Cryptosporidium Infection in Dairy Cattle

Prevalence, species distribution and associated management factors

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Cover: FITC-stained *C. parvum*-like oocysts
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Abstract

For almost 25 years, it has been known that *Cryptosporidium* parasites infect Swedish calves. This thesis explores how common these parasites are at herd level and at individual level in preweaned calves, young stock and periparturient cows. Species distribution and association with diarrhoeal problems are also highlighted. Two field studies were performed and in addition, existing clinical or cohort studies on the cryptosporidiostatic substance halofuginone were examined.

Cryptosporidium oocyst shedders were detected in 68 of 69 investigated herds. Calves had the highest prevalences followed by young stock and cows. The four common species in cattle, *C. parvum, C. bovis, C. ryanae* and *C. andersoni,* were all detected. *Cryptosporidium bovis* was most common in all age groups with an overall 77% prevalence, and the prepatent period was shown to be at least three days shorter than previously described. Overall, *Cryptosporidium* infection was not associated with disease in calves, but a higher percentage of calves infected with *C. parvum* had diarrhoea compared to calves infected with *C. bovis*. Nine different *C. parvum* subtypes were identified, of which three were novel. All subtypes belonged to the zoonotic subtype families IIa and IId. Several management factors were associated with shedding of oocysts. One management factor, 'disinfection of single pens', was associated with diarrhoeal problems at herd level, but several more management differences were indicated although they could not be shown statistically. Halofuginone had some beneficial effects on infection and diarrhoeal prevalences when used for prophylaxis, but mortality was not affected.

Cryptosporidium parasites were widely spread in the Swedish dairy cattle population, but because most animals were not infected with the zoonotic *C. parvum*, the potential for zoonotic transfer is fairly low. Management routines are important to decrease infection pressure and prevent infected calves from clinical disease. Halofuginone should be used with great care in a transition period when management routines are changed to improve calf health.

Keywords: *Cryptosporidium*, cattle, prevalence, *C. andersoni*, *C. bovis*, *C. parvum, C. ryanae*, subtypes, diarrhoea, halofuginone

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Dedication

To my beloved little monsters

"Use the difficulty"
Michael Caine

"Snälla flickor kommer till himlen. Stygga flickor kan komma hur långt som helst...."

Contents

List of Publications

I Silverlås, C., Emanuelson, U., de Verdier, K., Björkman, C. (2009). Prevalence and associated management factors of *Cryptosporidium* shedding in 50 Swedish dairy herds. *Preventive Veterinary Medicine* 90, 242-253.

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III Silverlås, C., de Verdier, K., Emanuelson, U., Mattsson, J.G., Björkman, C. (2009). *Cryptosporidium* infection and calf diarrhoea in dairy herds. (manuscript)

IV Silverlås, C., Björkman, C., Egenvall, A. (2009). Systematic review and meta-analyses of the effects of halofuginone against calf cryptosporidiosis. *Preventive Veterinary Medicine* 91, 73-84.

Abbreviations

18S rRNA	Small subunit ribosomal ribonucleic acid
28S rRNA	Large subunit ribosomal ribonucleic acid
AIDS	Acquired immunodeficiency syndrome
CD4+	Immunoglobulin marker of a specific T-cell population
CI	Confidence interval
COWP	Cryptosporidium oocyst wall protein
DNA	Deoxyribonucleic acid
ES	Pooled estimate
HIV	Human immunodeficiency virus
HSP70	70-kDa heat shock protein
kDa	kilo Dalton
MLG	Multilocus subtype (syn. multilocus genotype)
OPG	Oocysts per gram faeces
OR	Odds ratio
PCR	Polymerase chain reaction
PR	Prevalence ratio
SVA	National Veterinary Institute
T-cell	Lymphocyte that differentiate in the thymus
TP	Serum total protein

1 Background

1.1 History

The first *Cryptosporidium* species was described in 1907 by Edward Tyzzer. The parasite was found in the ventricular glands of mice and was named *Cryptosporidium muris* (*C. muris*). In 1912, a smaller species found in the small intestine of mice was also described by Tyzzer and named *C. parvum*. Since then, cryptosporidia have been identified in all vertebrate classes. *Cryptosporidium parvum* was first recognised as an important pathogen in the 1970's, when it was linked to chronic diarrhoea in an 8-month-old heifer (Panciera *et al.*, 1971) and a few years later to diarrhoea in humans (Meisel *et al.*, 1976; Nime *et al.*, 1976). Since these reports considerable research on *Cryptosporidium* spp. and cryptosporidiosis has been done. Until recently, species differentiation was based on oocyst morphology and host class. Oocysts ~5 μm Ø found in mammals were considered to be *C. parvum*, and over 150 host species including humans were reported. Therefore the parasite is zoonotic (i.e. can be transmitted between animals and humans). Today molecular analysis is used to identify different species of cryptosporidia. The first method was described in 1991 (Laxer *et al.*, 1991), and in 1995 a molecular method to distinguish between genotype I (anthroponotic or human adapted) and genotype II (zoonotic) of *C. parvum* was published (Morgan *et al.*, 1995). In 2002, genotype I was upgraded to a separate species, namely *C. hominis* (Morgan-Ryan *et al.*, 2002). It has now been shown that there are several species morphologically similar to *C. parvum*, and today this species is mainly considered to infect cattle and humans.

1.2 Taxonomy

Cryptosporidia are protozoan parasites historically classified as belonging to phylum Apicomplexa, class Coccidea, together with e.g. *Eimeria*, *Isospora* and *Toxoplasma*. This classification was based on similarities in life cycles, such as invasion of host epithelial cells. On the other hand, cryptosporidia also have several properties different from other coccidians. For example, intracellular stages are surrounded by a membrane (a parasitophorous vacuole) and have a feeder organelle, oocysts sporulate in situ, are auto infective to the host and are resistant to anticoccidial drugs. In addition, coccidian species are generally named based on unique oocyst morphology, but within the *Cryptosporidium* genus several species have similar oocyst morphology (Fayer, 2008).

In recent years, several studies have suggested that cryptosporidia might be more closely related to the gregarines, which are apicomplexan parasites of invertebrates, than to coccidia. This relationship was indicated by the identification of extracellular *Cryptosporidium* life cycle stages similar to those of gregarines both from in vitro cultures and from faeces or gut contents (Rosales *et al.*, 2005; Hijjawi *et al.*, 2002). In addition, molecular phylogeny based on the 18S rRNA and β–tubulin genes (Leander *et al.*, 2003; Carreno *et al.*, 1999) and recently on protein sequences, 28S rRNA and α- and β-tubulin genes (Templeton *et al.*, 2009) also indicate a close relationship with gregarines. Thus, it has been proposed that taxonomy should be changed to reflect the more distant relationship between *Cryptosporidium* and coccidians (Plutzer & Karanis, 2009).

To be considered a valid *Cryptosporidium* species, four criteria have to be fulfilled. First, a unique DNA sequence must be shown and deposited in GenBank. Second, oocyst morphology must be thoroughly described. Third, data on host specificity from experimental or natural infection must be recorded and fourth, the species must be named in accordance to the International Code of Zoological Nomenclature rules (Fayer, 2008).

1.3 Life cycle

Cryptosporidium parasites have direct life cycles, i.e. all life cycle stages take place within one host. *Cryptosporidium parvum* completes a lifecycle in approximately two days (Figure 1). The infection route is faecal to oral. When oocysts are exposed to the reducing environment in the small intestines they excystate and four sporozoites are released. The sporozoites

invade epithelial cells of the distal jejunum and ileum. Caecum, colon and even extraintestinal mucous membranes can also be infected depending on the host immune status. Within the epithelial cells, each sporozoite is quickly transformed into a trophozoite, retained within a membrane called a parasitophorous vacuole just below the cellular membrane. This means that although infection is intracellular, the parasite remains extracytoplasmic. Trophozoites go through an asexual cycle and develop into type I meronts, which releases six to eight merozoites into the intestinal lumen to infect new epithelial cells, and undertake either a new asexual cycle or turn into type II meronts and go through a sexual cycle. The mature type II meront contains four merozoites, which, after release and infection of new epithelial cells will develop into either a male microgamont or a macrogamont (ovum). Microgamonts release microgametes (sperm) that fertilize macrogamonts, producing zygotes which develop into infectious oocysts. Oocysts sporulate in situ and are infectious at release from the epithelial cells. Approximately 80% of the oocysts have thick walls and exit the host with faeces to infect new hosts. The thin-walled oocysts in turn can excyst while still in the same host and cause auto-infection.

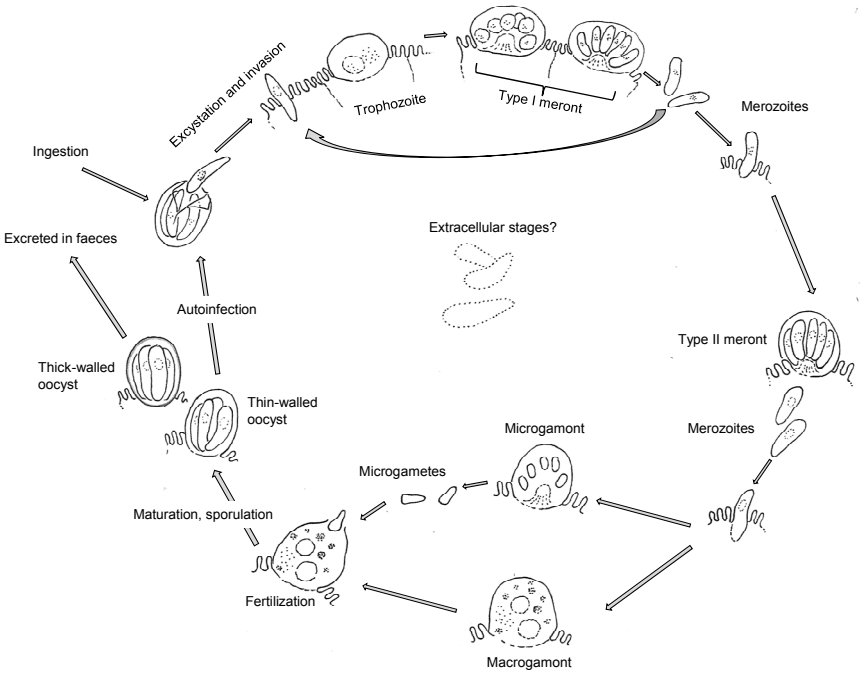

Figure 1. Life cycle of *C. parvum*. Drawing by Katarina Näslund

1.4 Methods for detection of infection

Cryptosporidium infection can be detected in several ways. A common method is microscopy of faecal samples, which can be mounted on slides either directly or after flotation or sedimentation and gradient techniques, which are used to remove faecal debris and concentrate oocysts. This facilitates detection of infection in animals shedding lower numbers of oocysts, thus increasing the sensitivity of analysis.

Different techniques for microscope visualisation also exist. Oocysts can be detected without staining, using phase contrast microscopy, but using a stain facilitates detection. Modified Ziehl Neelsen stain, where oocysts appear purple on a blue background, is commonly used. Immunofluorescence staining with monoclonal antibodies against oocyst wall antigens produces bright green oocysts at epifluorescence microscopy. Oocyst vitality can be controlled with 4,6´ diamino-2-phenylindole dihydrochloride (DAPI) that stains DNA, and nuclei of viable oocysts appear blue under UV-light.

Antigen ELISAs and rapid immunochormatographic (strip) tests can also be used. Other methods, such as histology of intestines, can be used to detect the different intracellular parasite stages in deceased animals.

1.5 Molecular analysis

1.5.1 Molecular tools for species identification

Molecular analysis is vital to determine species when oocyst morphology is compatible with several species. A number of highly preserved genes have been targeted for this purpose, including small subunit RNA (18S rRNA), 70 kilo Dalton (kDa) heat shock protein (HSP70), *Cryptosporidium* oocyst wall protein (COWP) and the actin gene. The 18S rRNA gene is useful because in addition to regions that vary between species, it contains several regions that are preserved within the *Cryptosporidium* genus. This makes it easy to develop primers that target most species. The HSP70, COWP and actin genes from different *Cryptosporidium* species are quite variable throughout their sequences. This means that they are of limited use for species identification (Xiao & Ryan, 2008).

DNA extracted from oocysts can be amplified by one of several methods, including standard or nested polymerase chain reaction (PCR) protocols. In standard PCR, one pair of primers is used to amplify a gene in forward (5´-)

and reverse (3´-) directions, whereas in nested PCR, two sets of primers are used, where the first (external) primer pair targets the gene of interest. A second (internal) primer pair is then used to amplify a shorter (internal) segment of the amplicons produced in the primary PCR. This method is especially useful if a sample contains small amounts of oocysts because it results in more DNA copies than standard PCR. PCR products (amplicons) are separated in an agarose gel using an electric field, and results are usually visualized by staining with ethidium bromide to identify presence of *Cryptosporidium* in the sample. Species differentiation can be done if restriction enzymes are used to digest amplicons in fragments of varying size depending on species (i.e. restriction fragment length polymorphism (RFLP) analysis), which causes the products to migrate different distances on the gel. Another way to determine species after PCR is to subject amplicons to DNA sequencing. Amplicon DNA is then purified and amplified again using the (internal) primers from the PCR protocol and colour-labelled nucleotide bases. These colour-labelled bases emit light at different wave lengths, and this property is used to analyse the gene sequence. The forward and reverse sequences produced can then be assembled to contigs (Figure 2A) and compared to sequences deposited in GenBank, using BLAST (Basic Local Alignment Search Tool, http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Mixed infections are hard to identify by PCR, because the dominating species or the species with highest affinity for the primer will be amplified to a much larger extent than the other(s), resulting in identification of only the dominant species (Xiao & Ryan, 2008). If more than one species amplifies successfully, this is indicated at gene sequencing as double spikes in many positions and thereby inability to assemble contigs (Figure 2B). For successful analysis of mixed infections, either a combination of several species/genotype-specific primers (Xiao & Ryan, 2008) or cloning of single amplicons produced in the PCR have to be used. Another possibility is to perform GP60 subtype analysis (see section 1.5.2) because the primer used is quite species specific. This was shown by Feng *et al.* (2007), who identified *C. parvum* subtypes in 10 samples positive for *C. bovis* by 18S rRNA PCR.

