Infection dynamics of *Cryptosporidium bovis* and *Cryptosporidium ryanae* in cattle

Malin Åberg

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Swedish University of Agricultural Sciences
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Malin Åberg
Faculty of Veterinary Medicine and Animal Science
Department of Clinical Sciences
Uppsala

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Abstract

In order to investigate the infection dynamics of the protozoan parasites *Cryptosporidium bovis* and *Cryptosporidium ryanae* in cattle, a Swedish dairy farm known to be free of *C. parvum* was recruited. Two sampling regimes were utilized; a cross-sectional study of pre-weaned calves over one year (study I), and a two-year prospective cohort study (study II). In study I, feces samples were collected from up to twenty calves once a month (13 occasions). In study II samples were collected from 16 heifers from birth to calving. They were sampled once a week for two months, and then monthly until calving. The samples were cleaned using a flotation method and examined with immunofluorescence microscopy to quantify the shedding. The *Cryptosporidium* positive samples were further processed with molecular species determination.

In study I, a total of 238 samples were examined and oocysts were found in 92 samples, of which 72 were successfully species determined: 87.5% were *C. bovis,* 9.7% were *C. ryanae* and 2.8% were a mix of both species. In the cohort (study II), a total of 455 samples were collected and for calves up to nine weeks old, *C. bovis* was found in 58.5% of the samples, *C. ryanae* in 9.2%, and both *C. bovis* and *C. ryanae* in 3.1%. No parvum was found in either study.

The prevalence of shedding calves was at its highest at ages four and five weeks in both studies: 54.8% and 56.7% in study I, 81.3% and 87.5% in study II. The cumulative incidence in the cohort reached 100% when the calves were five weeks old, which is earlier than what many international studies have shown for *C. bovis*.

The highest oocysts per gram feces count (OPG) were 1.1×10^6 and 3.6×10^6 in study I and study II, respectively. The youngest calf in which *C. bovis* was identified was 5 days old, and the youngest calf in which *C. ryanae* was identified was 15 days old. In four calves in study II, the detected species changed from *C. bovis* to *C. ryanae* or the other way around, and two samples were a combination of both species. Several individuals shed oocysts sporadically up to 16 months of age. Calf housing type and seasonality were not associated with differences in the shedding of oocysts (I). There was no association between the presence of diarrhea and oocyst shedding.

Keywords: Cryptosporidium, *C. bovis*, *C. ryanae*, cohort, infection dynamics

Author's address: Malin Åberg, SLU, Department of Clinical Sciences,
P.O. Box 7054, S-750 07 Uppsala, Sweden

Infektionsdynamiken för *Cryptosporidium bovis* och *Cryptosporidium ryanae* hos nötkreatur

Abstract

För att undersöka infektionsdynamiken hos parasiterna *Cryptosporidium bovis* och *Cryptosporidium ryanae* hos nötkreatur rekryterades en svensk mjölkgård som hade visat sig vara fri från *C. parvum*. Två provtagningsregimer användes; en tvärsnittsstudie av ej avvanda kalvar under ett år (studie I) och en tvåårig kohortstudie (studie II). I studie I samlades träckprov från upp till tjugo kalvar, en gång i månaden (13 tillfällen). Från de 16 kvigorna i kohortstudien samlades prover initialt en gång i veckan i två månader, och sedan varje månad under resten av tiden. Proverna renades med hjälp av en flotationsmetod och undersöktes med immunofluorescensmikroskopi för att kvantifiera utsöndringen. De kryptosporidiepositiva proverna undersöktes vidare med molekylärbiologiska metoder för att bestämma arterna.

I studie I undersöktes totalt 238 prover och oocystor hittades i 92 prover, varav 72 artbestämdes: 87,5 % var *C. bovis*, 9,7 % var *C. ryanae* och 2,8 % var en blandning av båda arterna. I kohorten (studie II) samlades sammanlagt 455 prover och för kalvar upp till nio veckor gamla så hittades *C. bovis* i 58,5 % av proverna, *C. ryanae* i 9,2 %, och både *C. bovis* och *C. ryanae* i 3,1 %. *C. parvum* hittades inte i någon av studierna.

Förekomsten av utsöndrande kalvar var som högst i åldrarna fyra och fem veckor i båda studierna: 54,8 % och 56,7 % i studie I, 81,3 % och 87,5 % i studie II. Den kumulativa förekomsten i kohorten nådde 100 % när kalvarna var fem veckor gamla, vilket är tidigare än vad många internationella studier har visat för *C. bovis*.

De högsta värdena av oocystor per gram träck (OPG) var $1{,}1 \times 10^6$ och $3{,}6 \times 10^6$, i studie I respektive studie II. Den yngsta kalven där *C. bovis* identifierades var 5 dagar gammal, och den yngsta kalven där *C. ryanae* identifierades var 15 dagar gammal. Hos fyra kalvar i studie II ändrades arten från *C. bovis* till *C. ryanae* eller tvärtom, och två prover var en mix av båda arterna. Flera individer utsöndrade oocystor sporadiskt upp till 16 månaders ålder. Varken kalvarnas boxtyper eller årstid var förknippade med utsöndring av oocystor (I). Det fanns inget samband mellan förekomsten av diarré och oocystutsöndring.

Keywords: kryptosporidier, *C. bovis*, *C. ryanae*, tvärsnittsstudie, kohort, infektionsdynamik

Författarens adress: Malin Åberg, SLU, Inst. för kliniska vetenskaper,
Box 7054, 750 07 Uppsala

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List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Malin Åberg*, Ulf Emanuelson, Karin Troell, and Camilla Björkman (2019). Infection dynamics of *Cryptosporidium bovis* and *Cryptosporidium ryanae* in a Swedish dairy herd. *Vet. Parasitology X*, vol 1(May).

II Malin Åberg*, Ulf Emanuelson, Karin Troell, and Camilla Björkman (2019). A single-cohort study of *Cryptosporidium bovis* and *Cryptosporidium ryanae* in dairy cattle from birth to calving. Manuscript.

* Corresponding author

1 Introduction

General introduction

The ever-fascinating protozoan parasites *Cryptosporidium* spp. have been found on all the world's continents, except Antarctica. They are single-celled organisms that infect a wide range of animals, including humans. Infection can either be asymptomatic or result in illness of differing severity. Symptoms often include watery diarrhea, abdominal pains, malnutrition and fever, especially in young and immunocompromised individuals. The infection is spread through direct contact or via contaminated food and water (Shirley, Moonah & Kotloff, 2012). Outbreaks occur regularly in humans and animals and can bring enormous costs and work hours (Chyzheuskaya *et al.*, 2017). Cryptosporidiosis is an important cause of diarrheal disease in humans and bovines worldwide, and both are susceptible to several *Cryptosporidium* species, *C. parvum* being the most common zoonotic pathogen (Shirley, Moonah & Kotloff, 2012).

