are needed for the control of infection in natural conditions. Next to BZ, paromomycin is a potential chemotherapeutic compound for treatment of giardiosis. Hence, the third objective of the present work was to evaluate the efficacy of different dosages of paromomycin against an experimental *Giardia* infection in calves, and to determine the efficacy of a combination of fenbendazole treatment and hygienic measures against a natural infection in calves (**objective 3**).

In **chapter 2**, the prevalence of *Cryptosporidium* and *Giardia* in both dairy and beef calves, and the test properties of three diagnostic assays for *Giardia* and six diagnostic assays for *Cryptosporidium* were estimated using Bayesian analysis. The prevalence of *Giardia* in dairy calves was 22% (95% CI: 12-34%) and in beef calves 45% (95% CI: 30-64%). Furthermore, 48% of the dairy and 64% of the beef farms had at least one *Giardia* positive calf. The test evaluation indicated that ELISA and IFA were both sensitive and specific diagnostic techniques for use in epidemiological studies, whereas ME was less sensitive. The *Cryptosporidium* prevalence was estimated to be 37% (95% CI: 7-70%) in dairy and 12% (95% CI: 1-30%) in beef calves. Furthermore, 32% of the dairy and 24% of the beef farms had at least one *Cryptosporidium* positive calf. A 4-test Bayesian approach included the test results of the four conventional assays (ME, IFA, Tetra and Techlab ELISA), and indicated that the specificity estimates of the IFA and both ELISAs were high compared to ME. The IFA and the Techlab were found to be most sensitive. In the six-test Bayesian model, the sensitivity estimates of the conventional techniques decreased, due to the inclusion of 2 PCR assays with a higher sensitivity compared to the conventional techniques.

The specificity estimates of these conventional assays were comparable in the four-test and in the six-test approach. The results in chapter 2 illustrate the potential of a Bayesian analysis in estimating prevalence and test characteristics in the abscence of a gold standard, but they also indicate that estimates of test characteristics should be used within the limits of the original study design. The need for sensitive diagnostic assays in epidemiological studies for *Cryptosporidium* is emphasized, especially for the identification of subclinicaly infected animals as the PCR assays identify these animals with reduced oocyst excretion, which the conventional techniques fail to identify.

In **chapter 3**, the molecular identification of 115 *Cryptosporidium* positive samples, based on the 18S rDNA and the HSP-70 gene, revealed that the majority of the isolates from dairy and beef calves was *C. parvum*, probably due to the age of the calves which was limited to a maximum of 10 weeks. The age limit also accounts for the low number of *C. bovis* infections, and for the absence of *C. andersoni* or the *Cryptosporidium* cervine genotype in the present study. In 1 beef calf *C. suis* was retrieved. In clinically affected calves, all under 6 weeks of age, solely *C. parvum* was identified, confirming that neonatal cryptosporidial diarrhea in calves is primarily caused by *C. parvum*. The subgenotype analysis on the gp60 gene identified all but one of the *C. parvum* positive calf isolates as the zoonotic IIa allele (mainly IIaA15G2R1), indicating the zoonotic reservoir function of calves. In 1 clinically affected calf, subgenotype IId was identified. In the 120 *Giardia* positive calf samples selected for genotyping, a high prevalence and wide distribution among farms of infections with the zoonotic assemblage A was found using the β-giardin gene. Furthermore, mixed assemblage A/E infections were identified using a new species specific PCR, based on the triose phosphate isomerase gene. The identification of zoonotic lineages in the majority (for *Cryptosporidium*) or in a substantial part (for *Giardia*) of their parasite population indicate that dairy and beef calves are a potential zoonotic reservoir for human infections.