Today 21 species have been confirmed using molecular analysis, and about 60 genotypes have been reported (Fayer & Santin, 2009; Plutzer & Karanis, 2009). Genotypes are isolates which differ in investigated DNA sequences compared to already described species, but further research on pathogenicity and host specificity is needed before it can be determined whether the genetic differences reflect separate species or just intra-species variations.

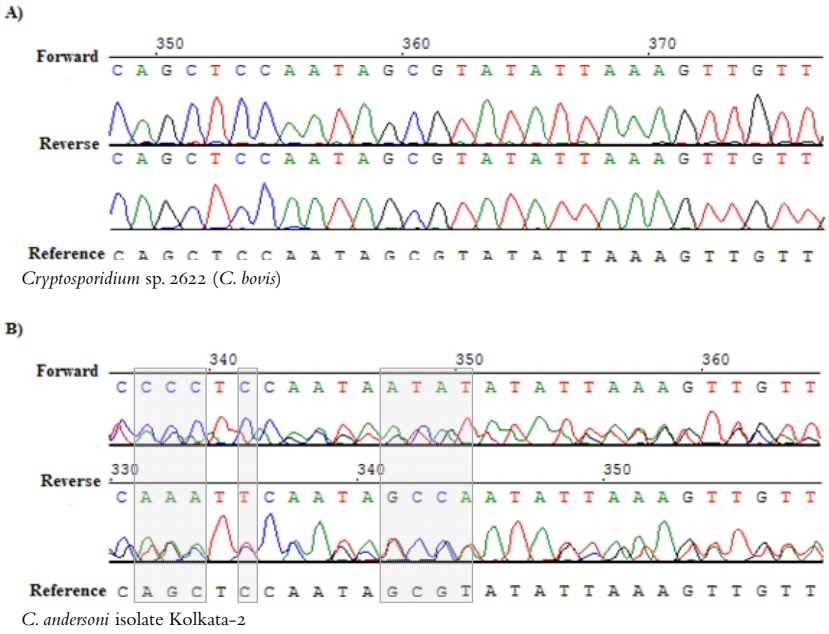

Figure 2. Chromatograms of partial *Cryptosporidium* sequences from a preserved region of the 18S rRNA gene

A) Chromatogram of a sample containing *C. parvum*-like oocysts and where sequences could be assembled to a contig. The 773 base pair sequence had 100% identity with a *C. bovis* isolate (*Cryptosporidium* sp. 2622).

B) Forward and reverse sequences from a sample containing both *C. parvum*-like oocysts and *C. andersoni* oocysts. Sequences contained double spikes throughout the 757 base pair (forward) and 744 base pair (reverse) sequences and could not be assembled to a contig. Both sequences had the highest similarity (85% and 91% respectively) with reference strain *C. andersoni* Kolkata-2 using BLAST. Regions of dissimilarities highlighted in grey.

1.5.2 Intra-species molecular analysis

PCR protocols to analyse intra-species differences have been developed primarily for *C. parvum* and *C. hominis*, because these are the main species important for human medicine. They are divided into subtypes based on the sequence of the 60-kDa glycoprotein (GP60) gene. This glycoprotein is expressed on the apical surface of invading stages (sporozoites and merozoites) and is a target for neutralizing antibodies (Cevallos *et al.*, 2000). Thus, GP60 subtyping may have a direct application for determining the virulence of different *C. parvum* and *C. hominis* subtypes. The GP60 gene has a highly polymorphic region of microsatellites in the 5´ end, consisting of trinucleotide repeats (TCA, TCG and TCT designated A, G and T respectively)

all coding for the amino acid serine, and subtypes are named based on the number of each present repeat. Some subtypes also have other short repetitive sequences (R) immediately after the trinucleotide repeats. In addition, large sequence variations outside this polymorphic region are used to determine subtype families. *Cryptosporidium hominis* subtype families have prefixes Ia, b and d-g, whereas *C. parvum* subtype families have prefixes IIa-l. Some subtypes have small variations outside the polymorphic region, and these differences are annotated by a, b etcetera after the subtype name. Examples of *C. parvum* subtypes are IIaA15G2R1, IIdA20G1a and IIiA10. The T repeat is only found in subtype family Ie. Zoonotic transmission of *C. parvum* is seen in subtype families IIa and IId, whereas families IIc and IIe are anthroponotic.

When performing population genetic studies, it is not enough to target one locus. Instead a number of minisatellites and microsatellites (including GP60) are targeted to identify multilocus subtypes (MLGs) (Xiao & Ryan, 2008).

1.6 Infection and disease

Different isolates and MLGs of *C. parvum* and different *C. hominis* GP60 subtype families have been associated with varying pathogenicity in both calves and humans (Cama *et al.*, 2007; Okhuysen *et al.*, 1999; Pozio *et al.*, 1992).

1.6.1 Infection and disease in cattle

Cryptosporidium parvum, C. bovis, C. ryanae and *C. andersoni* are the four major species identified in cattle (Fayer *et al.*, 2007; Feng *et al.*, 2007; Langkjaer *et al.*, 2006; Santín *et al.*, 2004; Peng *et al.*, 2003). Sporadic infection with *C. felis, C. hominis, C. suis*, *C. suis*-like genotype and *Cryptosporidium* pig genotype II have been reported (Fayer *et al.*, 2006; Geurden *et al.*, 2006; Langkjaer *et al.*, 2006; Smith *et al.*, 2005; Bornay-Llinares *et al.*, 1999). In addition, *C. canis* has been reported from experimental infection (Fayer *et al.*, 2001). Clinical infection is primarily seen in calves.

Cryptosporidium parvum, with a mean oocyst size of 4.5 µm x 5.5 µm, is common in young calves and has a predilection for the distal jejunum and ileum. The prepatent period, i.e. the period from infection until the host starts excreting oocysts, is 2 to 7 days. Shedding occurs for approximately 1-12 days (patent period) before the host immune system has cleared the

infection, and an infected calf can shed 10^{10} oocysts during this period. Clinical cryptosporidiosis is mainly seen in 1- to 4-week-old calves, and severity of the disease depends on several factors, including the host's immune system, the infection dose and if concurrent infection with pathogens such as rotavirus is present. *Cryptosporidium* infection can be asymptomatic or cause pasty to watery and profuse diarrhoea, dehydration, inappetence and even mortality. Diarrhoea is a combination of unusually high faecal water content and increased bowel movements, resulting in loose to watery faeces and an increased number of stools per day. *Cryptosporidium* associated diarrhoea is caused by two pathogenic mechanisms. Malabsorptive diarrhoea is caused by loss of enterocytes and blunting of villi, which reduces the intestinal surface and presence of mature cells, leading to decreased nutrient and water absorption (Foster & Smith, 2009; Klein *et al.*, 2008). In addition, prostaglandins (mainly PGE_2 and PGI_2) induce secretion of chloride and carbonate ions into the intestinal lumen and decrease absorption of sodium chloride. This produces an osmotic pressure that forces water into the lumen, resulting in secretory diarrhoea (Foster & Smith, 2009). Intestinal damage caused by massive infection may lead to reduced growth rates (Klein *et al.*, 2008). However, Klein *et al.* (2008) also showed that intestinal absorption was restored three weeks post infection, indicating that no prolonged or permanent damage occurs. When calves die, co-infection with other pathogens such as rotavirus or coronavirus is common (Moore & Zeman, 1991) but there have been lethal cases when *C. parvum* was the only pathogen isolated (Sanford & Josephson, 1982).

Cryptosporidium bovis and *C. ryanae* are morphologically similar to *C. parvum,* with approximate oocyst sizes of 4.6 µm x 4.9 µm for *C. bovis* and 3.2 µm x 3.7 µm for *C. ryanae*. The size differences between these two species and *C. parvum* are too small for reliable species determination by microscopy, and differentiation must be done by molecular analysis. The prepatent period is 10 days for *C. bovis* and 11-12 days for *C. ryanae* (Fayer *et al.*, 2005; Fayer *et al.*, 2008). They infect the small intestine, are associated with subclinical infection and are mainly found in weaned calves and older animals. They are considered cattle specific species and have not been shown to be involved in zoonotic transmission.

Cryptosporidium andersoni is a larger, cattle specific species, approximately 5.5 µm x 7.4 µm and morphologically similar to *C. muris*. The prepatent period is 18-45 days (Kvac *et al.*, 2008). This species infects the abomasum

and is mainly found in weaned calves and older cattle. The infection is chronic and subclinical in nature, but reduced growth rates and lower milk yields have been reported (Anderson, 1998; Esteban & Anderson, 1995). Infection has also been shown in camelids (Wang *et al.*, 2008). In a few human cases oocysts with similar morphology and an 18s rRNA sequence almost identical to *C. andersoni* were identified, opening the possibility for zoonotic transmission of this species (Leoni *et al.*, 2006).

The role of cows as a possible infection source for calves has been addressed. Such transmission could be facilitated by a periparturient rise in oocyst shedding in infected cows. Periparturient rises have been shown for *C. parvum*-like oocysts (Faubert & Litvinsky, 1999) and for *C. andersoni* (Ralston *et al.*, 2003). In contrast, Atwill *et al.* (1999) did not find evidence for a periparturient rise.

1.6.2 Infection and disease in humans

Humans can be infected with several species, but *C. hominis* and *C. parvum* are the major species. *Cryptosporidium hominis* is the only anthroponotic species shown so far. Except for *C. parvum, C. meleagridis* is the most common species in zoonotic transmission, followed by *C. felis* and *C canis*. Sporadic cases of infection with *C. muris*, *C. andersoni*, *C. suis*, *C. suis*-like *C. hominis* monkey genotype, *C. parvum* mouse genotype and *Cryptosporidium* sp. genotypes cervine, chipmunk, deer, horse, rabbit skunk and pig genotype II have also been reported (Chalmers *et al.*, 2009; Kvac *et al.*, 2009; Robinson *et al.*, 2008; Xiao & Feng, 2008; Feltus *et al.*, 2006; Leoni *et al.*, 2006; Mallon *et al.*, 2003b; Ong *et al.*, 2002).

The prepatent period of *C. parvum* is 3-14 days, and the patent period is 1-20 days (Fayer, 2008). As for cattle, infection can be asymptomatic. Clinical cryptosporidiosis is mostly acute and associated with watery diarrhoea, abdominal pain, vomiting, dehydration and mild fever in immunocompetent hosts. Because cryptosporidia are able to autoinfect their hosts, immunocompromised persons (e.g. HIV-infected individuals) are not able to fight the parasites. This causes chronic infection that may spread throughout the intestines and even extra-intestinally, and the disease may be life threatening. Extra-intestinal invasion is facilitated by the oocysts ability to excyst in the absence of the reducing environment of the intestines (de Graaf *et al.*, 1999). Studies in healthy volunteers have shown that previous *Cryptosporidium* infection provides some protection, reflected by a higher infection dose needed to induce shedding, fever shedders and less severe

diarrhoea at second exposure (Chappell *et al.*, 1999; Okhuysen *et al.*, 1998). None of the volunteers had detectable IgG antibody response 45 days after the first exposure, whereas 32% had seroconverted at the same time after the second exposure (Okhuysen *et al.*, 1998). This indicates that repeated exposures are needed for a long-lasting immunity. Several studies indicate earlier and more frequent exposure in developing countries, as seroprevalence was ~60% in 4- to 5-year-olds in Brazil (Teixeira *et al.*, 2007; Cox *et al.*, 2005) and 73% in 3-year-olds in Guatemala (Steinberg *et al.*, 2004), compared to 13% in children up to 5 years of age in Oklahoma (Kuhls *et al.*, 1994). In comparison, 14- to 21-year-olds had a seroprevalence of 58% (Kuhls *et al.*, 1994). Cryptosporidiosis is a common parasite cause of tourist diarrhoea when travelling to more rural areas (Yoder & Beach, 2009; Nair *et al.*, 2008; Weitzel *et al.*, 2006; Jokipii *et al.*, 1984).

1.7 Epidemiology

Several factors are critical to the epidemiology of *C. parvum* (Dillingham *et al.*, 2002). They facilitate spread of *C. parvum* and make control and eradication difficult.