Background

Cryptosporidium was first discovered in 1907 in the gastric glands of tame mice (*Mus musculus*). It was recognized as a coccidian and given the name *Cryptosporidium muris* (Tyzzer, 1910). Tyzzer reported various forms of the parasite and was the first to describe its different phases of development. Some years later, the smaller *C. parvum* was discovered in the intestinal mucosa of mice (Tyzzer, 1912).

The first report of cryptosporidiosis in bovines was in the 1970s; an 8-month-old calf suffered from diarrhea and debilitation (Panciera, Thomassen & Garner, 1971). Histopathologic findings in the small intestine were characterized by villous atrophy and presence of various developmental forms of *Cryptosporidium* in the microvillus border of the epithelium (Panciera,

Thomassen & Garner, 1971). Several publications on findings of the parasite in cattle, sheep, pigs, horses, and several other animal species were published during the 1970's (Fayer, 2008). In 1983, neonatal diarrhoea in experimentally infected calves was reported with *Cryptosporidium* species as the single infective agent (Tzipori *et al.*, 1983).

In 1976, the first two cases of human *Cryptosporidium* infection were reported in the USA. A 3-year-old child and a 39-year-old immunosuppressed patient were both suffering from watery diarrhea (Meisel *et al.*, 1976; Nime *et al.*, 1976). The interest for *Cryptosporidium* increased in 1982 after a report of 21 immunocompromised human patients with cryptosporidiosis suffering from enteritis (Fayer, 2008). In 1993, a large outbreak of waterborne cryptosporidiosis in humans took place in Milwaukee, USA, involving 403,000 persons, and focus was again put on *Cryptosporidium*, its biology and the prevention of similar outbreaks. It was also during this decade that new molecular techniques were developed for identifying the different *Cryptosporidium* species (Fayer, 2008).

Species and subtypes

Many *Cryptosporidium* species and genotypes have been discovered, and sometimes renamed, in the hundred years since Tyzzer's first discovery. Today, there are over 30 different species genetically determined and more than 40 genotypes (Certad *et al.*, 2017).

Some species of *Cryptosporidium* spp. can be zoonotically transmitted. Ruminants and humans are the most frequently reported hosts of *Cryptosporidium* spp., and share a vulnerability to the species *C. parvum* (Fayer *et al.*, 1998). In contrast, many species of *Cryptosporidium* have a very narrow host spectrum, and are infectious to one host only. Xiao (2002) proposes that this adaptation is due to the parasite's evolving alongside its host for millions of years. In contrast to this, *C. parvum* is thought to originally be a parasite of rodents that has been recently established in cattle and humans, and therefore has not had the time to readily adapt to a distinct host (Xiao *et al.*, 2002).

The identification of *Cryptosporidium* species was traditionally based on the size of the oocysts, the species of the host animal, and the site of infection within the animal. However, microscopy does not discriminate between species, and up until 2002 all oocysts with a size of approximately 4.5 × 5.0 µm found in humans and cattle were considered to be *C. parvum*. Because of this, studies and experiments prior to this point in time are limited in their application on infection dynamics of newly discovered and defined species.

In the mid-1990s, molecular techniques for the detection and identification of the parasite were developed, such as polymerase chain reaction (PCR) (Fayer,

2008; Xiao, 2010). The 18S rRNA gene, which codes for the cellular ribosome in eukaryotes, is remarkably consistent between all species and is often used for identifying *Cryptosporidium* species (Xiao, 2010). The sequence analysis of the highly heterogeneous 60 kDa glycoprotein (gp60) is a way to further subtype some species, such as *C. parvum* and *C. hominis* (Xiao, 2010). This subtyping has been essential in the tracking and mapping of zoonotic spread of *C. parvum* and *C. hominis* (Fayer, Santín and Trout, 2009). Subtyping have also been used on *C. cuniculus* in rabbits (Liu *et al.*, 2014), *C. meleagridis* in birds (Stensvold *et al.*, 2014), *Cryptosporidium* chipmunk genotype I in humans (Guo *et al.*, 2015) and many others. No such subtyping has yet been implemented on the bovine species *C. bovis* and *C. ryanae*.

Life cycle

Cryptosporidium spp. are monoxenous organisms, i.e. their lifecycle is completed within a single host. The infectious stage is the oocyst, which spreads to the environment through feces from infected animals (Fayer, 2008). Oocysts enter the digestive tract by ingestion of infected food or water. The thick oocyst wall endures the hostile environment of the stomach and allows for the excystation either in the gastric mucosa or intestinal epithelium, depending on species (Robertson, Campbell & Smith, 1992). The oocyst wall bursts open at a "seam" to expel four sporozoites, each with a haploid genome, that migrate over the microvillus surface of the host cell (Bouzid *et al.*, 2013). They release material from dense granules from their so called apical complex, which enables the attaching to and fusing with the host cell membrane. The sporozoites infect the crypts of the intestinal mucosa by creating an invagination of the host's cellular membrane, a so-called parasitophorous vacuole, which is an intra-cellular, but extracytoplasmic space (Bouzid *et al.*, 2013). This allows the parasite to develop within the host cells into the next stage, trophozoites, without triggering a defense mechanism against intracellular breach. Within the protected environment of this vacuole an asexual division and multiplication (merogony) occurs to develop merozoites, which are released into the gut lumen. The merozoites invade adjacent cell membranes and a majority of them develop into type I meronts, which release more infective merozoites. However, a small portion forms a type II meront, which matures into the sexual stages of the development, called microgamonts and macrogamonts. Male gametes (e.g., sperms) are called microgametes. Sixteen or more microgametes are released from the microgamont and each can fertilize a macrogamont to form a new diploid zygote. After this sexual fertilization, oocysts form and are released in a sporulated form to either auto-infect the hosts cells or to become thick-walled

oocysts excreted from the animal to begin a new cycle outside of the host (Bouzid *et al.*, 2013). Autoinfection provide an explanation for persistent chronic infection (Bouzid *et al.*, 2013). The shed oocysts are robust and can survive in the environment for long periods of time (Fayer, Morgan & Upton, 2000; Bouzid *et al.*, 2013).