In **chapter 4**, the treatment and control of clinical giardiosis was studied. In **chapter 4.1**, a controlled and blinded study was conducted to evaluate the efficacy and safety of a treatment with paromomycin sulphate against an experimental *Giardia* infection in calves. Animals were infected with 10^5 *Giardia* assemblage E cysts and were either

treated eleven days later with 25, 50 or 75 mg paromomycin /kg body weight /day during 5 consecutive days or not treated (control group). Efficacy was evaluated based on reduction in cyst excretion. Furthermore weight gain and diarrhea scores were monitored. In the group treated with 75 mg/kg there was a 100% reduction in cyst excretion until 9 days after the start of the treatment (D9) and a very high reduction (≥98%) until D13. There was a high reduction (≥93%) until D9 and D13 in the groups treated with 25 and 50 mg/kg respectively. The cumulative cyst excretion on D13 was significantly ($P<0.05$) lower in the groups treated with 75 and 50 mg/kg compared to the control group. Although there was a trend towards higher weight gain and less diarrhea in the treated groups, differences between groups were not significant. No adverse reactions to the paromomycin treatment were recorded.

In **chapter 4.2**, the efficacy of a combination of animal treatment and environmental measures against a natural *Giardia* infection in calves between the age of 1 and 6 months was evaluated. Ten commercial farms with a total of 94 calves suffering from chronic diarrhea, ill thriving and impaired growth, were included in 2 subsequent studies. The first study indicated that treatment of all animals with fenbendazole at 15mg/kg/day during three consecutive days combined with environmental measures on the final day of treatment, which were either removal of bedding, thorough cleaning and ammonia 10% disinfection or relocation of the treated animals to a *Giardia* free environment, resulted in a total reduction in cyst excretion for at least 2 weeks. In the second study the IFA was used quantitatively and confirmed that the combination of treatment and environmental measures resulted in a total reduction of cyst excretion during 2 weeks and in a significant (≥98.0%; $P<0.05$) reduction of the cyst excretion until at least 4 weeks after treatment. Furthermore there was a noticable improvement of the clinical symptoms in all animals towards D28 after treatment and 4 months after treatment health in all calves was normal.

Finally in **chapter 5**, the main difficulties encountered in the present thesis, and the advantages and disadvantages of the applied methodology are discussed. The general conclusions and future perspectives are presented.

The results of the present thesis revealed that *Cryptosporidium* and *Giardia* are highly prevalent in both dairy and beef calves in East Flanders (Belgium). Furthermore, calves should be considered as a reservoir for human cryptosporidiosis and giardiosis. To further elucidate the importance of the calf reservoir to zoonotic transmission and

the transmission dynamics, longitudinal studies in well defined endemic foci should be conducted using specific genotyping tools. For the treatment of giardiosis in calves several compounds are effective, although in natural conditions control solely based on animal treatment is not sufficient, but should be combined with environmental hygienic measures. Vaccination could provide an alternative to chemotherapeutic treatment but preliminary results show that vaccination did not result in a protective immune response in calves. Therefore, the immune respons against a *Giardia* infection in calves is in need of further research.

Samenvatting

Samenvatting

Cryptosporidiosis is een protozoaire infectie die vooral voorkomt bij kalveren jonger dan 1 maand, en een milde tot uitgesproken diarree veroorzaakt met eventueel gewichtsverlies en deshydratatie tot gevolg. In combinatie met andere, vooral virale infecties wordt mortaliteit beschreven. De klinische symptomen veroorzaakt door een infectie met *Giardia* worden meestal waargenomen bij dieren die ouder zijn dan 1 maand. Giardiosis wordt gekenmerkt door een chronische en intermitterende diarree, en door slechte groei. Zowel *Cryptosporidium* als *Giardia* komen ook vaak voor bij de mens, met als belangrijkste symptoom diarree. Chronische infecties bij kinderen kunnen leiden tot verminderde lichamelijke en intellectuele ontwikkeling. Ofschoon vooral jonge kalveren worden beschouwd als een belangrijk zoönotisch reservoir, is de rol van kalveren in de epidemiologie van humane infecties nog helemaal niet duidelijk.

