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# Cryptosporidiosis in Calves

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# Lay Summary

Cryptosporidiosis is a disease caused by the parasite *Cryptosporidium*. This parasite is zoonotic, meaning it can pass between animals and humans. Cryptosporidiosis in neonatal calves is commonly caused by *C. parvum* and the symptoms include watery diarrhoea, lethargy, reduced appetite and dehydration. This dehydration can be so severe that death can occur. Very little has been published about the transmission of this parasite to young calves and it is important to know this in order to reduce this transmission and therefore the impact that cryptosporidiosis has on farms. The potential routes examined in this thesis include transmission from the mother of the calf or other adult cattle on the farm, transmission from wild rabbits and transmission from pheasants. All of this has been done using very sensitive and the most up-to-date molecular techniques to diagnose the species and genotype of *Cryptosporidium* which is present. This thesis shows that adult dairy cattle are unlikely to play a major role in the transmission of *C. parvum* to their calves. Most of the adult cattle on the dairy farm were predominantly shedding *C. parvum* however calves on the same farm presented with different genotype. On the beef farm, however, many of the adult cattle did share the same genotype of *C. parvum* as their calves, and so pose more of a risk for *C. parvum* transmission to their calves.

Rabbits collected from 18 farms from across Scotland revealed *C. parvum* to be the most prevalent species; an unusual discovery it was previously believed that the species *C. cuniculus* was the most prevalent. The parasite load in the faeces of rabbits in this work appeared to be small and therefore rabbits are unlikely to be a major route of *C. parvum* transmission. The pheasants also had *C. parvum* as the most prevalent species, although very few shared the same type that was present in the calves at the pheasant samples location. On the other

hand, very few oocysts (the thick walled, infectious stage of the parasite which is found in the environment) are required to cause cryptosporidiosis in a calf, so even if co-located wildlife do not appear to be shedding high numbers of oocysts, there could still be a small risk of transmission of *C. parvum* to calves.

It is currently unknown whether infection of a calf with *Cryptosporidium* in the first few weeks of life will have a detrimental effect on the growth of the calf over time. This would affect both the production capability of the calf and the profitability of the farm. This thesis shows that infection in the first few weeks of a calf's life significantly reduces the weight gained over six months.

As this parasite is both costly to the farming community and a risk to animal and public health, it is essential that the right disinfectants are used to combat it. *Cryptosporidium* is resistant to many commonly used disinfectants that are used on farm and this thesis shows that Hydrogen peroxide based disinfectants are the most effective at inactivting the parasite oocysts and KENO$^{TM}$COX was the best at maintaining efficacy seven days post preparation. These disinfectants however must be made up fresh and the area cleaned before use for them to be at their most effective.

# Abstract

Cryptosporidiosis is a widespread zoonotic parasitic disease affecting livestock all over the world. Despite its prevalence, there is very little evidence about transmission routes to young calves, and how it could affect them long-term. Many commonly used disinfectants on farm are unable to inactivate *Cryptosporidium* oocysts, and some commercially available disinfectants, which claim to work, do not appear to have sufficient evidence available to the research and farming community. This work shows multiple commercial disinfectants which have been directly compared against each other for their efficacy against *Cryptosporidium* oocysts.

The idea that transmission could occur from adult cattle via direct contamination of calf pens with faecal material has been disputed in scientific literature. Older research suggests that adult cattle are not infected with the same species that the calves have, however more recent research with new oocyst concentration techniques has found this not to be the case. It is essential therefore that the genotypes of *Cryptosporidium* are determined to see if adult cattle pose a risk to their calves. Genotyping using microsatellite analysis gives a more in-depth look at the type of *C. parvum* present. The aim was, therefore, to determine the risk that adult cattle pose to their calves with regard to *C. parvum* oocyst transmission on both a dairy and a beef farm in Scotland. Using these methods, it was discovered that adult dairy cattle are unlikely to play a major role in the transmission of *C. parvum* to their calves. Most of the adult cattle on the dairy farm were predominantly shedding *C. parvum* however calves on the same farm presented with different multilocus genotype. On the beef farm, however, many of the adult cattle did share the same multilocus genotype as their calves, and so pose more of a risk for oocyst transmission to their calves.

The species and genotypes of *Cryptosporidium* present in Scottish wildlife has very few published studies, therefore the aim was to determine the prevalence of *C. parvum* in samples from rabbits and pheasants in Scotland. Rabbit faecal samples collected from 18 farms from across Scotland revealed *C. parvum* to be the most prevalent species; an unusual discovery as it was previosuly believed that *C. cuniculus* was the most prevalent species in wild rabbits. Despite this the DNA was very difficult to genotype which may indicate that the oocyst load in the faeces of rabbits was small, or that the PCR may have been affected by inhibition. If there is little DNA present, rabbits are unlikely to pose a major threat to calves with regard to *C. parvum* oocyst transmission. The pheasants also presented with *C. parvum* as the most prevalent species, although very few shared the same genotype that was present in the calves at the pheasant samples location. Very few oocysts are required to cause cryptosporidiosis in a calf, so even if co-located wildlife do not appear to be shedding high numbers of oocysts, there is still a small risk of transmission present.