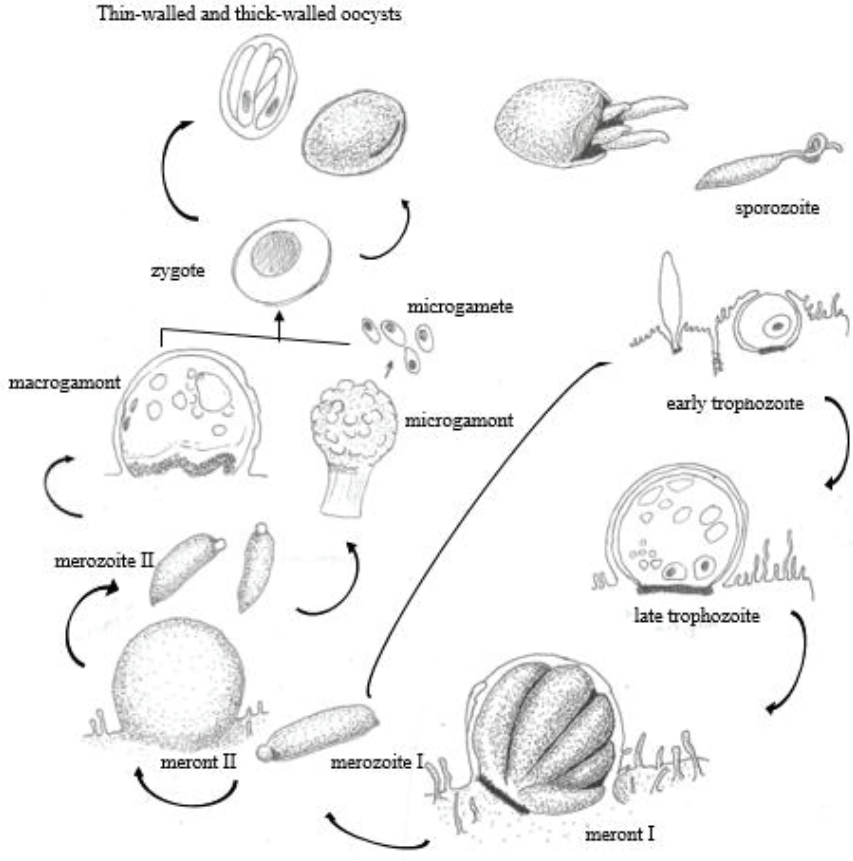

Figure 1. The life cycle of cryptosporidium, from oocysts in the environment, through the different stages in the enterocytes and to the development of new oocysts that either reinfect the host or are shed in feces.

Pathogenicity and immunity

A number of virulence factors are involved in the processes of adhesion, invasion, intracellular maintenance and cell damage for *Cryptosporidium*, however none seems to be singly responsible for the pathogenicity (Bouzid *et al.*, 2013). Further, *Cryptosporidium* spp. have the ability to rapidly change its phenotype, by so called contingency genes. They are genes which are subject to enhanced spontaneous mutation rates, enabling rapid switches in phenotype that are helpful to survival and proliferation in hosts (Barry *et al.*, 2003).

The immune response to *Cryptosporidium* involves T-cells and IFNγ (interferon-gamma), which are critical for the body's control of infection with *Cryptosporidium*, and immunocompromised individuals can sustain a chronic infection. In immunocompetent hosts, it seems that an ongoing infection may maintain the immunity to some extent, at least for the same species. Laboratory mice lacking the gene for IFNγ and those that lacked T-cells had more severe, longer-lasting infections than normal mice (Sateriale *et al.*, 2019).

The specific actions of *Cryptosporidium* spp. are, however difficult to study because of the major challenge of creating a long-term culturing system of parasites *in vitro*. Many microorganisms can be cultivated in different media, such as blood agar; however, since *Cryptosporidium* has intracellular stages it must be cultivated in cell-lines. The first successful in vitro culture of *Cryptosporidium* asexual life-cycle stages was described in 1983 (Karanis and Aldeyarbi, 2011). Obstacles remain in the maintenance of *Cryptosporidium* in in vitro cultures over extended periods of time, although the discovery of a new cell culture platform will probably facilitate the making of cures and vaccines (Karanis and Aldeyarbi, 2011; Miller *et al.*, 2018).

Transmission

The parasite's oocysts transmit through the fecal-oral route either by direct transmission from individual to individual, or by consumption of contaminated food or water. After testing the infectivity of *C. parvum* in human test subjects the median infective dose (ID_{50}) was calculated to be as few as 87 oocysts (Fayer, Morgan & Upton, 2000). As infected animals can release up to 10^{12} oocysts during the course of an infection (Jenkins *et al.*, 2004), each host contributes greatly to the spread of the parasite in the environment. The shedding of the organism's infective stage, the oocyst, can take place as early as day two of infection and lasts for about two weeks in immunocompetent hosts. As mentioned above, in immune deficient individuals the infection can even be chronic.

Cryptosporidium oocysts are extremely sturdy and can reside for a long time in the environment, although desiccation destroys oocysts after a few hours (Fayer, Morgan & Upton, 2000). Only temperatures of over 55°C result in loss of infectivity. Freezing oocysts to -20°C kills them within 24 hours and temperatures of -70°C kills them immediately. High resistance to chlorine, which is the most commonly used disinfectants of drinking water, makes it difficult to combat *Cryptosporidium* in cases of infected water wells (Korich *et al.*, 1990; Chalmers, 2014) however, UV-light can be used to kill oocysts in water (Rochelle *et al.*, 2005).

Cryptosporidium spp. in cattle

Cryptosporidium parvum and rotavirus are the two lead infectious causes of diarrhea in young calves in Sweden, while coronavirus is found sporadically and *E.coli* is an uncommon cause of calf diarrhea (Björkman *et al.*, 2003; Torsein *et al.*, 2011). The economic losses due to *Cryptosporidium* infections in neonatal calves have not been examined in detail, but relate to the diarrhea: treatment and management of sick animals, which are costly as well as time-consuming, growth retardation and to a lesser extent mortality (De Graaf *et al.*, 1999).

Cryptosporidium species commonly found in cattle are *C. bovis, C. parvum, C. ryanae* and *C. andersoni* (Ryan & Hijjawi, 2015). Not that long ago it was discovered that what had been known as *C. parvum* in cattle, were actually the three morphologically similar but genetically different species *C. parvum, C. bovis* and *C. ryanae.* Only *C. parvum* is considered to be pathogenic to cattle whereas the others are thought to cause subclinical infection (Santín *et al.*, 2004).

The overall picture reported from many countries is an age-related pattern in the species distribution. Almost all infections in calves 8 weeks and younger are caused by *C. parvum,* post weaned calves are infected by *C. bovis* and *C. ryanae,* and *C. andersoni* is found in young stock and adult animals (Santín et al., 2004; Fayer et al., 2006, 2007; Langkjær et al., 2007; Thompson et al., 2007). In Sweden however, *C. bovis* seems to be the dominating species in young calves, and Silverlås *et al*. (2010a) found high numbers of *C. bovis* oocysts but no *C. parvum* or other diarrheal agents in many samples from diarrheic calves. The question arose that perhaps *Cryptosporidium* naïve calves are more susceptible to *C. bovis* infection than previously thought, and that the parasite may in certain circumstances be pathogenic to calves (Silverlås *et al.*, 2010b).

Cryptosporidium bovis

Cryptosporidium bovis was first described as a new genotype (*Cryptosporidium* genotype Bovine B) in cattle in 2002 (Xiao *et al*., 2002). Only one induced infection trial has been published, where three calves were subjected to strains of genotype Bovine B. They excreted oocysts, but showed no clinical signs during the course of the infection (Fayer, Santín & Xiao, 2005). After the requisite determining qualities for species recognition were met, e.g. 3 unlinked loci difference from *C. parvum* and the propagation in calves, the genotype was recognized as its own species and renamed *C. bovis* (Fayer, 2010).