In de literatuurstudie (**hoofdstuk 1**), worden de meest recente ontwikkelingen in het wetenschappelijk onderzoek naar *Cryptosporidium* en *Giardia* besproken, met nadruk op de prevalentie, de moleculaire epidemiologie, de diagnose en de behandeling. In de conclusies van hoofdstuk 1 worden enkele lacunes aangehaald, die leiden tot het formuleren van de objectieven van deze thesis.

Door eerdere prevalentiestudies werd duidelijk dat zowel *Cryptosporidium* als *Giardia* veel voorkomen bij kalveren, hoewel de prevalentiecijfers sterk verschillen naargelang de studie. Die grote variatie in prevalentie is onder meer te wijten aan verschillen in de studieopzet, zoals de diagnosetechniek die wordt gebruikt. Hoewel de schatting van de prevalentie en een goed inzicht in de epidemiologie afhangen van een betrouwbare diagnose, zijn er geen referentie-of gouden standaard testen gekend voor *Cryptosporidium* en *Giardia.* Bovendien zijn de meeste testen nooit gevalideerd voor de diagnose van boviene infecties. De eerste doelstelling was dan ook een betrouwbare schatting te bekomen van de sensitiviteit en specificiteit van de gebruikte diagnosetechnieken, en van de prevalentie van *Cryptosporidium* en *Giardia* bij kalveren in Oost-Vlaanderen, door middel van Bayesiaanse analyse (**doelstelling 1**).

Recent werd het gebruik van moleculaire technieken in epidemiologische studies bepleit om de rol van runderen en andere dieren in de epidemiologie van humane infecties te bestuderen, daar niet alle species van *Cryptosporidium* of *Giardia* een zoönotisch belang hebben. In Noord-Amerika en in Australië wijzen moleculaire epidemiologische studies erop dat runderen een beperkte rol spelen in de humane epidemiologie. In Europa zijn geen gelijkaardige studies gekend, maar de hogere bevolkingsdichtheid en de intensieve veeteelt zou kunnen leiden tot een meer frequente overdracht tussen mens en dier. Voorts werd voor *Giardia* het voorkomen van gemengde infecties met zoönotische en diersoortspecifieke species bij het rund nog niet onderzocht. De tweede doelstelling betracht dan ook het zoönotisch potentieel van *Cryptosporidium* en *Giardia* in een selectie van kalverstalen na te gaan, waarbij aandacht wordt besteed aan identificatie tot op het niveau van subgenotypes voor *Cryptosporidium* en aan het voorkomen van gemengde infecties bij *Giardia* (**doelstelling 2**).

Verschillende benzimidazole preparaten hebben een goede efficaciteit voor de behandeling van *Giardia* infecties bij kalveren, vooral onder experimentele omstandigheden. In natuurlijke condities was de efficaciteit minder, hetgeen te wijten is aan de herinfectie van de dieren kort na de behandeling. Daarom zijn voor de controle van giardiosis in natuurlijke omstandigheden bijkomende managements-en omgevingsmaatregelen noodzakelijk. Daarnaast werd de werkzaamheid van een combinatie van behandeling met febendazole en omgevingsmaatregelen tegen een natuurlijke infectie met *Giardia* onderzocht. Naast benzimidazoles wordt paromomycine gezien als een efficiënt geneesmiddel voor de behandeling van giardiose bij kalveren. De derde doelstelling was het nagaan van de efficaciteit van verschillende doseringen van paromomycine tegen een experimentele besmetting met *Giardia* bij kalveren (**doelstelling 3**).