1. The oocysts are extremely resistant, and survive e.g. freezing at -10°C for one week and up to 4 days in drying faeces. Oocysts withstand most disinfectants at doses that are safe to work with (Fayer, 2008).
2. The small oocyst size makes it difficult to filter them from contaminated water.
3. The infective dose is low, and as few as nine oocysts of one *Cryptosporidium* isolate have proven infectious for humans (Okhuysen *et al.*, 1999). In calves, 50 oocysts have been shown to cause infection (Moore *et al.*, 2003). In contrast, one infected host can shed as many as 10^{10} oocysts, contributing to a huge infection pressure.
4. The oocysts are sporulated and infectious at shedding, which means that a new host can immediately be infected.
5. Zoonotic transmission can easily take place through direct contact or contamination of water, food, tools or surfaces.

1.7.1 Epidemiology in cattle

A large number of epidemiological studies have been performed to estimate the prevalence of *Cryptosporidium* infection in cattle. The infection has been found worldwide, but reported prevalences range from 0-100%, and vary with the age of sampled animals (summarized in Table 18.2, (Santín &

Trout, 2008)). Point prevalence studies show an age related pattern, with the highest prevalence in calves, and then infection becomes less common with increasing age (Fayer *et al.*, 2007; Fayer *et al.*, 2006; Maddox-Hyttel *et al.*, 2006; Santín *et al.*, 2004). Cumulative prevalence in calves has been estimated to 92% at 21 days of age (Atwill *et al.*, 1998), indicating that whenever cryptosporidia are present in a herd, all animals will become infected before weaning. Older studies, in which species identification was based on microscopy, probably overestimate the prevalence of *C. parvum* in weaned animals because recent studies that applied molecular analysis showed that this species is rare after weaning. Instead, the *C. parvum*-like species *C. bovis* and *C. ryanae* together with *C. andersoni* dominated, with *C. bovis* being most common in young stock (Fayer *et al.*, 2006; Langkjaer *et al.*, 2006; Santín *et al.*, 2004) and *C. andersoni* most common in adult cattle (Fayer *et al.*, 2007). Subclinical infection and lower *Cryptosporidium* prevalence in older animals could be due to several factors, such as an age-related resistance due to maturation of the intestinal mucosa (Harp *et al.*, 1990). This was further shown by Harp (2003) and Akili *et al.* (2006), who found that a 54-kDa protein present in intestinal mucosa from adult mice and bovines prevented *Cryptosporidium* infection in mouse pups. Infection could provide species-specific resistance (Fayer *et al.*, 2005; Harp *et al.*, 1990) and partial resistance to other *Cryptosporidium* species, or repeated *Cryptosporidium* exposure could result in natural vaccination. Another explanation might be that *C. bovis*, *C. ryanae* and *C. andersoni* are truly less pathogenic than *C. parvum*, resulting in low grade infection and lower oocyst output, which in turn reduces the infection pressure among these animals.

Risk factors for infection and disease in dairy calves vary between studies, which could reflect variations in herd management in different parts of the world. For example, Trotz-Williams *et al.* (2007) found a higher risk for infection with increasing age and in calves born during summer, whereas calves given feed with coccidiostats or calves born to dams vaccinated against rotavirus, coronavirus and *E. coli* F5+ to prevent calf diarrhoea had decreased risk of shedding oocysts. In the same study, diarrhoea was associated with *Cryptosporidium* infection and high oocyst shedding rates, increasing age, calves being born in summer and calves staying more than one hour with the dam. Maddox-Hyttel *et al.* (2006) found higher shedding rates in calves from organic herds, and lower shedding rates when pens had an empty period between calves. In beef cattle, higher prevalences were

found in herds with many calves, high stocking density and a long calving season (Atwill *et al.*, 1999).

The effect of herd management strategies has been considered a cause for variation in *C. parvum* subtype distribution. Studies from areas with closed herd management (few animal movements between herds) have shown a high number of GP60 subtypes in the calf population, but only one subtype within any herd (Brook *et al.*, 2009; Soba & Logar, 2008; Thompson *et al.*, 2007; Misic *et al.*, 2006). In contrast, only a few subtypes were identified in areas with higher exchange rates between herds, but several subtypes could be present in a herd (Brook *et al.*, 2009; Trotz-Williams *et al.*, 2006; Peng *et al.*, 2003). Multilocus subtyping of calf samples have shown the same within-herd pattern, with more mixed subtype infections and more MLGs per herd in Turkey, where animal movement between herds occurs frequently, than in Israel, where closed herds are more common (Tanriverdi *et al.*, 2006).

1.7.2 Epidemiology in humans and zoonotic transmission

Contaminated drinking water, food or recreational water and direct contact with infected persons or animals are examples of common infection sources for humans. Poor water supply and poor water quality is associated with higher seroprevalence in children (Teixeira *et al.*, 2007). A higher seroprevalence has also been associated with the use of surface water as a drinking water source compared to an underground source (Frost *et al.*, 2002). Heavy rainfall is a risk factor for contamination of surface water. A number of waterborne outbreaks have occurred, and cattle are often suspected as a primary source of water contamination by effluents from farms or run-off from grazing areas (Hunter & Thompson, 2005; Meinhardt *et al.*, 1996). However, in the largest reported outbreak, with >400,000 persons in Milwaukee affected, *C. hominis* was the species identified by molecular analysis (Sulaiman *et al.*, 2001).

Multilocus subtype analysis of human and cattle isolates have shown that anthroponotic as well as zoonotic *C. parvum* are present in human cases (Leoni *et al.*, 2007; Ngouanesavanh *et al.*, 2006; Mallon *et al.*, 2003a; Mallon *et al.*, 2003b). Mallon et al. (2003a, b) found that most human and cattle *C. parvum* MLGs were identical, and there was evidence of panmixia (random mating) in these *C. parvum* populations, which indicate frequent zoonotic transfer. Zoonotic *C. parvum* transfer was also supported by the results of Hunter *et al.* (2007) who found a higher frequency of pre-infection animal

contact in humans with certain alleles at two loci. Further, one of the "anthroponotic" alleles was more common in urban areas and the "zoonotic" allele at this locus was more common in more rural areas (Hunter *et al.*, 2007). Fewer MLGs were present in *C. hominis* and anthroponotic *C. parvum* populations compared to zoonotic *C. parvum* populations, which is indicative of a higher host specificity (Hunter *et al.*, 2007; Leoni *et al.*, 2007; Ngouanesavanh *et al.*, 2006; Mallon *et al.*, 2003a; Mallon *et al.*, 2003b). In addition, *C. hominis* populations had completely different MLGs than *C. parvum*, indicating that no genetic exchange occurred between this species and *C. parvum* (Leoni *et al.*, 2007; Ngouanesavanh *et al.*, 2006; Mallon *et al.*, 2003a). There are reports of human *C. parvum* cases caused by contact with infected calves (Kiang *et al.*, 2006; Robertson *et al.*, 2006; Preiser *et al.*, 2003; Pohjola *et al.*, 1986). However, it is important to note that even when zoonotic *C. parvum* GP60 subtypes or MLGs are isolated, cattle are not necessarily the infection source, but these subtypes might circulate in the human population in addition to the anthroponotic ones. This was indicated by the results of Hunter *et al.* (2007) since less than 50% of infected persons with "zoonotic" alleles reported animal contacts.

1.8 Treatment of cryptosporidiosis

Supportive care is the basis for treatment of clinical cryptosporidiosis. Diarrhoeic calves should not be deprived of their ordinary milk feeds and in addition they should be offered oral electrolyte solutions (McGuirk, 1998; Roenfeldt, 1995; Garthwaite *et al.*, 1994). Calves that are too depressed to drink should be given intravenous fluids. Parenteral nutrition can be used in humans in addition to fluid therapy, and antimotility drugs (e.g. loperamide) can improve intestinal absorption (Pantenburg *et al.*, 2009).

Halofuginone is the only substance approved for use against calf cryptosporidiosis. The drug affects invading parasite stages, but the exact mechanism of action is unknown. The drug is approved for prophylaxis and therapy (http://www.ema.europa.eu/vetdocs/PDFS/EPAR/halocur/V-040-PI-en.pdf). Nitazoxanide is registered for use in immunocompetent humans (Pantenburg *et al.*, 2009; Rossignol, 2009). AIDS patients suffering from chronic cryptosporidiosis have improved when highly active antiretroviral therapy is used to normalize CD4+ T-cell levels (Pantenburg *et al.*, 2009). Several other drugs, including paromomycin and hyperimmune bovine colostrum have been tested in both humans and calves with varying effects.

1.9 *Cryptosporidium* infection in Sweden

1.9.1 Infection in cattle

Cryptosporidium oocysts were first documented in diarrhoeal calf faecal samples in 1985 (Viring *et al.*, 1985). Since then these parasites have been identified by microscopy in other studies investigating causes for diarrhoea in calves (Björkman *et al.*, 2003; Viring *et al.*, 1993; Tråvén *et al.*, 1989) and the persistence of one *C. parvum* MLG in a herd over time has also been shown (Björkman & Mattsson, 2006). *Cryptosporidium* analysis is incorporated in the routine diagnostics of calf diarrhoeal samples sent to the National Veterinary Institute (SVA) through "kalvpaketen". These are diagnostic packages including a number of analyses for samples from herds that have problems with diarrhoea or respiratory disease in calves. However, no studies have investigated how common *Cryptosporidium* infection is in Swedish cattle and it has thus not been known how common these parasites are in different age groups or in animals without clinical signs of infection. Accordingly, the distribution of different species has so far also been unknown.

1.9.2 Infection in humans

Human cryptosporidiosis has been notifiable in Sweden since 1 July 2004 (http://www.smittskyddsinstitutet.se). From August 2004 and forward, trends and statistics on reported cases are available on the website of the Swedish Institute for Infectious Disease Control (SMI) (http://www.smittskyddsinstitutet.se/statistik/cryptosporidiuminfektion).
On average, 1.3 cases/100,000 citizens were diagnosed in 2005-2009, with an increase from 0.8 to 1.7 cases/100,000 citizens. Approximately 1/3 of reported cases each year are domestic, with a peak of 44% in 2008. Much of the overall case increase is probably due to an improved awareness of the disease. Still, reported cases most likely provide an underestimation of the true occurrence because not all infected persons develop clinical signs, and not all of those with diarrhoea need medical help. In addition, physicians have to ask most laboratories specifically for *Cryptosporidium* analysis, and cryptosporidiosis is perhaps still considered a cause of tourist diarrhoea rather than a domestic infection. Therefore, many cases may be missed. Cryptosporidiosis is expected to be one of several emerging infectious diseases due to anticipated climate changes (Anonymous, 2007). A number of outbreaks have occurred, associated with contamination of surface water (Hansen and Stenström, 1998), public pools (Mattsson *et al.*, 2008; Insulander *et al.*, 2005), and a day care centre pool (Persson *et al.*, 2007). A

restaurant outbreak with 16 confirmed cases (Insulander *et al.*, 2008), another outbreak at a day care centre and an increase in sporadic cases (http://www.smittskyddsinstitutet.se/statistik/cryptosporidiuminfektion/?t=com#statistics-nav) probably contributed to the peak of domestic cases in 2008.

2 Aims

The overall aim of this thesis was to evaluate the presence of *Cryptosporidium* in Swedish dairy cattle.

The specific aims were:

- To estimate *Cryptosporidium* prevalence at herd level and in different age groups (paper I)
- To identify factors that affect herd prevalence as well as infection in individual animals (paper I)
- To investigate the distribution of *Cryptosporidium* species in the dairy cattle population (paper II)
- To estimate the role of *Cryptosporidium* in herds with calf diarrhoeal problems (paper III)

In addition, it was decided to estimate the effects of the substance halofuginone on *Cryptosporidium*-associated diarrhoea in calves (paper IV)

3 Materials and Methods

Two field studies were performed (papers I, II and III). Paper IV was based on a number of international studies on calves. Detailed descriptions of materials and methods used in each study are given in the respective papers (I - IV).

3.1 Study populations

In paper I, a stratified random sampling was performed to include 50 herds. Stratification was made for the five regions Skåne, Västergötland, Östergötland, Uppland and southern Norrland (Figure 3). These regions were selected so that areas with different herd density across Sweden would be represented, and herds with ≥50 cows per year were eligible for inclusion. These herds were assigned random numbers, separately for each region. Starting with the lowest random numbers, farmers were contacted by mail and asked to participate until all 50 herds were recruited. The number of herds sampled in each region was proportional to the source population, e.g. 10% of all eligible herds were situated in Uppland, thus 5 (10%) of all 50 herds were sampled in this region.

In paper II, *Cryptosporidium* positive samples from paper I were used to determine the species distribution in the different age categories, regions and in calves with diarrhoea or not.

Paper III had a matched case control design, where 10 herds with diarrhoeal problems in calves were compared to 10 herds without calf health problems. Problem herds were identified through contacts with veterinary practitioners in the field, except for one case herd where the farmer herself contacted SVA. These veterinary practitioners also identified possible control herds and

conducted samplings. Matching was done for sampling conductor to avoid personnel bias within the same pair, and for herd size. No regional limitations were used. The approximate location of each case control pair is shown in Figure 3.