Oocysts measure 4.76–5.35 × 4.17–4.76 µm, with a mean size of 4.89 × 4.63 µm (Fayer, Santín & Xiao, 2005). The prepatent period was determined by experimental infection to be 10 days (Fayer, Santín & Xiao, 2005), however some studies suggest a shorter period of 7 or 9 days (Silverlås, *et al.*, 2010a; Silverlås & Blanco-Penedo, 2013). Few cases of human infection with *C. bovis* have been reported (Ng *et al.*, 2012).

Many studies conclude that post-weaned calves mainly shed *C. bovis* and *C. ryanae*, however, this pattern is not seen in most Swedish herds. In a study including pre-weaned calves in 50 dairy herds, Silverlås *et al*. (2010b) found *C. bovis* in 52% of calves <14 days, 70% of calves 15-21 days and 89% of calves 22-28 days. Similar findings have been reported from another Swedish study (Silverlås and Blanco-Penedo, 2013). This pattern has been reported also in Canada, Sri Lanka and China (Budu-Amoako *et al*., 2012; Abeywardena *et al.*, 2014; Gong *et al*., 2017).

Cryptosporidium ryanae

Cryptosporidium ryanae is one of the smallest *Cryptosporidium* species, measuring 2.94–4.41 µm × 2.94–3.68 µm, with a mean of 3.16 × 3.73 µm (Fayer, Santín &Trout, 2008). It was first described in the USA in 2004, as the *Cryptosporidium* deer-like genotype (Santín *et al.*, 2004), but has later been found around the world (Fayer, Santín & Trout, 2008; Feng *et al.*, 2012). Although it has never been found in deer, the name was given because of the 99.5% homology in the 18S rRNA gene locus with the previously reported deer genotype (Xiao *et al.*, 2002).

In 2008 *Cryptosporidium* deer-like genotype was recognized as a species and received the name *C. ryanae* (Fayer, Santín & Trout, 2008). It was found to propagate in two experimentally infected calves of the age two and a half weeks, which began shedding *C. ryanae* oocysts after 11 days and excreted them for two weeks. It was found non-infectious to BALB/c mice and lambs. None of the

individuals showed any clinical disease during the infection (Fayer, Santín & Trout, 2008).

In a longitudinal study of 30 cattle, *C. ryanae* was examined alongside *C. parvum, C. bovis* and *C. andersoni* for two years. During the longitudinal study, 60% of the calves were excreting oocysts of *Cryptosporidium* deer-like genotype at some point. The age of the calves was 2.5 to16 months, but the greatest number of infected calves was found at 18-20 weeks of age, the same age period as *C. bovis*. The calves were all previously infected with *C. parvum*. (Fayer, Santín & Trout, 2008).

Cryptosporidium ryanae seems to have a narrow host spectrum, but has been found in zebu cattle and buffalos besides domestic cattle (Feng *et al.*, 2012). The species has shown no zoonotic potential (Khan, Shaik & Grigg, 2018).

Cryptosporidium parvum

The discovery of C*ryptosporidium* oocysts in mouse intestines in 1912 was the first time the name *C. parvum* was used. It is now believed that the species Tyzzer found in mice was actually *C. tyzzeri*, since the strain was highly infectious to mice and the *C. parvum* strains recognized today are not (Ren *et al.*, 2012). In the literature, the name *C. parvum* has been used for a number of strains of *Cryptosporidium* that were later recognized as another species, for example *C. bovis* and *C. ryanae,* as described previously.

It has a mean size of 5.41 µm × 4.93 mm (Fayer, Santín & Trout, 2008). *Cryptosporidium parvum* is the most commonly found species in diarrheic calves (Fayer, 2008; Holzhausen *et al.*, 2019), but clinical disease is not always observed in infected calves (Geurden *et al.*, 2007; Silverlås et al., 2010a). The pathogen has been shown to induce severe villus atrophy of the intestines and, in response to this, elongation of the enteric crypts. Systemic infection has never been described, but infection may extend to the biliary tract (Laurent & Lacroix-Lamandé, 2017). Clinical symptoms are mostly seen in calves up to six weeks of age and can include watery diarrhea, anorexia, fever, depression, and sometimes death (Robertson *et al*., 2014). The cause of clinical disease is not yet fully understood, but is multifactorial and depend on both host factors and the *Cryptosporidium* itself (Certad *et al.*, 2017). Calves seem to generally become infected with oocysts directly after birth and symptoms can start as early as three days post infection (Fayer, 2008).

The main source of infective oocysts are other calves (Atwill *et al.*, 1998; Santín, Trout & Fayer, 2008). Whether periparturient cows contribute to the contamination of the environment and in effect, the spread of infectious oocysts to newborn calves has been debated. There was no such increase when fecal

samples from 43 periparturient dairy cows were tested for *Cryptosporidium* oocysts (Atwill, Pereira & Teaching, 2003). On the other hand, a slight but measurable increase in oocyst excretion at calving was shown by De Waele *et al.* (2012). Whether or not the periparturient shedding is responsible for the infection of neonatal calves, the period prevalence of calf hood infection can be as high as 90% (Atwill *et al.*, 1998; Holzhausen *et al.*, 2019).

Cryptosporidium parvum is also pathogenic to many animal species, including humans, and is, together with *C. hominis*, the most commonly found species in humans (Chalmers *et al.*, 2005).

Cryptosporidium andersoni

Cryptosporidium andersoni was first described in a feedlot cattle herd, where animals were reported to eat less and have a lower than expected daily weight gain. The parasite oocysts recovered were larger than the *C. parvum* oocysts linked to infection in calves and its site for replication was in the epithelial lining of the abomasum (Anderson, 1987). It was first reported as *C. muris*, which is the species of similar size that infects mice. However, the oocysts from cattle are not infective to mice and analysis of the 18s rRNA and HSP70 gene have shown that the forms occurring in cattle are genetically distinct (Morgan *et al*. 2000). In 2000, the species was named *C. andersoni* (Lindsey, 2000) and has now been reported from all over the world (Enemark *et al*., 2002; Fiuza *et al.*, 2011; Kváč *et al.*, 2011, Qi *et al.*, 2016). Its size is 6.0-8.1 × 5.0-6.5 µm, with a mean of 7.4 × 5.5 µm ((Lindsey *et al.*, 2000), noticeably larger than the other three species found in cattle, which makes it easier to identify by microscopy.

The parasite is reported mainly in young stock and adult animals (Santín *et al.*, 2004), however some countries report findings in pre-weaned calves as well (Kváč *et al.*, 2011). It is reported as common in China, even in calves where a prevalence of 8.7% was reported in calves 3 to 12 months (Wang *et al.*, 2011). The parasite has been associated with drops in milk yield of dairy cows (Esteban, 1995) and reduced weight gain in feedlot cattle (Anderson, 1990). In one case it was related to decrease in dry matter intake of feedlot cattle, however the weight gain was not significantly reduced (Ralston *et al.*, 2003). The infection is commonly known to progress without any symptoms at all, and neither are post mortem changes (Kváč & Vitovec, 2003; Ralston *et al.*, 2003).