In **hoofdstuk 2** werd de prevalentie van *Cryptosporidium* en *Giardia* in melkvee-en vleesveekalveren onderzocht, en werden de sensitiviteit en de specificiteit van 3 diagnostische testen voor *Giardia* en van 6 technieken voor *Cryptosporidium* geschat aan de hand van een Bayesiaanse analyse. De prevalentie van *Giardia* was 22% (95% CI: 12-34%) in melkveekalveren en 45% (95% CI: 30-64%) in vleesveekalveren. In 48% van de melkveebedrijven en in 64% van de vleesveebedrijven werd minstens 1

Giardia positief kalf gevonden. De evaluatie van de testen toonde aan dat zowel ELISA als IFA gevoelige en specifieke diagnosetechnieken zijn voor gebruik in epidemiologische studies. De ME was minder gevoelig.
De prevalentie van *Cryptosporidium* werd geschat op 37% (95% CI: 7-70%) in melkveekalveren en op 12% (95% CI: 1-30%) in vleesveekalveren. In 32% van de melkveebedrijven en in 24% van de vleesveebedrijven werd minstens 1 *Cryptosporidium* positief kalf gevonden. Een 4-testen Bayesiaan model voor de evaluatie van de conventionele diagnosetechnieken (ME, IFA, Techlab en Tetra) toonde aan dat de specificiteit van de IFA en beide ELISA testen hoger was dan die van ME. De IFA en de Techlab waren de meest gevoelige technieken. In het 6-testen model was de schatting van de gevoeligheid van de conventionele technieken duidelijk lager dan in het 4-testen model. Dit was vooral te wijten aan de hogere gevoeligheid van de bijkomende PCR testen. De schatting van de specificiteit van de conventionele technieken was vergelijkbaar in het 4-testen en in het 6-testen model. De resultaten van hoofdstuk 2 illustreren het potentieel van een Bayesiaanse analyse voor het bekomen van een betrouwbare schatting van testkarakteristieken en prevalentie in afwezigheid van een gouden standaard, maar geven aan dat de schattingen van de sensitiviteit en de specificiteit moeten worden gebruikt binnen de grenzen van de proefopzet. Voor *Cryptosporidium* werden de voordelen van de meer gevoelige PCR technieken duidelijk aangetoond in deze epidemiologische studie, vooral voor de diagnose van dieren met een lage uitscheiding van oocysten, die door de conventionele technieken moeilijk of niet worden aangetoond.

In **hoofdstuk 3** gebeurde de moleculaire identificatie van 115 *Cryptosporidium* positieve melkvee-en vleesveestalen op basis van het 18S rDNA en het HSP-70 gen. De hoge prevalentie van *C. parvum,* de lage prevalentie van *C. bovis* en de afwezigheid van *C. andersoni* en het cerviene genotype van *Cryptosporidium* zijn vooral te wijten aan de leeftijd van de dieren in deze studie, die maximaal 10 weken bedroeg. In 1 vleesveekalf werd een infectie met *C. suis* gevonden. Bij kalveren met klinische symptomen, alle jonger dan 6 weken, werd enkel *C. parvum* aangetoond, hetgeen het belang illustreert van dit species in het ontstaan van klinische cryptosporidiose. De subgenotypering op basis van het gp60 gen toonde aan dat alle *C. parvum* positieve stalen het zoönotische allel IIa (voornamelijk subtype IIaA15G2R1) waren, behalve in 1 kalf met klinische symptomen met allel IId.

In de 120 *Giardia* positieve stalen geselecteerd voor genotypering, werd op basis van het β-giardin gen een hoge prevalentie en wijde verspreiding op bedrijfsniveau gevonden van het zoönotische assemblage A. Daarnaast werden ook gemengde assemblage A en E infecties gevonden met de nieuwe species specifieke tpi PCR. De identificatie van zoönotische isolaten in het merendeel (*Cryptosporidium*) of in een aanzienlijk (*Giardia*) deel van hun parasietpopulatie, duidt aan dat melkvee-en vleesveekalveren een reservoir zijn voor humane infecties.