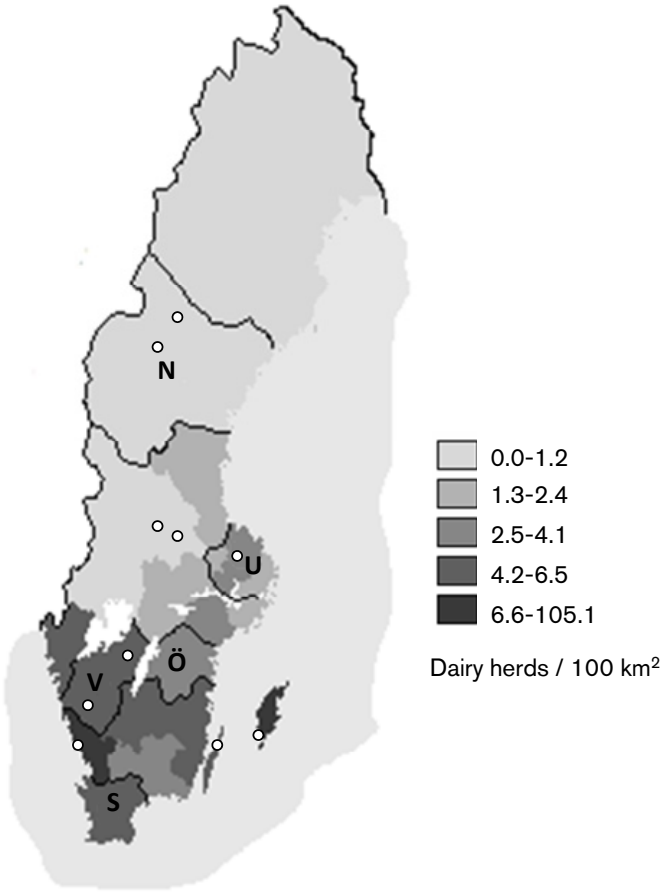

Figure 3. Dairy herd densities in different areas of Sweden and location of samplings in paper I and III. Sampled regions in paper I marked by letters: S - Skåne, V -Västergötland, Ö - Östergötland, U - Uppland, N - southern Norrland. Approximate locations of case control pairs in paper III are marked by white circles.

The systematic review and meta-analysis in paper IV utilized already performed studies from a number of different calf populations.

3.2 Sample and data collection

In paper I and III, each herd was visited once and 25 animals, including 10 preweaned calves (<2 months), 10 young stock animals (4-12 months) and 5 periparturient cows (1 week ante partum to 2 weeks post partum) were to be sampled. This sample size would enable detection of at least one randomly selected shedding animal at a herd prevalence of 10% with 95% confidence assuming a perfect test (Dohoo *et al.*, 2003). Additional diarrhoeal calves in the herds were sampled. Blood was collected from 1- to 8-day-old calves for analysis of serum total protein (TP). A number of variables concerning the health status of sampled animals (e.g. faecal consistency and body condition score) and the environment in herds were recorded. In paper III, it was also recorded whether sampled calves had been medically treated, and in that case what drug, against which symptoms and when in time compared to sampling. Questionnaires were used to interview farmers about management routines at the visit. Samplings were performed during the stable seasons (mid October to end of March) 2005-2006 (paper I), 2006-2007 (paper I, III) and 2007-2008 (paper III). In total, 69 herds were sampled, as one of the herds in paper I also participated as a control herd in paper III.

For paper II, a random selection of two samples positive for *C. parvum*-like oocysts in each age category and herd from paper I was done to select samples for molecular analysis. As a first option, random selection was only done from samples estimated to contain at least 250 oocysts to increase chance of successful analysis (DVM Charlotte Maddox-Hyttel, personal communication). However, if such samples were not available, samples with lower oocyst counts were used so that all infected herds would be represented. In addition, all *C. andersoni* positive samples were subjected to molecular analysis. In cases when PCR or sequencing failed, another positive sample from the same age group and herd was chosen.

In paper IV, five databases on the internet and the library catalogue of SLU were searched to find cohort studies or clinical trials performed on calves and investigating the substance halofuginone. Search terms were 'cryptosporid* AND halofuginone' (PubMed, Scirus, Web of Science, Agricola), 'cryptosporidium halofuginone' (IVIS) and 'cryptopsoridios*', 'parvum' and 'halofuginone' (library catalogue of SLU). In addition, references used in identified papers and conference abstracts were searched, posters were collected on site at one conference and personal contact was made with researchers when interesting references were identified but full

texts could not be retrieved through the internet or prints. Data from identified studies were extracted and entered into a database. To be included in the meta-analysis, studies should use the recommended treatment regimen of ~100 μg halofuginone/kg and day for 7 consecutive days as either prophylactic or therapeutic treatment. Studies were excluded if original data could not be retrieved (i.e. only abstract was available) or if calves were not followed in parallel.

3.3 Laboratory analyses

3.3.1 Detection of *Cryptosporidium* oocysts

Faecal samples were cleaned and concentrated using a saturated sodium chloride flotation method. Briefly, 1 g of each sample was suspended in saturated sodium chloride and centrifuged to separate oocysts from faecal debris. Supernatants (containing the oocysts) were transferred to new tubes and further cleaning was done by addition of water, followed by centrifugation and then vacuum was used to remove supernatant until 5 mL remained. This cleaning step was repeated three times, but the last time only 1.5 mL of samples was left in the tubes. Cleaned samples were stored at 4-8°C. Subsamples of 60 μL were put on teflon printed microscope diagnostic slides (Immuno-Cell, Belgium) dried, fixed and stained with 20 μL fluoresceine isothiocyanate conjugated anti-*Cryptosporidium* monoclonal antibody (FITC-mAb; Crypto Cel IF test kit, CelLabs, Australia). Samples were evaluated by epifluorescence microscopy at 200 and 400 x magnifications. The method enables detection of oocysts at shedding rates of 50-100 OPG (Andersson, 2004), and is described in more detail in paper I.

3.3.2 DNA analysis of *Cryptosporidium* positive samples

For determination of *Cryptosporidium* species, oocyst DNA was extracted using a combined freeze-thawing and QIAamp DNA stool mini kit (Qiagen) protocol (Quilez *et al.*, 2008). A ~800 base pair fragment of the 18S rRNA gene was amplified by a nested PCR protocol (Santín *et al.*, 2004). Samples with verified *Cryptosporidium* presence by PCR were purified and subjected to gene sequencing in both directions. Samples positive for *C. parvum* were further analysed at the GP60 locus by a nested PCR protocol (Chalmers *et al.*, 2005) followed by gene sequencing in both directions to determine subtype. Contigs of forward and reverse sequences were assembled and aligned using modules ContigExpress and AlignX of the Vector NTI 10 software (Invitrogen). Contigs were then compared to sequences deposited in GenBank using BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

3.3.3 Additional analyses

In paper III, rotavirus, coronavirus, *Escherichia coli* (*E. coli*) F5+ and TP were analysed using in-house methods from SVA. Faeces from all calves were investigated for presence of rotavirus and coronavirus by indirect antigen ELISAs. *Escherichia coli* F5+ was analysed in calves up to two weeks of age and was detected through cultivation on blood agar and agglutination tests for F5 adhesin. Total protein was measured by refractometry.

3.4 Statistical methods

All data were entered into a Microsoft Access database (© 1989–1997 Microsoft Corporation) and transferred to Stata 9 (paper I, IV) or Stata 10 (paper II, III) (© 1984-2008, StataCorp, College Station, Texas) for data work up and statistical calculations.

3.4.1 Descriptive statistics

Depending on data distribution, descriptive statistics were done using Fisher's exact test, χ^2 test or the Mann-Whitney test to compare proportions of species, OPG and diarrhoea and to compare *Cryptosporidium* prevalence in case and control herds (paper III). For the thesis, *Cryptosporidium* prevalences in paper I and III were compared using the non-parametric equality-of-medians test, TP levels were compared using ttest, age in *Cryptosporidium* positive/negative calves with TP values was compared by the Mann-Whitney test, and sequenced calf *Cryptosporidium* samples (paper II, III) were analysed for the association of diarrhoea with *C. parvum* or *C. bovis* (χ^2 test).

3.4.2 Multivariable modelling

Multivariable modelling was done in paper I and III. Poisson regression was used to evaluate factors associated with prevalence of *C. parvum*-like oocyst shedders in sampled animals within a herd (paper I). Logistic regression was used to evaluate factors associated with *C. parvum*-like oocyst shedding in individual calves, young stock animals and cows, with herd as random effect to adjust for clustering within herds (paper I). Logistic regression was also used to evaluate factors associated with being a calf in a case herd, using robust standard errors to adjust for clustering within herds (paper III). Multivariable modelling was preceded by univariable modelling, using $p>0.2$ as an exclusion criteria from further modelling. Spearman rank correlations were used to detect collinearity between variables with $p\leq0.2$. If variables were correlated ≥60%, one was chosen for further analysis. For the poisson model, submodelling was performed based on questionnaire

categories to decrease the number of variables to include in multivariable modelling. Variables with $p \leq 0.05$ in submodels were used in the multivariable model. Manual backward elimination was used in paper I and manual forward selection was used in paper III. Confounding was assessed for every variable deleted from or entered into a model, and considered to be present if any prevalence ratio (PR) or odds ratio (OR) changed ≥25%. Any confounding variable was retained in the respective model. Once a main effects model was achieved, two-way interactions of significant variables were investigated. Graphing of poisson and negative binomial probabilities indicated that data in the poisson model were overdispersed and followed an approximate negative binomial distribution and the model was changed accordingly. Model diagnostics were performed by visual evaluation of Anscombe, Pearson and deviance residuals, Cook's distances (negative binomial model), Hosmer-Lemeshow and Pearson goodness-of-fit tests and plotting of residuals against predicted probabilities (logistic models). Detected outliers were investigated to look for data errors.

3.4.3 Meta-analysis

Results were compared for those days from where most studies had reported data collection, i.e. day 0 (study/treatment start), 4, 7, 14, 21 and day 28. Data from all studies could not be included all investigated days, and it was decided that at least three studies should be included on a single day to enable valid data interpretation. For each day and study, relative risks (RRs) with confidence intervals (CIs) between treated and control groups were calculated by the Mantel-Haenszel method. These data were then used to perform the meta-analysis using a random effects approach to adjust for differences in study populations to calculate pooled estimates (ESs) (DerSimonian & Laird, 1986). Heterogeneity, i.e. variation in treatment effect across studies, was assessed by the Q and I^2 statistics. If present (Q $p<0.1$ or $I^2>50\%$), the cause of heterogeneity was investigated through visual exploration of influence plots and metaregression of factors (e.g. number of calves in trial) that varied across studies. Subgroup meta-analysis was performed if metaregression gave significant results. Publication bias indicates that small studies have too large effects on the estimates, and was assessed by Egger's regression asymmetry test (Egger *et al.*, 1997), Begg's adjusted rank correlation test (Begg & Mazumdar, 1994) and visual exploration of funnel plots. Bias was considered to be present if at least two of these three tests indicated this.

4 Results

4.1 Prevalence of *Cryptosporidium* shedders

4.1.1 *Cryptosporidium parvum*-like oocyst shedders

Cryptosporidium positive animals were detected in 48 of 50 herds in paper I and in all 20 herds in paper III. Shedders were detected in all age groups in 12 herds from paper I and 10 herds from paper III. In paper I, 11 herds only had shedders identified in one age group. In nine of these herds, shedders were only detected in the calf group, whereas shedders were only detected in the young stock group in two herds. In both papers, similar age related prevalence patterns were seen (Figure 4), with a prevalence peak in the 3rd to 5th week of life, followed by a second but lower peak in the 8th week of life. Neither age-specific prevalences in individual animals, nor median within-herd prevalences differed in case and control herds (paper III, $p>0.05$). Age-specific prevalences in individual animals (paper I vs. paper III) were 52% vs. 66% in calves, 29% vs. 37% for young stock and 6% vs. 14% for cows ($p=0.001$ for calves, $p<0.01$ for cows and $p<0.05$ for young stock).

Median within-herd prevalences were 35% (range 0-71%) and 43% (range 23-64%) in paper I and paper III respectively. In addition, median within-herd prevalence in paper I was higher in the second than in the first year (24% vs. 38%, $p=0.01$), but the prevalence range was wider in the first year (0-71% vs. 23-58%). Median within-herd prevalences by age group (paper I vs. paper III) were 56% (range 0-100%) vs. 65% (range 30-100%) in calves, 25% (range 0-100%) vs. 32% (range 10-80%) in young stock and 0% (range 0-40%) vs. 10% (range 0-60%) in cows ($p<0.05$ for cows).

The youngest positive calves were two days old (n=3). The herd that participated in both paper I and III was negative for cryptosporidia in paper I but positive two years later when sampled for paper III.