Other species found in bovines

Occasionally, *C. hominis* is found in calves. A limited number of observations have been reported in Australasia, Asia, Africa and Europe. In France, infections

were found in calves from 3 weeks to 7 weeks of age, however no *C. hominis* infected calves exhibited clinical disease (Razakandrainibe *et al.*, 2018).

Other species that are occasionally found in cattle are *C. felis*, *C. suis*, *C. suis*-like genotype and *C. scrofarum* (Khan, Shaik & Grigg, 2018) while *C. canis* has been detected in experimental infection (Fayer *et al*., 2001).

Detection and species determination

The most commonly used diagnostic method is the detection of oocysts or antigens in fecal samples. The samples are often concentrated to enrich oocysts using flotation, such as sugar or salt solutions (Chalmers *et al.*, 2005; Leitch & He, 2011). Different staining methods e.g. Ziehl-Neelsen dye and auramine phenol staining, can be used to visualize the oocysts and facilitate the microscopic detection. Staining with monoclonal antibodies coupled with a fluorescent dye produces brightly colored oocyst walls, and are used in immunofluorescence microscopy (Silverlås *et al.*, 2010a). In order to species determine the oocysts, PCR was used to sequence a part of the S18 rRNA gene to be compared with other species already listed in the GenBank database using BLAST.

Prevention and treatment

Preventive hygiene measures and good management are currently the most important tools to control cryptosporidiosis. The infection load must be reduced as much as possible and it is therefore vital to thoroughly clean pens between calves. The oocysts are sturdy and resists most disinfectants commonly used in Sweden. Disinfection of calf pens with hydrated lime has proven to delay the onset of diarrhea and improve calf body condition score of calves, however it seems to not affect diarrhea incidence or duration (Björkman *et al.*, 2018). Further, recent trials in the UK show that hydrogen peroxide 3% is an effective disinfectant for *Cryptosporidium* oocysts (Wells, Shaw & Thomson, 2019).

No vaccines are available, for either humans or bovines (Thomson *et al.*, 2017). The treatment of calves with clinical disease of cryptosporidiosis is to first and foremost treat the symptoms, such as diarrhea and fever. Depending on the status of the animals, the treatment could be with fluids and electrolytes and, if necessary, antipyretics (Fayer, 2008; Meganck, Hoflack & Opsomer, 2014).

In Sweden, the only registered product for the prevention and treatment of cryptosporidiosis in animals is halofuginone (Silverlås, Björkman & Egenvall, 2009). Halofuginone lactate is a synthetic quinazolinone with cryptosporidiostatic, but not cryptosporidicidal, activity on the sporozoite and

merozoite stages. It does not completely inhibit the infection, but can lower the oocyst output and delay the onset of shedding (Lefay *et al.*, 2001; Jarvie *et al.*, 2005; De Waele *et al.*, 2010). One report found it to reduce clinical signs (De Waele *et al.*, 2010).

Calves are born essentially without antibodies, and depend on the uptake of maternal immunoglobulins from colostrum. Total serum protein content in blood collected 2-7 days after birth can be used as an estimate of the colostrum uptake, and concentrations below 55 g/l are considered to indicate an inadequate colostrum management, which can lead to susceptibility to disease (Tyler *et al.*, 1999).

2 Aims of the thesis

The overall aim of this thesis was to illustrate the infection dynamics of *C. bovis* and *C. ryanae* in a dairy herd without the influence of *C. parvum*.
More specific aims were to investigate:

- The species distribution pattern and infection intensity over one year in pre-weaned calves.
- How the distribution pattern and infection intensity varies in cattle from birth to calving.

3 Material and methods

Detailed descriptions of the material and methods used are given separately in the two papers I and II.

The farm and animals

The farm was selected because of previous findings of *C. bovis,* but no *C. parvum*. It was an organic farm with 140 milking cows held in a loose housing system, with two automatic milking stations (AMS). In Sweden, there were 2427 dairy herds in the year 2018 and automatic milking systems were used in a third (738) of those (Cattle statistics 2018, Växa, https://www.vxa.se/fakta/styrning-och-rutiner/mer-om-mjolk/). The cows were of the Swedish Red breed, which is one of the two most common breeds in Sweden, the other being the Swedish Holstein.

Newborn calves were kept with their dams for the first day to get access to the dam's colostrum, and then moved to single pens, where they were given additional pooled colostrum from older cows. They were fed whole milk until weaning, which started at 12 weeks. The weaning period started later than in most international studies, where calves are usually weaned at six to eight weeks. When coming across the terms pre- and post-weaned calves, one must take this difference into consideration.

The herd was declared free of bovine viral diarrhea virus (BVDV) and *Salmonella* spp., both a cause of diarrhea. A general regime of hygiene for visitors included means for handwashing and disinfection of boots with Vircon®. The claw trimmer and herd veterinarian, often accompanied by veterinary students, were regular visitors.

Study design

In order to establish what *Cryptosporidium* species were present in the herd, a pre-study was made in January 2012 to collect 50 fecal samples from cattle of all age categories (paper I). *Cryptosporidium parvum* was still absent from the herd, and the sampling for the cohort study (paper II) started in February 2012. In total, 16 heifer calves born in succession over seven weeks were enrolled. In their first two months, the samples were taken every week, then once a month for the rest of the two-year study, that finished in July 2014. The cross-sectional study (I), started in June 2012, after the first two months of intense sampling in the cohort were finished, and the two studies ran in parallel until late April 2013. Up to twenty individuals, of the most affected age category (<2 months), were randomly selected each month for the cross-sectional study (paper I), and that was approximately the number of calves available of this age.

Sampling

Rectal fecal samples were collected and immediately put into plastic jars that were marked with the individual's ear tag number. The consistency and color of feces were noted on the jar. The scoring of the feces was made according to the scale: normal, pasty, loose or diarrheic.

To assess the colostrum management in the herd, a blood sample was taken from each calf in the cohort study (paper II) during its first week of life for analysis of blood serum protein.

Clinical examination

Calves were given a brief clinical examination during feces sample collection where body condition score, noted as thin, normal or fat, and any nasal discharge and coughing were noted.

Laboratory methods

Each fecal sample was cleaned using a saline-glucose flotation method, as described in Maddox-Hyttel *et al*. (2006).

Two ml of each cleaned sample were stored in buffer solution at 4 °C before microscopy and DNA extraction. Storing oocysts in this manner ensures their viability for at least 24 weeks (Fayer, Trout & Jenkins, 1998).

Microscopy

Briefly, ten microliters of each sample suspension were transferred to a 12 mm Ø well of a 3-well slide and stained with a fluorescent dye. The entire well area was examined by epifluorescence microscopy at 200x and 400x magnification. The detection limit was one oocyst per sample, which translates into 200 OPG. When the recapture of oocysts from the flotation was determined in three spiked samples, it was estimated to be about 50% (results not shown). This was somewhat lower than the 50-90% found in a previous testing (unpublished, Sofia Lundström, 2010). As to not overestimate the OPG, the estimated losses were not considered when analyzing data.