In het vierde hoofdstuk (**hoofdstuk 4**), werd de behandeling en de controle van klinische giardiose onderzocht. In het eerste deel (**hoofdstuk 4.1**), werd de efficaciteit en de veiligheid van verschillende doseringen van paromomycine tegen een experimentele infectie met *Giardia* nagegaan. Elf dagen na besmetting met 10^5 *Giardia* assemblage E cysten, werden de dieren gedurende 5 opeenvolgende dagen dagelijks behandeld met 25, 50 of 75 mg paromomycine per kg lichaamsgewicht. De controlegroep werd niet behandeld. De efficaciteit werd geëvalueerd op basis van de reductie in cyst-uitscheiding, gewichtstoename en optreden van diarree. In de groep die werd behandeld met 75mg/kg werd een reductie in cyst-uitscheiding van 100% waargenomen tot 9 dagen na de start van de behandeling (D9) en een zeer hoge reductie (≥98%) tot D13. In de groepen behandeld met 25mg en 50mg/kg, werd een hoge reductie waargenomen tot respectievelijk D9 en D13. De cumulatieve cyst excretie was significant ($P<0.05$) lager in de groepen behandeld met 50 en 75mg/kg in vergelijking met de controlegroep. Hoewel een hogere gewichtstoename en minder diarree werd waargenomen in alle behandelde groepen in vergelijking met de controlegroep, waren deze verschillen niet significant. Er werden geen bijwerkingen gezien tijdens en na behandeling met paromomycine.
In het tweede deel van hoofdstuk 4 (**hoofdstuk 4.2**), werd een combinatie van fenbendazole behandeling en omgevingsmaatregelen tegen een natuurlijke *Giardia* infectie geëvalueerd bij kalveren tussen 1 en 6 maand oud. Er werden 10 bedrijven bezocht met in totaal 94 dieren met giardiose, chronische diarree en slechte groei. Op de eerste 5 bedrijven werden alle dieren dagelijks behandeld met 15mg fenbendazole per kg lichaamsgewicht gedurende 3 dagen en werden omgevingsmaatregelen getroffen. Deze bestonden erin de mest weg te halen, en de stal grondig te kuisen en te desinfecteren met een 10% ammonia oplossing, of de dieren te verplaatsen naar een

Giardia vrije stal. De omgevingsmaatregelen werden op de derde dag van de behandeling toegepast. Deze maatregelen leidden tot een totale reductie in cyst-uitscheiding gedurende tenminste 2 weken. In een tweede studie op 5 bedrijven werd de diagnostische techniek kwantitatief gebruikt. De resultaten van deze studie bevestigden dat er een totale reductie in cyst-uitscheiding was gedurende twee weken na behandeling, en gaven aan dat de uitscheiding significant (≥98.0%; $P<0.05$) was gedaald tot minimum 4 weken na de behandeling. Daarenboven werd bij alle dieren een klinische verbetering vastgesteld binnen de 4 weken na behandeling, en was de gezondheidstoestand normaal bij alle dieren 4 maanden na behandeling.

Tot slot worden in het vijfde hoofdstuk (**hoofdstuk 5**) de belangrijkste voor-en nadelen van de gebruikte methodologie, de algemene conclusies en de toekomstperspectieven besproken.
De resultaten van deze thesis tonen aan dat zowel *Cryptosporidium* als *Giardia* frequent voorkomen bij melkvee-en vleesveekalveren in Oost-Vlaanderen. Voorts bleek dat kalveren wel degelijk dienen beschouwd te worden als een reservoir voor humane infecties. Om het belang van kalveren in de humane epidemiologie verder uit te diepen zijn longitudinale studies nodig in welomschreven endemische locaties. Daarbij dienen moleculaire technieken gebruikt te worden die identificatie tot op het subgenotypeniveau voor *Cryptosporidium* en het opsporen van gemengde infecties voor *Giardia* mogelijk maken. Voor de behandeling van giardiose in kalveren zijn verschillende produkten, zoals fenbendazole en paromomycine beschikbaar, maar voor de controle van een infectie in natuurlijke omstandigheden, is enkel behandeling van de dieren niet voldoende en moet die worden gecombineerd met omgevingsmaatregelen. Vaccinatie zou een alternatief kunnen zijn, maar preliminaire gegevens tonen aan dat vaccinatie geen protectieve immuunrespons genereert bij runderen. De immuunrespons tegen *Giardia* bij runderen dient daarom verder bestudeerd te worden.