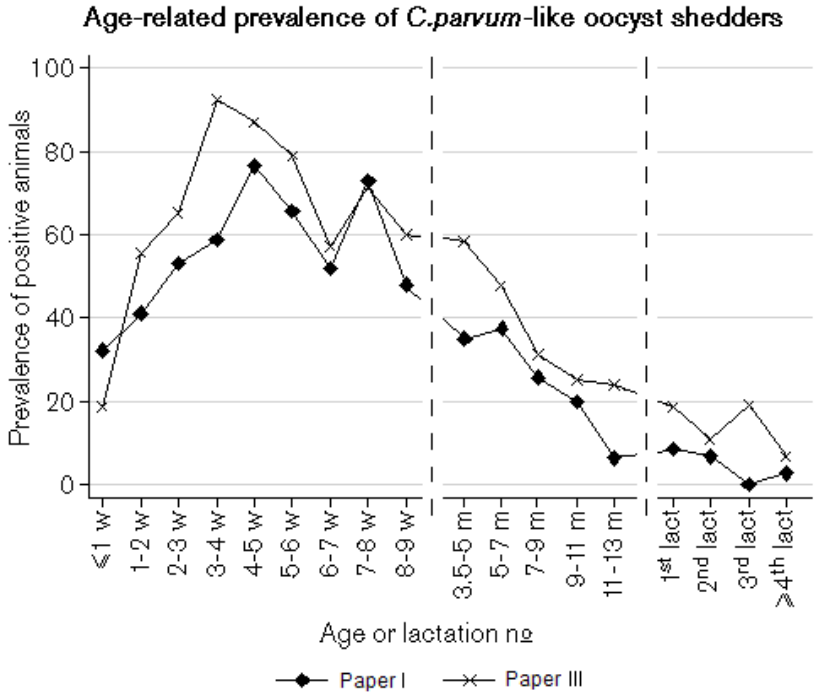

Figure 4. Age related prevalences of *C. parvum*-like oocyst shedders in paper I and paper III. **w**: age in weeks (preweaned calves); **m**: age in months (young stock); **lact**: lactation number. The curve for paper I is based on 459 calves, 493 young stock animals and 249 cows. The curve for paper III is based on 196 calves, 198 young stock animals and 100 cows.

4.1.2 *Cryptosporidium andersoni* oocyst shedders

Cryptosporidium andersoni oocysts were detected in both paper I (n=7) and paper III (n=9). This is the first time *C. andersoni* has been reported in Sweden (paper I). Six animals had mono infection by microscopy and 10 animals also shed *C. parvum*-like oocysts. *Cryptosporidium andersoni* oocysts were found in four calves aged 7-34 days, eight young stock animals aged 174-376 days, two periparturient heifers and two cows (parity 3 and 5 respectively). Shedding rates were 100-550 OPG, except for one periparturient heifer that shed ~1.65 x 10^6 OPG. This heifer calved three days after sampling. Due to the high shedding rates, she was further sampled one and two weeks after the first sampling for follow up. The shedding rates had then declined to ~500,000 and ~250,000 OPG respectively, and at the last

sampling, approximately 50% of the oocysts appeared fragile and less fluorescent. She was sampled again approximately one week after her next calving a year later. This time no oocysts were detected.

4.2 *Cryptosporidium* species and subtype distribution

4.2.1 *Cryptosporidium* species

Species could be determined in 186 of 269 (69%) samples from 66 of the 68 infected herds. Of these 186 sequenced samples, 115 were from calves, 59 from young stock and 12 from cows. The lowest estimated oocyst count in successfully sequenced samples was 25 oocysts (n=2), but only 30 of 75 samples (40%) containing <250 oocysts were successfully sequenced compared to 156 of 194 (80%) of those with ≥250 oocysts.

All four species known to commonly infect cattle were identified, with *C. bovis* being most common (76.9%), followed by *C. parvum* (12.4%), *C. ryanae* (8.6%) and *C. andersoni* (2.1%). An age-related pattern in species distribution was seen, but *C. bovis* was still the most prevalent species in all age groups (Figure 5). *Cryptosporidium parvum* was detected from 4 days of age, *C. bovis* from 7 days of age and *C. ryanae* from 12 days of age. Species distribution did not differ between case and control herds in paper III. *Cryptosporidium parvum* was only identified in preweaned calves and this was the most prevalent species during the first week of life. In the second week, *C. bovis* and *C. parvum* prevalences were equal and after that *C. bovis* dominated (Figure 6). *Cryptosporidium ryanae* was identified in calves and young stock. Presence of *C. andersoni* was confirmed in young stock in paper II, and in cows in paper III, but could not be confirmed in any of the four calves positive by microscopy. For cows, the two successfully analysed samples in paper II both contained *C. bovis*, whereas eight samples in paper III contained *C. bovis* and two contained *C. andersoni*.

Mixed infections were indicated in nine samples that produced double spikes at sequencing (Figure 2B, p 14). Of these, three had been diagnosed with mixed *C. andersoni* and *C. parvum*-like infection at microscopy. Despite the high number of double spikes, sequences from eight of the samples matched sequences in GenBank, but only one species per sample could be confirmed.

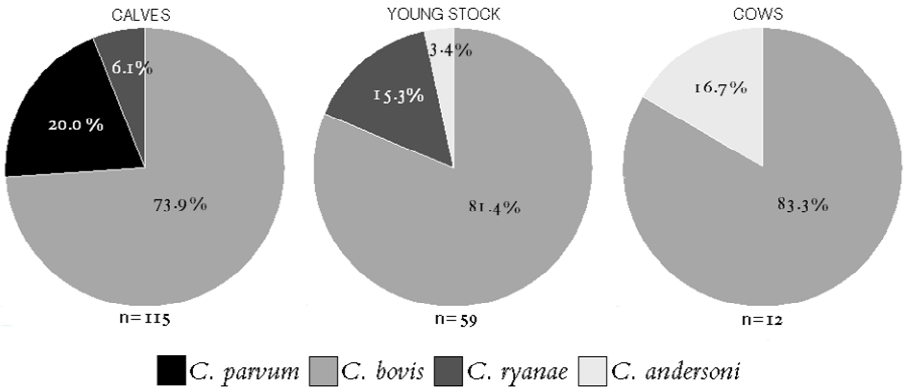

Figure 5. Species distribution in all successfully sequenced samples from calves, young stock and cows in paper II and paper III. **n:** number of successfully sequenced samples within each age group.

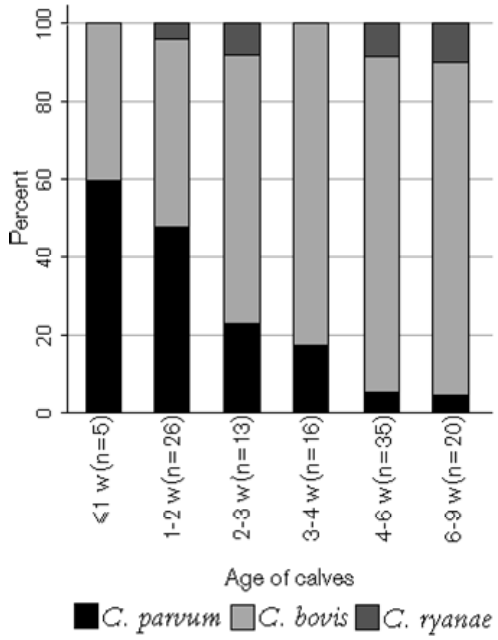

Figure 6. Species distribution in successfully sequenced samples from preweaned calves of different ages in paper II and paper III. **w:** week(s) in life, **n:** number of calves included in each category.

There was no obvious spatial pattern in species distribution when summarizing results from paper II and III. *Cryptosporidium parvum* showed a geographically limited distribution to southern counties with high herd densities in paper II, with most isolates (10 of 15) identified in Skåne, but this species was identified in low herd density regions further north

(Dalarna, southern Norrland) in paper III. *Cryptosporidium andersoni* was confirmed in four herds from three regions (Skåne, Uppland and southern Norrland) by molecular analysis. These regions represent different parts of the country as well as different herd densities. In addition, samples from Östergötland and Västergötland were diagnosed with *C. andersoni* by microscopy.

4.2.2 *Cryptosporidium parvum* subtypes

Nine subtypes were identified in 21 of 23 samples determined to contain *C. parvum* (paper II and III). All subtypes belonged to the zoonotic families IIa (n=5) and IId (n=4). Three subtypes were novel, IIaA21G1R1 (n=3), IIdA16G1 (n=1) and IIdA23G1 (n=2). Two previously identified subtypes, IIdA20G1 (n=2) and IIdA22G1 (n=1), had variations outside the repetitive regions compared to the reference sequences in GenBank, and were named IIdA20G1e and IIdA22G1c. These five unique sequences were subsequently reported to GenBank (accession numbers FJ917372-FJ917376). The other isolates belonged to subtype IIaA15G1R1 (n=2), IIaA16G1R1 (n=6), IIaA17G1R1 (n=2), and IIaA18G1R1 (n=2). When two *C. parvum* isolates from a herd were sequenced, only one subtype was identified.

4.3 Factors associated with shedding of *C. parvum*-like oocysts

At herd level, five investigated factors were associated with prevalence among sampled animals in the multivariable model (paper I). Placing of young stock close to calves or close to calves and cows, using a continuous system or mixing continuous and all-in all-out systems when moving young stock, and herds sampled in the second year (2006-2007) were associated with higher PRs. Weaning calves at 9-12 weeks of age compared to weaning before 9 weeks or after 12 weeks of age, and cleaning single pens a few times per year compared to cleaning several times per calf were associated with lower PRs. No confounders or significant two-way interactions were detected.

The multivariable model for calves included four significant variables. OR for infection in calves increased with age. In similarity to the herd model, placing of young stock close to calves or close to calves and cows and using a continuous system or mixing continuous and all-in all-out systems were associated with a higher OR for infection. Leaving the calf with the dam for at least 12 h decreased OR for infection compared to separation before 5 h of

age. The average time cows spent in maternity pens was identified as a confounder and was thus retained in the model although non-significant. No significant two-way interactions were detected and the model had a good fit (p=0.86).

Although univariable logistic regression identified six variables associated with shedding in young stock, age was the only significant factor that remained after multivariable modelling, with decreasing OR as age increased. The model had a poor fit (p<0.01).

The multivariable cow model included two significant variables. Cows from organic herds had a higher OR of infection compared to cows from conventional herds, and cows from herd with ≥30 calves at sampling had a higher OR than cows from herds with ≤15 calves. No significant two-way interactions were detected. Standard errors were large and CIs were wide, indicating unstable estimates. The model had a moderate fit (p=0.20).

4.4 Factors associated with diarrhoea and diarrhoeal problems

Data on oocyst output in diarrhoeic and non-diarrhoeic calves from paper II and III are given in Table 1. When comparing calf samples from paper II and III, diarrhoea was more common in calves infected with *C. parvum* than in calves infected with *C. bovis* (p<0.05). In contrast, there was no association between any of the *Cryptosporidium* species and diarrhoea or oocyst output in paper III. Diarrhoea was however more common in case herd calves (p<0.05). Only 31 of 196 sampled calves in paper III presented with diarrhoea (22 of 104 case calves and 9 of 92 control calves). Of these 31 calves, 2 had *C. parvum*, 6 had *C. bovis* and 12 were infected with undetermined *Cryptosporidium* spp. In addition, none of the other pathogens analysed in paper III were significantly associated with diarrhoea. Rotavirus and coronavirus were both detected in 2 diarrhoeic calves and *E. coli* F5+ was only detected in one non-diarrhoeic calf. Rotavirus was detected in case herds as well as control herds, whereas coronavirus and *E. coli* F5+ were only detected in control herds. Only one diarrhoeic calf was diagnosed with more than one pathogen (coronavirus and undetermined *Cryptosporidium* spp.). Full information on pathogen detection in paper II and III is given in Table 2.

Table 1. Oocyst output ranges by *Cryptosporidium* species in diarrhoeic vs. non-diarrhoeic calves in paper II and III.

Cryptosporidium	Oocyst output per gram faeces (OPG)	
	Paper II [a]	Paper III [a]
Cryptosporidium positive	100-4 x 10^7/100-2.0 x 10^8	100-4 x 10^6/100-1 x 10^8
Cryptosporidium spp. [b]	100-4 x 10^7/100-1 x 10^7	100-4 x 10^6/100-7 x 10^6
C. parvum	1 x 10^5-1 x 10^7/1500-2 x 10^8	12,750-3 x 10^6/2800-2 x 10^8
C. bovis	9300-1 x 10^6/300-2 x 10^7	9250-2 x 10^6/1200–2 x 10^6
C. ryanae	4300/1300-101,300	- /3650-500,000
C. andersoni [c]	- /300	- /100-150

[a] diarrhoeic/non-diarrhoeic calves; [b] samples containing *Cryptosporidium* oocysts of undetermined species; [c] positive for *C. andersoni* oocysts at microscopy, none of these samples could be verified by DNA analysis.

Table 2. Distribution of *Cryptosporidium* species and other pathogens in calves in paper II and III.

Detected pathogens	Number of calves					
	Total		No diarrhoea		Diarrhoea	
	II [a]	III [b]	II [a]	III [b]	II [a]	III [b]
Calves in paper	94 (459)	104/92	75 (371)	82/83	19 (88)	22/9
Cryptosporidium positive [c]	94 (241)	129	75 (196)	52/57	19 (45)	17/3
Cryptosporidium spp. [d]	21	90	12	40/38	8	10/2 [f]
C. parvum	15	9	9	5/2	6	2/0
C. bovis	54	27	50	5/16	4	5/1
C. ryanae	4	3	3	2/1	1	0/0
C. andersoni [e]	1	0/3	1	0/3	0	0/0
Rotavirus	ND	16	ND	4/10	ND	1/1
Coronavirus	ND	6	ND	0/4	ND	0/2 [f]
E. coli	ND	1	ND	0/1	ND	0/0

[a] Number in parenthesis represents calves in paper I (samples in paper II were a random subset of the 241 positive samples from paper I); [b] case/control calves; [c] Some samples positive for both *C. andersoni* and *C. parvum*-like oocysts; [d] samples containing *Cryptosporidium* oocysts of undetermined species; [e] positive for *C.andersoni* oocysts at microscopy, none of these samples could be verified by DNA analysis; [f] one diarrhoeic calf had co-infection with coronavirus and *Cryptosporidium* spp. ND: not done.