DNA extraction, gene amplification, and sequencing

All samples positive for *Cryptosporidium* spp. by microscopy were further analyzed by extracting DNA by 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 as described by Xiao et al. (1999).

To verify the absence of *C. parvum,* all positive samples were also analyzed according to Chalmers *et al*. (2005), at the 60 kDa glycoprotein (gp60) gene, a highly variable gene which is useful in the identification and subtyping of *C. parvum.*

All sequences obtained were compared with published sequences in the GenBank database using BLAST (Basic Local Alignment Search Tool, NCBI [http://www.ncbi.nlm.nih.gov/BLAST]).

Statistical analysis

All data were recorded in Excel (Microsoft). All statistical analyses were done with STATA® (StataCorp LP, College Station, TX). Fisher's exact test, Pearson's chi-squared test, and t-tests were used to analyze differences in the prevalence of shedding and in OPG between groups of animals. Results were considered significant when $p \leq 0.05$.

4 Results

Pre-study

Cryptosporidium spp. oocysts were found in 18 of the 50 (36%) samples collected in the pre-study (described in paper I). Only *C. bovis* and *C. ryanae* were detected, but no *C. parvum*. It was then decided to continue with the two long-term studies.

Cross-sectional and cohort studies

In the cross-sectional study (paper I), a total of 238 feces samples were collected at 13 occasions from calves ≤65 days old. Ninety-two samples (38.7%) were positive for oocysts and species could be determined in 72 (30.3%) of these. *Cryptosporidium bovis, C. ryanae* and a mix of the two species were found in 63 (87.5%), seven (9.7%), and two (2.8%) samples, respectively.

In the cohort study (paper II), 455 feces samples were collected from 15-16 heifers from birth to calving, resulting in 99 (21.8%) positive samples. Of these, 55 were successfully species determined; 46 (83.6%) were *C. bovis*, seven (12.7%) were *C. ryanae,* and two (3.6%) were a mix of the two species. Two samples had a single *C. andersoni* oocysts identified by microscopy due to their larger size; however this was not confirmed by sequencing.

In II, 143 samples were from calves ≤65 days. Sixty five were positive for oocysts, and of those 46 (70.8%) were species determined, by PCR 38 (82.6%) were *C. bovis,* six (13.0%) were *C. ryanae,* and two (4.3%) were a mix of both species. One single *C. andersoni* oocyst was identified by microscopy in one sample due to its larger size; however, this could not be confirmed by sequencing.

Cryptosporidium parvum was not found in either study I or II.

The youngest calf in which oocysts were detected, was two days old and sampled in study I. The sample contained 200 OPG, and species could not be determined. The earliest confirmed shedding of *C. bovis* was on day 5 (study I), and of *C. ryanae* on day 15 (in both studies). There was a difference in shedding between calves of different age, the highest percentage of shedding animals seen in weeks 4 and 5 in both studies (54.8% and 56.7% in paper I, 81.3% and 87.5% in paper II). In the cohort study (II), the cumulative incidence of *C. bovis* reached 100% when the calves were five weeks old.

The OPG of *C. bovis,* in calves ≤65 days old ranged between 200 and 1.1×10^6 (geometric mean 1.56×10^4) and between 400 and 3.6×10^6 OPG (geometric mean 1.9×10^4) in study I and study II, respectively. For *C. ryanae,* the OPG ranged between 200 and 1.0×10^5 OPG (geometric mean 7.5×10^3) and between 1.2×10^3 and 4.2×10^5 OPG (geometric mean 7.8×10^3) in study (I) and study (II), respectively. In four calves (paper II), the *Cryptosporidium* species detected changed from *C. bovis* to *C. ryanae* or the other way around over the weeks.

In the cross-sectional study (I), feces samples were classified as firm (37%), pasty (36%), loose (22%), and diarrheic (5%). No correlation between shedding and diarrhea was seen, in either (I) or (II). All but two calves (II), which suffered from pneumonia, were in normal body condition throughout the study. The two calves were treated with antimicrobials and NSAID and recovered, but were never inseminated. One calf in (II) died unexpectedly of what was believed to be pneumonia at two months of age, leaving 15 calves for the rest of the study.

In study I, no difference was seen in prevalence between the two types of group pens, i.e. indoors vs. outdoors. Further, there was no difference in prevalence of shedding calves between seasons or between female and male calves.

In study II, oocysts were found in 29 of 165 samples (17.6%) collected from individuals between 10 weeks and 12 months. Nine of the 29 positive samples (31.0%) were successfully analyzed molecularly. The species distribution was *C. bovis* in eight (88.9%) and *C. ryanae* in one sample (11.1%). *C. andersoni* was identified, by microscopy, only in two samples. In individuals older than one year, five samples of 147 (3.0%) were positive, and of these, one was species determined as *C. bovis.* No individual shed oocysts after the age of 16 months and thus no shedding was detected post-partum.

The mean total serum protein values in the thirteen calves in study II, varied between 40 and 70 g/L. Five of the 13 calves had values below 55 g/L, indicating suboptimal colostrum feeding and that the colostrum feeding routines could be in need of evaluation.

5 Discussion

The farm in this study was selected not only because of previous detection of *C. bovis*, but also because of the apparent absence of *C. parvum,* to enable the unique long term investigations of *C. bovis* and *C. ryanae* without the influence of that species. Overall, the results correlate with previous reports from Sweden, where *C. bovis* is the most common species in pre weaned calves in herds without ongoing diarrhea problems (Silverlås et al., 2009; 2010).

Sampling

Two different study designs were used. The cross-sectional study (I) focused on what the infection pattern for pre-weaned animals looks like. In the cohort study, the long term infection patterns in individual animals were studied e.g. the coming and going of species, longevity of the infections, if they were associated with clinical disease, and if the infection dynamics differed from previous reports on *C. bovis* and *C. ryanae*. Further, to answer questions such as if and how the infections were influenced by housing systems, season and gender.

A difference between the studies was that in study I, individuals of the most predisposed age i.e. pre weaned calves were targeted and in study II, the same individuals were repeatedly sampled from birth until calving. Therefore, the results of a larger proportion of positive samples in study I were not surprising.

Because the feces samples from the calves were collected once a week, the beginning of oocyst shedding could not be determined to the exact day.

Species

No *C. parvum* was introduced over the two years of the study, which shows that a herd can stay free from introduced pathogens for an extended time.

In both studies, the excretion of *C. bovis* and *C. ryanae* oocysts started sometime in the first three weeks of life. This is in contrast to many international reports, where *C. bovis* is rarely found in calves younger than three to four weeks and *C. parvum* is the predominant species in that age-category (Geurden *et al*., 2007; Santín *et al*., 2008; Brook *et al*., 2009; Karanis *et al*., 2010; Follet *et al*., 2011). The shedding pattern is, however, similar to previous reports from Sweden (Silverlås *et al*., 2010; Silverlås & Blanco-Penedo, 2013; Björkman *et al*., 2015). Also in some reports from China, Canada and India, *C. bovis* is the most common species found in pre-weaned calves (Feng *et al*., 2007; Wang *et al*., 2011; Budu-Amoako *et al*., 2012; Fan *et al*., 2017).

In the cohort study (II), the cumulative incidence of *C. bovis* reached 100% when the calves were five weeks old. This is considerably earlier than what was found in the first long-term cohort study of *Cryptosporidium* spp., where the incidence of *C. bovis* reached at most 80% on week 16 (Santín *et al*., 2008). However, in that study all of the 30 calves were infected with *C. parvum* before any other *Cryptosporidium* species was detected, which may explain the delayed onset of shedding and possibly also why not all calves eventually shed *C. bovis*.

In four samples, the sequencing chromatograms of the 18S rRNA gene showed overriding peaks at specific sites, indicating that two species were present. The chromatograms were compared to those of *C. bovis* and *C. ryanae* and it was revealed that it was those two species in a mixed infection.

In four heifers in study II, the detected *Cryptosporidium* species shifted between *C. bovis* and *C. ryanae* at several occasions. Sometimes, *C. bovis* was detected first and sometimes *C. ryanae*. For example, heifer number 6 excreted *C. bovis* in week 2, and only *C. ryanae* in week 3. Later on, in week 5, she excreted *C. bovis* again and week 7 and 9 it was *C. ryanae* again. These shifts in species could be either a new infection or indicate a mixed infection that was not detected. Something similar was seen by Follet *et al*., when two different *Cryptosporidium* species were detected on consecutive weeks in 34.0% of the calves. Furthermore, two individuals were infected with different species (either *C. parvum*, *C. bovis* or *C. ryanae)* at the three different sampling occasions (Follet *et al*., 2011), which suggests that the immunity against one species does not extend to the other two.

When a new species first appear, it is probable that it is in fact a new infection. However, when the species switch from one week to the next it could either be explained by the coming and going of the species or by a mixed infection where the PCR only amplified one of the species. Mixed infections are hard to identify by PCR, because the dominating species or the species with the highest affinity for the primer will be amplified to much larger extent than the

other, resulting in identification of only the dominant species (Xiao & Ryan, 2008).

Cryptosporidium andersoni was identified by microscopy in one 9-day-old calf and two young heifers (II). Even though they were not confirmed by the PCR, their identification by microscopy is made easier by the much larger size of its oocysts, and the findings were thought to be accurate. In most parts of the world it has been reported that *C. andersoni* is found predominantly in young stock and adult animals (Anderson, 1987). However, in the Czech Republic, *C. andersoni* was found in 21 of 750 samples from calves < 2months (Kváč *et al.*, 2011b). Further, *C. andersoni* was the dominant species in weaned dairy calves and heifers in a Chinese study (Wang *et al.*, 2011). The detection of this species in young animals was perhaps unusual, but even more so the lack of findings in older animals. It can be difficult to find low excretion of oocysts in adult shedders, with the methods that were used in these studies. Wells *et al*. (2016), found that a combination of acid flocculation and salt flotation enhances the detection of oocysts in adult cow feces, however this was after the sampling was finished in our studies. Perhaps even more of the older animals would be found shedding.

In our studies, the earliest finding of oocysts was in a 2-day-old calf. Since the prepatent period of *Cryptosporidium* spp. is at least a few days it seems unlikely that it was anything other than a passing oocyst and not an active infection. The earliest shedder with confirmed *C. bovis* infection was five days old, which is much earlier than what was found in previous investigations (Santín *et al.,* 2004; 2008). The earliest shedding of *C. ryanae* was at day 15 in both study I and II, which agrees with other reports where it was excreted at 11 days (Fayer, Santín & Trout, 2008) and 17 days (Rieux *et al*., 2013).

Clinical signs

Our results corroborate previous findings that neither *C. bovis* nor *C. ryanae* cause clinical disease in cattle (Santín, Trout & Fayer, 2008; Khan, Shaik & Grigg, 2018). This negates the proposition made by Silverlås *et al*. (2010b) that *C. bovis* may cause clinical signs in *Cryptosporidium* naïve calves in Sweden. Diarrhea in calves is often a multifactorial disease, depending on infectious pathogens and environmental and physical traits. Even though *C. bovis* is found in a diarrheic calf, it does not necessarily mean that it is the cause of the clinical symptoms, especially as *C. bovis* seems to be so common in Swedish herds.

The few investigations of naturally infected calves have not found any association between diarrhea and shedding of *C. bovis* or *C. ryanae.* However, those calves had usually been through an infection with *C. parvum*, which might

affect the later infection with *C. bovis* and *C. ryanae* (Santín, Trout & Fayer, 2008; Rieux *et al.*, 2014). In this study, where no *C. parvum* was present in the herd, the results show that when *C. bovis* and *C. ryanae* are the only *Cryptosporidium* species involved in infection of young calves with no previous exposure to another *Cryptosporidium* species, there is no development of clinical disease. Few experimental studies are reported; in the first compilations of *C. bovis* and of *C. ryanae* by Fayer *et al*. (2005; 2008), experimentally challenged calves showed no signs of diarrhea.

It has been shown that the innate immunological response in the intestines involving dendritic cells and macrophages is important for controlling early acute infection of *C. parvum*, and for activating T-cells, which are necessary for clearing of infection (Laurent & Lacroix-Lamandé, 2017). It seems that an ongoing infection may maintain the immunity to some extent, at least for the same species, protecting from severe clinical symptoms (Sateriale *et al.*, 2019).

Housing

There was no difference in shedding prevalence between the two types of group pens for calves, i.e. indoors and outdoors, in study I. This is in accordance with results from 15 U.S. farms, where no association between housing type and infection with *Cryptosporidium* spp. was found (Santín *et al.*, 2004). However, results vary between studies, and housing calves in single pens in a cow barn has been found to be associated with a higher prevalence of *Cryptosporidium* infection than housing in individual outdoor calf huts (Quigley *et al*., 1994). The seemingly different results from many studies confirm that it is hard to assess the impact of for example housing on the multifactorial reasons for disease.

6 Conclusions

These two studies are, to my knowledge, the first to examine pre-weaned calves and a cohort of cattle, infected only with *C. bovis* and *C. ryanae*. Oocysts were found at the earliest on day five for *C. bovis* and day 15 for *C. ryanae*, and excreting calves peaked in weeks four and five, which is earlier than in most other studies. Housing type and seasonality were not associated with differences in the shedding of oocysts. Oocyst excretion was found sporadically during the first 16 months in 13 of the 16 calves. Furthermore, there was no association between the presence of diarrhea and oocyst shedding.

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Popular science summary

The parasites *Cryptosporidium* spp. are found all over the world. They are single-celled organisms that can infect a wide range of animals, including humans. Infection can either be asymptomatic or result in illness of differing severity. Symptoms include watery diarrhea, abdominal pains, malnutrition and fever. The parasites spread through direct contact with infected animals or by intake of contaminated food and/or water.