Descriptive analysis of herd data in paper III showed that disinfection of single pens between calves was associated with having diarrhoeal problems ($p<0.05$). Other investigated factors were not significant or could not be used for statistical analysis, but several factors seemed differently distributed between case herds and control herds when looking at data distribution.

Three significant variables were included in the model for factors associated with being a case herd calf. In agreement with descriptive statistics at herd

level, disinfection of single pens gave a higher OR of being from a case herd. Calves from herds where diarrhoeal consistency usually varied considerably had a higher OR of belonging to a case herd compared to when pasty diarrhoea was most common. Calves infected with *C. bovis* had a lower OR of belonging to a case herd compared to *C. bovis* negative calves. Whether *C. parvum* had been identified or not in a herd was also included in the model. This variable was not significant but acted as a confounder to the diarrhoeal consistency and disinfection variables.

4.5 Total protein in 1-to 8-day-old calves

Blood was obtained from 141 calves, and faeces were obtained from 121 of these calves. Total protein levels ranged from 42-80 g/L, and mean TP for all calves was 55.7 g/L. *Cryptosporidium* positive calves had a mean TP below the acceptable value, and mean TP was significantly lower than in *Cryptosporidium* negative calves ($p<0.01$, Table 3). There was also a significant age difference in these calves ($p<0.05$) with a median age of 4 days in *Cryptosporidium* negative calves (10 - 90 percentiles 1 - 8 days) vs. 5 days in *Cryptosporidium* positive calves (10 - 90 percentiles 2 - 8 days). There were no significant differences in mean TP level between calves from paper I and III, between bull- and heifer calves or between calves with diarrhoea or not (Table 3). The same pattern was seen when samples for each of the papers were analysed separately, with a $p<0.05$ for lower mean TP and a mean TP below the acceptable value (53.8 g/L, n=28 (paper I) vs. 50.4 g/L, n=7 (paper II)) in *Cryptosporidium* positive calves, but no significant mean TP differences by sex or diarrhoeal status. However, comparing case and control herd calves (paper III), mean TP approached statistical significance ($p=0.07$, single-sided ttest) with 54.3 g/L (95% CI 51.4-57.1 g/L) in the 23 case herd calves vs. 57.0 g/L (95% CI 54.6-59.5 g/L) in the 22 control herd calves.

At herd level (paper III), median TP was comparable in case and control herds and situated around the acceptable value (55 g/L) for sufficient passive immunity (Radostits, 2000) although all control herds reported to feed colostrum by bottle and routines varied in case herds. Two case and two control herds reported to measure colostral quality. The two case herds had median TPs of 50 and 52 g/L, whereas the two control herds had medians of 58 and 64 g/L.

Table 3. Total protein in serum samples from 141 1-to 8-day-old calves in paper I and III.

Category	Calves	Mean Total Protein g/L	95% Confidence Interval g/L (range g/L)
Study			
paper I	96	55.7	54.4 - 57.1 (42 - 80)
paper III	45	55.6	53.7 - 57.5 (42 - 71)
Sex			
male	67	56.4	54.7 - 58.1
female	74	55.0	53.6 - 56.5
Cryptosporidium			
positive	35	53.2 [a]	51.1 - 55.2
negative	86	56.8 [a]	55.3 - 58.2
Diarrhoea			
yes	14	57.6	53.8 - 61.3
no	107	55.5	54.2 - 56.8

[a] $p<0.01$, double-sided ttest

4.6 The effect of halofuginone on calf cryptosporidiosis

Seven studies on halofuginone treatment could be used to investigate the effects of prophylactic use of halofuginone on infection prevalence, diarrhoeal prevalence and overall mortality. Only three studies on therapeutic use had enough information to allow meta-analysis but much heterogeneity was observed and valid interpretations of results could not be performed. All ten included studies were randomised. Two prophylactic studies were reported as double blinded and two were reported as blinded. One of the therapeutic studies was reported as blinded. Data on oocyst output, diarrhoeal intensity and weight gain were presented in so many different ways that it was not possible to make reliable comparisons. Data on general condition and dehydration were too sparse to analyse.

Prophylactic use delayed infection and diarrhoea, as indicated by lower prevalences in treated groups compared to control groups on study days 4 (ES 0.45, 95% CI 0.32; 0.64 for infection prevalence and ES 0.5, 95% CI 0.35; 0.71 for diarrhoeal prevalence) and day 7 (ES 0.51, 95% CI 0.42; 0.62 for infection prevalence and ES 0.51, 95% CI 0.40; 0.65 for diarrhoeal prevalence). However, after treatment was terminated, infection and diarrhoeal prevalence increased in the treated groups and on day 21 infection prevalence was significantly higher in the treated groups compared to control groups (ES 2.13, 95% CI 1.07; 4.25). Heterogeneity was present days 14 and 21 for infection prevalence. Two large studies (>100 calves) were identified as highly influential but were kept in the overall estimate.

Metaregression identified sponsor as a significant variable day 14 ($p<0.001$), with non-sponsored studies having a negative coefficient (-0.82; 95% CI 1.23; 0.41) compared to sponsored studies, indicating a better effect of halofuginone treatment in non-sponsored studies. Subgroup meta-analysis confirmed these differences with ES 1.01 (95% CI 0.62; 1.64) in sponsored studies compared to ES 0.42 (95% CI 0.22; 0.81) in non-sponsored studies. Sponsor also showed a non-significant trend in the same direction day 21. Publication bias was indicated for days 7 and 14 and was caused by two small, non-blinded studies. Overall mortality was not affected by prophylactic treatment.

5 Discussion

5.1 *Cryptosporidium* shedding and species distribution

The identification of *Cryptosporidium* in 68 of 69 sampled herds indicates that these parasites are ubiquitous in Swedish dairy herds of average herd size and more. Herd and calf *Cryptosporidium* prevalences in paper I were of similar magnitude to those found in a Danish study of similar design (Maddox-Hyttel *et al.*, 2006). In the two herds that were negative in paper I, 8 of 10 and all 9 preweaned calves respectively were sampled, covering most of the two-month age interval. Based on *Cryptosporidium* infection dynamics and the sensitive detection method used, it is probable that these herds were negative at that time. One of these herds thereafter became infected and *Cryptosporidium* oocysts were identified in calves and young stock when it was sampled for paper III two years later.

The age distribution of *C. parvum*-like oocyst shedders was approximately the same in paper I and paper III although both median within-herd and age group-specific prevalences were significantly higher in paper III. Median within-herd prevalence in paper I was also significantly higher in the second year compared to the first year but not significantly different from the median within-herd prevalence in paper III. This could reflect a prevalence increase, e.g. due to weather conditions that benefited *Cryptosporidium* more in the second and third year of sampling. A recent rapid spread is unlikely because the parasite is a frequent finding in calf diarrhoeal samples at SVA.

The two prevalence peaks observed in preweaned calves (Figure 4, p 34) indicate that repeated, or perhaps more probably constant, exposure occurs and that primary infection is not completely protective. Similar prevalence results have been shown by Santín *et al.* (2004) and Santín *et al.* (2008).

However, in both these studies the first prevalence peak occurred at two weeks of age and was due to *C. parvum* infection. A much smaller peak was associated with *C. bovis* infection at four weeks of age before a second *C. parvum* peak occurred at seven weeks of age (Santin *et al.*, 2008). Based on our molecular analysis results, *C. parvum* is probably not the main cause of either peak seen in the Swedish calves (Figure 6, p 36). In most herds in both paper I and II, calves were moved to group pens at the latest by three weeks of age. Taking the prepatent periods into account, grouping could explain the timing of the first peak. The second peak is difficult to explain. Re-grouping or pen relocation was done at weaning or soon thereafter, but only two herds weaned calves as early as in the 8th week of life. However, some herds moved calf groups several times before weaning, which would expose calves to new infection pressures from the previous pen inhabitants.

Even since molecular analysis became an important tool in species determination and two additional species similar to *C. parvum* were identified in cattle, *C. parvum* has been the dominant species isolated from calves (Brook *et al.*, 2009; Broglia *et al.*, 2008; Soba & Logar, 2008; Plutzer & Karanis, 2007; Thompson *et al.*, 2007; Langkjaer *et al.*, 2006; Trotz-Williams *et al.*, 2006; Santín *et al.*, 2004). Thus, it was surprising to find such a dominance of *C. bovis* in our preweaned calves, even in calves from herds with diarrhoeal problems. However, *C. bovis* has been identified as the dominant species in extensively reared calves in Zambia (Geurden *et al.*, 2006) and also in calves in some herds in the US and in Asia (Feng *et al.*, 2007). The sensitive method used for oocyst detection facilitates the identification of low OPG shedders. Because *C. bovis* is associated with lower shedding rates than *C. parvum*, this could have affected species distribution compared to studies using less sensitive methods. Still, shedding rates were similar in samples identified as *C. bovis* and *C. parvum*. The species distribution in young stock and cows was approximately as could be expected based on the results from previous studies. No samples from these age groups were positive for *C. parvum*, showing that grazing cattle should be of minor importance for zoonotic transmission, even if water contamination with *Cryptosporidium* oocysts occurs.

Cryptosporidium parvum-like oocysts were detected from two days of age, which is in agreement with the *C. parvum* prepatent period, and indicates transmission either from the dam or from contamination of calving pens. *Cryptosporidium parvum* was confirmed by molecular analysis from four days of age. *Cryptosporidium bovis* was identified in two 7-day-old calves, one 8-

day-old and one 9-day-old calf, showing that the prepatent period is shorter than the previously described 10 days (Fayer *et al.*, 2005). This prepatent period was determined based on experimental infection in two calves previously infected with *C. parvum*. Thus, our results indicate a shorter prepatent period in *Cryptosporidium*-naïve calves, perhaps due to a partial resistance to other species stimulated by an earlier *C. parvum* infection. *Cryptosporidium ryanae* was identified from 12 days of age, which is in agreement with the described prepatent period.

The identification of high numbers of *C. andersoni* oocysts in one heifer, with subsequent decline in shedding rates in the weeks post partum could be an indication of the periparturient rise previously described (Ralston *et al.*, 2003; Faubert & Litvinsky, 1999). Unfortunately we do not have enough data on ante partum shedding to confirm this theory. A 7-day-old calf was positive for one *C. andersoni* oocyst in addition to *C. parvum*-like oocysts. Even if this calf was infected on the day of birth, shedding occurred much earlier than the shortest prepatent time described (18 days). It is thus uncertain whether this oocyst really reflects infection, or was just "passing through" or if the sample was contaminated. One other calf and two young stock animals in the same herd also shed single oocysts of *C. andersoni* but these low rates do not facilitate contamination. Except for this 7-day-old calf, shedding was detected in 25- to 34-day-old calves, which is well above the lowest prepatent time limit.

Mixed infections were only identified in 10 samples by microscopy, and indicated in an additional 6 samples at sequencing. It is likely that more samples contained mixed infections in such different proportions that PCR and sequencing produced evidence of mono infection, but this was not further investigated.

5.2 *Cryptosporidium parvum* subtypes

The many subtypes in examined samples and the indicated subtype clonality within herds is in agreement with what has previously been shown for areas that use closed herd management. Five unique GP60 sequences were identified, including three novel subtypes and two subtypes with small sequence variations compared to reference sequences. Sweden has a quite isolated geographical location, with a long coastal line and a mountain chain and wilderness on the border to neighboring countries. This, together with closed herd management, has enabled successful control programmes against

some serious infectious diseases such as bovine viral diarrhea, and could also contribute to an isolated and distinctive *C. parvum* subtype population. The persistence over time of a unique *C. parvum* MLG in a Swedish dairy herd has been reported (Björkman & Mattsson, 2006), and GP60 subtyping has now shown that this isolate belongs to subtype IIdA20G1e (unpublished), which was one of the unique sequences identified in paper II. Population uniqueness has previously been shown in e.g. Ireland, where subtype IIaA18G3R1 dominated in calves as well as in human cases whereas an otherwise widespread zoonotic subtype, IIaA15G2R1 (Alves *et al.*, 2006; Trotz-Williams *et al.*, 2006; Sulaiman *et al.*, 2005) was much less prevalent (Zintl *et al.*, 2008; Thompson *et al.*, 2007). Neither of these two subtypes was identified in our material, further indicating an isolated population. Because only 21 *C. parvum* isolates were subtyped, many more samples need to be subjected to molecular analysis in order to get a complete picture of the *C. parvum* population. More samples per herd also need to be subtyped because a maximum of two *C. parvum* samples per herd were used. This could of course contribute to a false clonal pattern.

All subtypes identified belonged to zoonotic subtype families, and four of the subtypes have previously been identified in humans, suggesting a strong zoonotic potential in *C. parvum* infected calves. Due to the apparent dominance of *C. bovis* in Swedish dairy calves, the zoonotic potential of a *Cryptosporidium* infected calf might still be low.

5.3 Factors associated with shedding of *C. parvum*-like oocysts

The fact that two variables were significantly associated with shedding at both herd and calf level in paper I was probably due to the large influence calf prevalence would have on within-herd prevalence. These two variables concerned young stock management, but young stock should not affect calf prevalence because *Cryptosporidium* species distribution was expected to differ between these groups. Thus, the identification of the "all in all out" variable and the higher PR/OR for keeping calves and young stock close together seemed odd. Nonetheless, when adding the results from molecular analysis, the similar species distributions in these age groups indicate that there is indeed an association between calf and young stock prevalences.

Cleaning routines of single pens and age at weaning were other calf associated variables that were significant in the herd model (paper I). Deep litter bedding has been associated with a lower OR of oocyst shedding (Maddox-Hyttel *et al.*, 2006). If farmers cleaning a few times per year use deep litter bedding, infection pressure could be reduced by frequent administration of new straw, and frequent cleaning could be associated with the exposure of viable oocysts. However, it was not recorded how farmers performed cleaning, and bedding types were not recorded in such a way that this could be further investigated. The fact that a medium age (9-12 weeks) at weaning was associated with lower PR for within-herd prevalence compared to weaning at younger or older age seemed odd. However, from Figure 4 (p 34), it is clear that prevalence decreases around 9 weeks of age. It could perhaps be beneficial to wean and move animals in this period. Because no animals were sampled between 9 weeks and 3.5 months of age, we are unaware of prevalences in this period. It is possible that yet another prevalence peak appears between 9 and 13 weeks of age. If these calves are kept with the younger calves they would contribute to the infection pressure and overall calf prevalence detected here. The effect of year of sampling could, as discussed above (section 5.1, paragraph 2), be due to better weather conditions from a *Cryptosporidium* perspective.

In addition to the two young stock variables, both age and the time a calf was allowed to stay with the dam affected the calf OR for shedding oocysts (paper I). There was a higher OR of shedding with increased age, which is associated with prolonged exposure to oocysts. Cows have been implied as a *Cryptosporidium* infection source for calves (Huetink *et al.*, 2001; Faubert & Litvinsky, 1999), but our results indicate that this is not an important infection route in Swedish dairy calves. Instead, it seems that staying with the dam is protective, because it delays exposure to the high infection pressure in the calf facilities.

In contrast to calves, young stock had a lower OR for being an oocyst shedder with increased age (paper I), as can also be seen in Figure 4 (p 34). The model fit was poor, indicating that more variables probably affect shedding status although none of the other five variables significant in univariable modelling could be included at multivariable modelling.

Interestingly, organic cows had a higher OR of being infected than conventional cows (paper I). The association between infection and management system could be due to chance since few organic cows were

sampled (30 compared to 219 conventional). Moreover, organic cows were by chance sampled closer to calving, and if a periparturient rise is indeed present, this could be an explanation for the higher OR for organic cows. Some organic herds use nursing cows for their calves, but these are generally cows that cannot be used in the daily milk production, e.g. due to high somatic cell counts, and do not include periparturient cows. Less use of drugs in organic herds should not affect *Cryptosporidium* prevalence. Maddox-Hyttel *et al.* (2006) also identified an association between organic management and oocyst shedding because organic calves had a higher OR for high shedding rates compared to conventional calves. Larger herds are expected to have higher infection pressures than smaller herds, and thus the higher OR of cows shedding oocysts when many calves were present could reflect higher infection pressures. The unstable estimates were caused by the few positive cows (14 of 249).

5.4 Factors associated with diarrhoea and diarrhoeal problems

The conflicting results regarding an association between *C. parvum* and diarrhoea, where an association was found when comparing all samples in paper II and III but not for samples in paper III, could be due to a low number of samples sequenced in paper III. A lack of association with diarrhoea, however, does not mean that *C. parvum* is apathogenic, but rather that additional factors are needed to produce clinical disease. *Cryptosporidium bovis* positive samples were not associated with diarrhoea. Indeed, *C. bovis* was identified as a protective factor in the multivariable model for case herd calves because infected calves had a lower OR of belonging to a case herd compared to those not infected with *C. bovis* (i.e. *Cryptosporidium* negative or infected with *C. parvum* or *C. ryanae*). This also indicates that *C. parvum* is the species associated with diarrhoea although no significant association was found. Still, because *C. bovis* was identified in a total of 11 diarrhoeal samples, it seems that this species also has a pathogenic potential in young calves. Further indications of a pathogenic potential of *C. bovis* were high shedding rates, indicating massive infection and the lack of co-infections in the *C. bovis* positive diarrhoeal samples from paper III. *Cryptosporidium* positive diarrhoeal samples (irrespective of species) containing few oocysts are harder to interpret because diarrhoea can already be manifest at the beginning of the patent period (Harp *et al.*, 1989), when massive intestinal infection might be present but not reflected in shedding rates.

If other diarrhoeal pathogens are not examined, an association with diarrhoea might be found although the *Cryptosporidium* infection, especially at low shedding rates, might just be an accidental finding or a secondary cause. However, none of the investigated pathogens in paper III were significantly associated with diarrhoea, and co-infection was only identified in one diarrhoeic calf. Management factors seem to have an important role in the development of diarrhoea. Several discrepancies in management routines were identified although only one produced significance (paper III). Disinfection of single pens was significantly associated with case herds both in descriptive analysis and modelling. The same association has previously been identified in weaners (Maddox-Hyttel *et al.*, 2006). Since disinfection is not effective against cryptosporidia, the association could indicate that cryptosporidia are a substantial part of the problems with diarrhoea. The association could also indicate that farmers rely on disinfection and do not perform proper cleaning before administering the disinfectant, in which case the disinfectant will not work and pathogens continue to thrive. On the other hand, the association could be an effect of the problem rather than a cause because farmers might not use disinfectants in the absence of diarrhoeal problems. Median TP levels indicate that approximately half of both case and control herd calves had not achieved sufficient passive immunity. The TP differences in the four herds measuring colostral quality indicate differences in implementation of knowledge about colostral quality because the two control herds gave all calves colostrum by bottle, whereas the two case herds only gave calves colostrum by bottle if the farmers considered it necessary.

In addition to calf *C. bovis* status and disinfection of single pens, modelling indicated that diarrhoeal consistency was more variable in case herds. This could be due to fluctuating infection pressures caused by differences in e.g. calving intensity and crowding. The confounding effect caused by the variable '*C. parvum* identified in herd' is difficult to interpret, but this variable was associated with both disinfection and faecal consistency, indicative of a role in diarrhoeal problems.

5.5 Total protein in 1- to 8-day-old calves

Cryptosporidium positive calves had significantly lower mean TP level than *Cryptosporidium* negative calves, which could indicate a protective factor in sufficient passive transfer. Passive transfer does not protect against *Cryptosporidium* infection per se (Fayer *et al.*, 1989; Harp *et al.*, 1989), but

duration and intensity of shedding is dampened in calves with sufficient passive transfer (Lopez *et al.*, 1988) and in calves fed hyperimmune colostrum (Fayer *et al.*, 1989). However, Harp *et al.* (1989) showed that prolonged colostrum feeding for seven days did not affect timing of onset of oocyst shedding or diarrhoea. Because the positive calves in our studies were older than negative ones, it could be the prepatent period that caused or at least contributed to the relation between a low TP and infection.

5.6 The effects of halofuginone of calf cryptosporidiosis

Prophylactic halofuginone treatment had no effect on mortality but lowered infection and diarrhoeal prevalence as long as calves were treated. However, it is clear that infection or diarrhoea cannot be completely prevented even during treatment, and both infection prevalence and diarrhoeal prevalence increased in treated calves once treatment had stopped. Unfortunately, only one study reported complete prevalence data on *Cryptosporidium* associated diarrhoea. Thus, overall diarrhoeal prevalence had to be used for the interpretation of effects on *Cryptosporidium* associated diarrhoea. The effects of this on our results depends both on how much of the diarrhoea that was really *Cryptosporidium* associated and whether there is an even distribution of other diarrhoeal causes between groups in a study. However, when comparing data of overall diarrhoeal prevalence and infection prevalence, they peaked at approximately the same time (Lallemond *et al.* 2007; Jarvie *et al.*, 2005; Joachim *et al.*, 2003: Lefay *et al.*, 2001), and it is thus probable that *Cryptosporidium* infection contributed to most of the diarrhoeal cases.

Because three therapeutic studies had sufficient data to allow meta-analysis each investigated day, statistics were produced. However, heterogeneity was present on most investigated days, and on some days data from only two of the studies could be included. This made interpretations difficult. Specifically, heterogeneity was present on the first study day for diarrhoeal prevalence, and when investigated further, one of the included studies, although randomized, had a significantly higher RR for diarrhoeal prevalence and an almost significantly higher RR for infection prevalence in the control group this day. Irrespective of in which direction a case distribution is skewed, it may persist throughout the study, producing false negative or positive results. Thus, the results of this study were not considered reliable and with data from only two studies remaining, a valid meta-analysis could not be performed.

In addition to infection and diarrhoeal prevalence it would have been valuable to interpret the effects on oocyst output and diarrhoeal intensity. This could not be done because these variables were reported in many different ways, and calculations of comparable RRs were not possible. For example, oocyst output could be reported as mean oocyst output or scored on 0- to 3-grade or 0- to 5-grade scales. In addition, these data could be reported as a mean for *Cryptosporidium* positive animals in each group or for all animals in each group (i.e. including *Cryptosporidium* negative animals). It would also have been useful to evaluate effects on other parameters such as dehydration, general condition and weight gain to estimate if there was an overall improvement in performance of treated calves.

Since halofuginone is toxic at twice the recommended dosage, precise dosage is required. This means that the drug has to be administered to each calf individually, which is time-consuming. Intoxication symptoms are similar to those associated with cryptosporidiosis. Farmers without knowledge about these intoxication signs might misinterpret symptoms as a lack of effect, and perhaps administer more drugs, causing detrimental effects. It is therefore imperative that farmers are comprehensively informed about the side-effects of the drug to avoid negative treatment effects.

It is possible to induce drug resistance in protozoans, as in the malaria parasite *Plasmodium falciparum* (Kokwaro, 2009). *Cryptosporidium* parasites are quite insensitive to drug treatment and disinfection, and the effect of halofuginone is cryptosporidiostatic, i.e. it depresses rather than kills the parasites. Based on the only partial efficiency against cryptosporidia, an extensive use could perhaps induce resistance to halofuginone.

Taking into consideration the meta-analysis results, the cryptosporidiostatic effect and the narrow therapeutic window, this drug should be reserved for herds with severe problems and only be used in a transitional period in conjunction with improved management routines to decrease infection pressure.

5.7 Methodological considerations

Comparison of prevalence estimations between studies are not only affected by age of sampled animals or study design (point/repeated measurements), but also by the methods used for oocysts detection. A method that detects lower grades of shedding produces better prevalence estimates than less

sensitive methods. The proportion of the different species detected can also be affected because *C. bovis* and *C. ryanae* are associated with low shedding levels compared to *C. parvum*. A method that is only able to detect >1000 OPG might thus overestimate the proportion of *C. parvum* positive samples compared to a method that detects ~100 OPG.

5.7.1 Study designs

To get an optimal estimate of the *Cryptosporidium* herd prevalence in Sweden, a simple random sample from all dairy herds across Sweden is needed. However, this was not possible for logistic reasons, and stratification was made so that areas with different herd densities across Sweden would be represented. Further, the representativeness of herds from each region was optimized by randomly sampling a number of herds equal to the percentage in the total source population. We needed to ensure that at least 5 and preferably 10 calves would be present in a herd at time of sampling. Thus, small herds were excluded. The specific cut-off of a ≥50 cows herd size was used because we believed that Swedish average herd size would exceed this number during this PhD project. A disadvantage is that as a result we could not estimate prevalence in small herds. In paper III, field practitioners were engaged in samplings. This meant that personnel bias was more likely to be present compared to paper I, where only one investigator performed samplings. To decrease personnel bias, herds were matched so that the same person would sample a case and its control. The small study size (10 + 10 herds) was due to practical limitations, e.g. that external investigators had to be used. Although within-herd sample size was calculated based on a simple random sampling structure, groups with expected or possibly higher prevalences (i.e. preweaned calves and periparturient cows in the cow group) were targeted to facilitate detection of shedders. The age span of sampled young stock probably also facilitated detection of shedders because prevalence decreased with age.

For a true estimate of species distribution in a sampled population, all positive samples must be subjected to molecular analysis. However, these procedures are costly, and with many samples unlikely to be successfully sequenced due to low oocyst counts, the second best option (random selection within herds and groups) was chosen.

Meta-analysis was considered the best method to review existing studies on halofuginone because it provides an objective review of previous results. Other possible ways of comparing results would be to perform a regular

review (i.e. without including statistical analysis and based on the reviewers subjective opinion of the results) or to make a single dataset of all studies and treat them as one study. However, this approach demands that original data for individual calves can be retrieved from the researchers.

5.7.2 Laboratory methods

Although the flotation method used combined with immunofluorescence staining allows detection of oocysts at only 50-100 OPG, false negative animals are inevitably present in these studies. Animals still in the prepatent period cannot be detected and most animals shedding lower numbers of oocysts will certainly be missed. The lowest detection limit of 50 OPG would correspond to 25 oocysts in each flotated 1.5 mL sample (estimating 50% loss during the cleaning process), and one oocyst should be present in a 60 µL subsample (1/25 of 1.5 mL) analysed by microscopy. Thus, if oocysts were not evenly distributed in samples, animals shedding around 50-100 OPG could have been interpreted as *Cryptosporidium* negative. Clearly animals shedding lower than 50 OPG could be identified if at least one oocyst ended up in the analysed subsample. False positives could be present if contamination occurred sometime from sampling to microscopy analysis. However, care was always taken to avoid contamination, and if this was suspected, e.g. if a drop from one sample accidentally contaminated another sample, this was noted and considered during microscopy. If suspicion of contamination remained after microscopy, the sample was re-analysed from the step prior to the suspected contamination. The skill of personnel performing microscopy analysis also affects the outcome.