The whole lifecycle of *Cryptosporidium* spp. takes place within one host animal. The *Cryptosporidium* organisms invade the mucosa of the digestive tract, where they proliferate and are shed with feces into the environment ready to infect new hosts. They are robust in the environment and can withstand most commonly used disinfectants.

In bovines four species are commonly found, namely *C. bovis, C. parvum, C. ryanae* and *C. andersoni. C. parvum* is the only species known to cause diarrhea in calves. In most studies from around the world, *C. parvum* is the predominant species infecting pre-weaned calves, whereas *C. bovis* and *C. ryanae* are mostly found in post-weaned calves and heifers.

In Sweden, however, *C. bovis* is the most common species in young calves and we find high numbers of *C. bovis*, but no *C. parvum* or other diarrheal agents in many samples from diarrheic calves. These results suggest a gap of knowledge regarding the behavior and importance of *C. bovis.* Only two long-term studies have been performed in cattle and both were conducted in herds where *C. parvum* was also present.

The aims of the thesis were to clarify how the *C. bovis* and *C. ryanae* distribution pattern and infection intensity varies over time on a Swedish dairy farm. Two studies were conducted in a Swedish dairy herd with approximately 140 cows, which had previously been found to be infected with *C. bovis,* but not *C. parvum*. Two different sampling strategies were applied. In study I, fecal samples were taken once a month from 20 pre-weaned calves in the herd during one year. In study II, a group of 16 animals was followed from birth until calving.

Fecal samples were collected, the general health of each calf was assessed, and the state of the bedding was recorded. The samples were cleaned, concentrated and evaluated by microscopy to detect *Cryptosporidium*. DNA was extracted from all samples containing oocysts to determine the *Cryptosporidium* species.

In study I, a total of 238 feces samples were collected from dairy calves. *Cryptosporidium* was detected in 92 samples of which: 87.5% were *C. bovis,* 9.7% *C. ryanae* and 2.8% a mix of both species. The youngest calf in which *C. bovis* was identified was 5 days old, and the youngest calf in which *C. ryanae* was identified was 15 days old.

In study II, which followed 16 heifers over two years, fecal samples were collected weekly until calves were nine weeks old, then monthly until calving or culling. In calves up to nine weeks, *C. bovis* was found in 58.5% of the samples, *C. ryanae* in 9.2%, and both *C. bovis* and *C. ryanae* in 3.1%. The occurrence of shedding calves was at most 87.5% in week five, which is earlier than many international studies have shown for *C. bovis*. In four calves, the species detected changed from *C. bovis* to *C. ryanae* or the other way around, and two samples were a combination of both species. Several individuals shed parasites sporadically up to 16 months of age.

These results show a different infection pattern for *C. bovis* and *C. ryanae* than what has been described in other countries, where infection is rarely seen in the first two months. Individuals were excreting small amounts of the parasites sporadically for over a year after the initial infection. No seasonal differences were seen regarding the number of shedding calves, and housing did not influence shedding. There was no correlation between infection and diarrhea.

Populärvetenskaplig sammanfattning

Kryptosporidier är encelliga parasiter som återfinns över hela världen och kan infektera både djur och människor. Infektionen kan vara symtomlös eller orsaka diarré, buksmärta, feber och undernäring. Parasiterna sprids genom direktkontakt mellan infekterade individer eller genom intag av förorenad mat eller vatten. Parasiterna invaderar slemhinnan i matsmältningskanalen, där de sprider sig och utsöndras med avföringen för att sedan infektera nya värdar. De är robusta och tål de vanligaste desinfektionsmedlen.

Fyra arter är vanliga hos nötkreatur, nämligen *Cryptosporidium bovis*, *C. parvum*, *C. ryanae* och *C. andersoni*. Av dessa är *C. parvum* den enda som anses orsaka diarré, och det är en av de vanligaste smittsamma orsakerna till kalvdiarré. I de flesta studier från olika delar av världen är *C. parvum* den dominerande arten hos ej avvanda kalvar, medan *C. bovis* och *C. ryanae* oftast hittas bland lite äldre djur. I Sverige är emellertid *C. bovis* den vanligaste arten även hos unga kalvar. Dessutom har man här hittat *C. bovis*, men inga *C. parvum* hos ett antal kalvar med diarré. Kalvarna hade stora mängder parasiter i avföringen vilket indikerar att även *C, bovis* kan vara patogen. Dessa resultat visar på en brist på kunskap om *C. bovis* beteende och betydelse.

Syftet med avhandlingen var att klarlägga hur förekomst och mängd av *C. bovis* och *C. ryanae* varierar över tid i en svensk mjölkbesättning och hur patogena arterna är för nyfödda kalvar. Två studier genomfördes i en besättning med cirka 140 kor, som tidigare visat sig vara smittade med *C. bovis*, men inte *C. parvum*. Två olika provtagningsstrategier tillämpades. I studie I togs fekala prover en gång i månaden från 20 ej avvanda kalvar i besättningen under ett år. I studie II följdes en grupp av 16 djur från födsel till kalvning. Fekala prover samlades in, allmäntillståndet hos varje kalv bedömdes och underlaget i boxen registrerades. Proverna renades, koncentrerades och utvärderades genom mikroskopi för att detektera kryptosporidierna. DNA extraherades från alla prover innehållande parasiter för att artbestämmas dessa.

I studie I samlades totalt 238 avföringsprover från mjölkraskalvar. Kryptosporidier hittades i 92 prover varav: 87,5 % var *C. bovis*, 9,7 % var *C. ryanae* och 2,8 % var en blandning av de båda arterna. Den yngsta kalven där *C. bovis* identifierades var 5 dagar gammal, och den yngsta kalven där *C. ryanae* identifierades var 15 dagar gammal.

I studie II, som följde 16 kvigor under två år, samlades fekala prover in varje vecka tills kalvarna var nio veckor gamla, sedan månadsvis tills kalvning eller slakt. Hos kalvar upp till nio veckor hittades *C. bovis* i 58,5 % av proverna, *C. ryanae* i 9,2 % och både *C. bovis* och *C. ryanae* i 3,1 %. Förekomsten av parasitutsöndrande kalvar var som högst 87,5 % under vecka fem, vilket är tidigare än många internationella studier har visat för *C. bovis*. Hos fyra kalvar skiftade den detekterade arten från *C. bovis* till *C. ryanae* eller tvärtom, och två prover var en kombination av båda arterna. Flera individer utsöndrade parasiter sporadiskt upp till 16 månaders ålder.

Resultaten visar ett annat infektionsmönster för *C. bovis* och *C. ryanae* än vad som har beskrivits i andra länder, där infektion med dessa arter sällan ses under de första två månaderna. Individer utsöndrade små mängder parasiter sporadiskt i över ett år efter den första infektionen. Inga säsongsskillnader sågs med avseende på antalet parasitutsöndrande kalvar, och inhysning påverkade inte utsöndringen. Det hittades inget samband mellan infektion och diarré.

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