We first used a standard COWP PCR protocol. However, there were low success rates with samples containing less than 3×10^5 oocysts. Besides from low oocyst counts and mixed infections, failure to isolate DNA, debris and presence of inhibitors in a sample can affect PCR and sequencing results. Presence of DNA in some PCR negative samples was confirmed by DAPI staining. We also brought five samples to Denmark to compare our DNA extraction method to the one they used (Langkjaer *et al.*, 2006). We found that our DNA extraction method was more efficient, because the Danish standard 18S rRNA PCR protocol was positive for samples with ~650 oocysts after using our extraction method, compared to 3×10^5 oocysts using the Danish DNA extraction method (QIAamp DNA stool mini kit only). This 18S rRNA PCR protocol was then run in our laboratory on the same samples and another eight samples, but although one sample with ~150 oocysts was positive, none of the other five samples with <3500 oocysts came out

positive. Thus, this protocol was not considered sensitive enough for our study, because 62% of all positive samples contained <3500 oocysts. To increase the possibility of amplification in samples with lower oocyst counts, we switched to a nested 18S rRNA protocol. This improved the sensitivity, but we were still unable to produce positive PCR results in eight samples with >1000 oocysts (maximum 50,000 oocysts). Presence of inhibitors was investigated in PCR negative samples containing >100 oocysts, but no such presence was indicated.

5.7.3 Statistical analyses

To account for clustering within herds (i.e. animals within a herd are more alike than animals from different herds), random effect models or robust standard errors were used when modelling at individual animal level. Submodels were used to decrease the number of variables to be included in multivariable modelling, based on the questionnaire categories. Other methods, such as decreasing the "significant" p-value in univariable modelling to e.g. 0.1 could have been used, but this could result in the deletion of important variables. Another possible approach would be to use forward selection instead of backward elimination because this introduces one variable at a time instead of trying to force all variables in at the same time. Still, this would mean that a high number of variables had to be tested, increasing the chance for spurious effects.

The design with stratified sampling within herds made it impossible to make valid interpretations of overall within-herd prevalences and prevalences in young stock and cows. Because all or most calves were sampled in almost every herd, calf prevalence estimations should not have been affected by stratification.

The small sample size in paper III did not facilitate statistical analysis, and instead of a matched case-control analysis approach at herd level, modelling had to be done at calf level. Some apparent differences between case and control herds could still be seen when looking at distribution of questionnaire results although these could not be verified statistically.

More rigorous results are needed before a drug is approved for human use compared to animal use. Thus, veterinary drug trials usually include quite a few study objects, reducing the power of finding significant differences. Even if several trials are conducted, it might be difficult to compare results based on differences in trial design, analysis method sensitivities and data

presentation. Moreover, a regular review is based on the subjective impression of the reviewer. Meta-analysis can be used to make an objective review, including a statistical analysis that produces an overall estimate based on the results of all included studies. This approach increases the power to make correct interpretations about the drug's effects. It is required that all studies report data in a similar manner, but it is not required that the analysis methods used differ in sensitivity because meta-analysis first produces an estimate (e.g. RR) that compares groups within a study to each other. These estimates can then be used to compare studies to each other because they are not affected by between-study variability in analysis methods. The assessment of heterogeneity is important because heterogeneity indicates that data variation is larger than by mere chance. Influential studies identified on days with heterogeneity in prophylactic analysis were kept in the overall estimates since these studies were large, randomized and blinded, which should indicate that data quality is high. However, the identification of heterogeneity in the therapeutic analysis was considered to be too important to ignore based on the few included studies as well as the cause for heterogeneity.

6 Conclusions

- *Cryptosporidium* parasites are common in Swedish dairy herds and all four species previously reported as common in cattle (*C. parvum, C. bovis, C. ryanae* and *C. andersoni*) are present.

- Infection and species distribution are age related although species distribution in calves differs from previous studies.

- The prepatent period of *C. bovis* is not longer than 7 days, in contrast to the 10 days that has previously been shown.

- Several management factors were associated with oocyst shedding at herd level and in individual animals. The only factor associated with diarrhoeal problems at herd level was disinfection of single calf pens, whereas two additional factors were associated with being a calf in a case herd.

- *Cryptosporidium parvum* seems to be the species associated with calf diarrhoea although *C. bovis* was also identified in a number of diarrhoeal samples. The *C. parvum* subtype distribution implies a zoonotic potential, although the zoonotic potential of a randomly selected *Cryptosporidium* infected calf is low.

- Halofuginone has some beneficial effects on calf cryptosporidiosis, at least when used for prophylaxis. However, it is recommended that this drug is used with great care because of its toxic effects and a potential for induction of drug resistance.

7 Future research

- To get a better estimate of *Cryptosporidium* species distribution in diarrhoeal calves, a large number of diarrhoeal samples positive for oocysts need to be subjected to molecular analysis. To evaluate pathogenicity, the analysis should include complete oocyst counts, subtyping of *C. parvum* and (if possible) *C. bovis* as well as an evaluation of any history of diarrhoeal problems present in the herd of origin. In addition, other pathogens such as rotavirus, coronavirus, *E. coli* F5+ or other pathogenic *E. coli* strains should be investigated to exclude or include these as a contributing cause of diarrhoea.

- A way of estimating the pathogenicity of *C. bovis* to *Cryptosporidium*-naïve calves would be to perform an infection trial, following *C. bovis* and *C. parvum* infected calves in parallel to compare the course of infection. Pathological examinations of intestines of some calves should also be done to compare intensity of enterocyte invasion. To investigate immunity after infection, calves previously infected with *C. bovis* could be challenged with *C. parvum* and vice versa.

- The association between *Cryptosporidium* and organic management should be investigated further to see if there is a true association between shedding and different management systems. This could be done as a cohort study of conventional and organic farms, perhaps targeting cows and calves as these are the groups where an association has been implied.

- Although the few *C. parvum* isolates identified all belonged to zoonotic subtype families, more samples need to be analysed to get a complete picture of the subtype distribution and the zoonotic potential. This

could be achieved partially by analysis of samples from the organic cohort study, and also by analysis of diarrhoeal samples. These calves are important because they might need veterinary care and thus are more likely to have contact with persons other than the owners, who are vaccinated due to constant exposure.

- Because beef cattle management is generally more extensive than dairy cattle management, the results in this thesis cannot be extrapolated to this cattle population. Thus, separate studies have to be designed to estimate prevalences and species distribution.

8 Populärvetenskaplig Sammanfattning

8.1 Bakgrund

Kryptosporidier är encelliga parasiter som tillhör gruppen protozoer. De har en direkt livscykel. Parasiterna (oocystorna) är smittsamma redan vid utsöndring i avföringen, och en infekterad individ kan utsöndra miljarder oocystor, medan infektionsdosen är mindre än 100 oocystor. Kryptosporidier är dessutom tåliga i miljön samt mot desinfektionsmedel och läkemedel, vilket ytterligare underlättar spridning. Artbestämning byggde länge på oocystornas utseende och då ansågs *Cryptosporidium parvum* (*C. parvum*) infektera över 150 däggdjursarter. Idag används molekylära metoder för artbestämning, och hittills är 21 olika arter är beskrivna. Numera anses *C. parvum* främst infektera och orsaka diarré hos unga kalvar och människor, och smitta mellan djur och människa förekommer. Hos nötkreatur ses ytterligare tre vanliga arter: *C. bovis*, *C. ryanae* och *C. andersoni*. Dessa arter är vanligare hos äldre nötkreatur, ger symptomfri infektion och lägre oocystutsöndring. Oocystorna hos *C. parvum*, *C. bovis* och *C. ryanae* är nästan identiska och infekterar tunntarmen, medan *C. andersonis* oocystor är större och infekterar löpmagen.

Hos svenska kalvar har kryptosporidier påvisats i diarréprover sedan 1980-talet, men det har inte varit känt hur vanligt förekommande kryptosporidieinfektion är, och inte heller vilka arter som förekommer.

Syftet med denna avhandling var att undersöka förekomsten av kryptosporidier och vilka arter som finns i svenska mjölkbesättningar, påverkan på kalvhälsa samt om särskilda faktorer bidrar till spridning inom besättningar. Dessutom undersöktes effekten av halofuginon, den enda registrerade substansen mot kryptosporidios hos kalv.

8.2 Sammanfattning av studier och resultat

Fyra studier genomfördes, varav två fältstudier. En studie var rent laborativ och en studie gjordes på resultat från tidigare genomförda kliniska studier.

8.2.1 Utbredning och faktorer associerade med förekomst

Utbredning samt faktorer som har samband med förekomst undersöktes i en studie med 50 mjölkbesättningar (studie I). Avföring togs från kalvar, ungdjur och kor och skötselrutiner noterades. Kryptosporidier hittades i 48 av 50 besättningar, hos 52 % av kalvarna, 29 % av ungdjuren och 6 % av korna. Det fanns inget samband med diarré. Riskfaktorer för hög förekomst inom besättningar var att placera kalvar och ungdjur nära varandra, att ha ungdjur i kontinuerligt system samt att rengöra ensamboxarna ofta. Även avvänjningsålder och provtagningsår påverkade förekomsten. Kalvar hade högre infektionsrisk om de var placerade nära ungdjuren och om ungdjuren gick i kontinuerligt system. Kalvar som stannade hos kon i minst 12 timmar hade lägre infektionsrisk. Ungdjur hade minskad risk för infektion med ökad ålder. Kor i ekologiska besättningar och i besättningar med mycket kalvar hade högre risk för infektion.

8.2.2 Identifierade *Cryptosporidium*-arter

I studie II användes två positiva prover per ålderskategori och besättning från studie I för att undersöka förekomst av arter och så kallade subtyper. Alla fyra arter som beskrivits som vanliga på nötkreatur identifierades. *Cryptosporidium bovis* var vanligast, och kunde påvisas hos 7 dagar gamla kalvar, vilket är tidigare än enligt den 10 dagar långa inkubationstiden. *Cryptosporidium parvum* förekom bara hos kalvar, och var associerad med diarré. Nio olika subtyper av *C. parvum* hittades, varav tre inte har beskrivits tidigare och två hade små sekvensskillnader mot tidigare rapporterade isolat. Dessa fem subtyper rapporterades som unika till GenBank. Alla subtyper tillhörde de zoonotiska familjerna IIa och IId, vilket innebär att de kan infektera människor. *Cryptosporidium ryanae* hittades hos kalvar och ungdjur, medan *C. andersoni* hittades hos ungdjur.

8.2.3 Kryptosporidier i besättningar med diarréproblem

Kryptosporidiers roll vid kalvdiarré undersöktes i en fall-kontrollstudie omfattande 10 problembesättningar och 10 kontrollbesättningar (studie III). Provtagning gjordes som i studie I, men inkluderade även artbestämning, förekomst av andra diarréagens samt totalprotein hos en till åtta dagar gamla kalvar. Kryptosporidier hittades i alla besättningar, och även i denna studie dominerade *C. bovis*. Förekomsten i de olika åldersgrupperna var högre än i

första studien, men skiljde inte mellan fall- och kontrollbesättningar. Förekomsten av rotavirus var lika i fall- och kontrollbesättningar, medan coronavirus och *E. coli* F5+ bara hittades i kontrollbesättningar. Inget av undersökta agens var associerat med diarré. Infektion med *C. bovis* verkade vara vanligare hos kalvar i kontrollbesättningar, medan en kraftig variation i diarrékonsistens hos sjuka kalvar och desinfektion av ensamboxar mellan kalvar ökade sannolikheten för att en kalv skulle tillhöra en fallbesättning. Desinfektion av ensamboxar var också associerat med diarréproblem vid analys på besättningsnivå.

8.2.4 Halofuginons effekt mot kalvkryptosporidios

Slutligen gjordes en meta-analys (studie IV) på kliniska studier av substansen halofuginon. Meta-analys är ett verktyg för att objektivt jämföra studier som undersökt samma faktor, och ger ett säkrare resultat än de ingående studierna var för sig. Förebyggande behandling gav lägre andel kalvar med infektion och/eller diarré under behandlingstiden. När behandlingen avslutats ökade andelen kalvar med infektion och/eller diarré i de behandlade grupperna och kunde till och med bli högre än i obehandlade grupper. Dödligheten påverkades inte. Effekterna av terapeutisk användning kunde inte undersökas. Då kryptosporidierna inte kan elimineras helt och substansen är relativt toxisk bör dock halofuginon användas med försiktighet.

8.3 Slutsatser

Kryptosporidier är vanligt förekommande i svenska mjölkbesättningar. Alla fyra arter som är vanliga hos nötkreatur i utländska studier finns i svenska mjölkbesättningar. Avsaknaden av ett samband mellan diarré och kryptosporidieinfektion i dessa studier kan bero på att *C. bovis* var dominerande art även hos kalvarna. Arten *C. parvum* var dock associerad med diarré. Desinfektion är inte verksamt mot kryptosporidier och kan sannolikt bidra till diarréproblematik om man förlitar sig på dess verkan och inte har noggranna skötselrutiner i övrigt. Förebyggande behandling med halofuginon bör endast användas under en kortare period i besättningar med stora problem och tillsammans med förändrade skötselrutiner som kan minska smittrycket.

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