Young calves affected with cryptosporidiosis tend to make a full recovery under the right management, and the clinical signs clear up within a couple of weeks. It is not known whether or not there is a long-term effect on the calves ability to gain weight following infection with the parasite. Therefore the aim was to compare calves with different levels of clinical cryptosporidiosis to calves with no signs of clinical disease and weigh these animals periodically until they went to market at 6 months of age. It was found that calves with severe disease gained significantly less weight than those with no clinical disease and even animals with mild cryptosporidiosis suffered reduced weight gain over 6 months. This result demonstrates the economic cost that the parasite could have to the farming community on a long-term basis.

Commonly used disinfectants are typically ineffective against *Cryptosporidium* oocysts, and those that are on the market have very little evidence to support their efficacy. Therefore, seven commercial disinfectants were tested for their efficacy to inactivate *Cryptosporidium* oocysts based on excystation rate and sporozoite to shell ratio. It was identified that hydrogen peroxide and hydrogen peroxide-based disinfectants are the most successful at inactivating oocysts, but only when the disinfectant is freshly prepared. Testing the efficacy of disinfectants once the disinfectant had been made up for 7 days showed that the best performing disinfectant with regard to having the least degradation over seven days was KENO$^{TM}$COX. As many farmers are unlikely to make disinfectant up fresh every time it is used, it is useful to know that despite the high efficacy of some products, time since the product was prepared significantly reduces this. It was also found that pens contaminated with faecal material are likely to reduce the efficacy of hydrogen peroxide-based disinfectants and so it is important to clean pens before disinfection.

Therefore, this PhD has addressed the knowledge gaps in the literature regarding the role of adult cattle, rabbits and pheasants in the transmission of *C. parvum* to calves. Neither one poses a major risk due to the low oocyst output and mixed *C. parvum* genotypes present. It is more likely therefore that calves maintain infection through widespread environmental contamination caused by other infected calves. This work has shown how infection with *C. parvum* in the first few weeks of life has a significant effect on the weight gain achieved over a 6-month period and so cryptosporidiosis has a significant effect on livestock production and on the profitability of the farm business. The efficacy of commercial disinfectants has provided the advice that disinfectants should be made up fresh and used on an area that has already been cleaned of faecal material in order to inactivate as many of the oocysts as possible.

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# Author's Declaration

I declare that the work that is presented in this thesis is my own work unless otherwise stated. This work has not been previously submitted for any other degree or professional qualification.

Hannah Jade Shaw

# Acknowledgements

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Lastly, I would like to thank my brother Kieran, who was always so interested in what I did and wanted to hear all about it. He inspires me to keep going, no matter what life throws at you, because there is always a light at the end of the tunnel and all we can really do is live a good life.

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# Chapter 1: Introduction

## 1.1 Discovery of *Cryptosporidium*

*Cryptosporidium* is an obligate intracellular protozoan parasite that causes the disease cryptosporidiosis. The parasite, originally called *Cryptosporidium muris (C. muris)* was first described in 1907 by an American parasitologist; Ernest Edward Tyzzer (Tyzzer, 1907). It was not properly assigned as a new genus until 1910, where the parasite was found in the stomach of Japanese waltzing mice, white mice and coloured mice (Tyzzer, 1910). Tyzzer described the parasite as similar to a coccidian but with key differences that allowed him to assign it as a new genus. A coccidian is a subclass of parasite that is a single celled obligate intracellular parasite which forms spores. The key differences are that *Cryptosporidium* does not penetrate host cells but instead carries out its entire lifecycle either in the epithelium or free living in the lumen. This parasite does not form sporocysts, with the sporozoites instead residing free within the parasite oocyst. In 1912, Tyzzer discovered *Cryptosporidium parvum (C. parvum)* which was different to the previously described *C. muris* as it was found in the small intestine rather than the stomach (Tyzzer, 1912). The size of the parasite differed from *C. muris,* with *C. parvum* measuring at 5.0 µm x 4.5 µm compared with a size of 7.4 µm x 5.6 µm for *C. muris*. This, along with feeding experiments to determine the true location of both parasites, allowed Tyzzer to describe *C. parvum* as a separate species to *C. muris*.

*Cryptosporidium* parasites were only recognised as having the potential to cause disease in farm animals in 1955, after the parasite was attributed as the cause of diarrhoea in turkeys. The farmer reported moderate losses of turkeys between 10 and 14 days of age. This was the first description of the species *Cryptosporidium meleagridis (C. meleagridis)* (Slavin, 1955).

*Cryptosporidium* in calves was reported for the first time in the early 1970s where oocysts were found post-mortem in an 8-month-old calf (Panciera, Thomassen & Garner., 1971). However, these oocysts were present as a mixed infection with other gastrointestinal pathogens and so was not a proven primary enteropathogen (causing disease on its own). The fact that *Cryptosporidium* is a primary enteropathogen was not shown until 1980, where a study showed that all but six calves suffered diarrhoea intermittently from 5 days of age for a two-week duration (Tzipori, Campbell, Sherwood, Snodgrass & Whitelaw., 1980). Oocysts, which were collected from these calves, were used to inoculate a calf under experimental conditions, which began to shed oocysts at 9 days post-infection and suffered diarrhoea that was not attributed to any other gastrointestinal pathogen (Tzipori et al., 1980). This was followed by further work that demonstrated that the parasite was able to cause clinical disease on its own (Heine, Pohlenz, Moon & Woode., 1984; Tzipori et al., 1983). At the time, the two species thought to infect cattle were *C. muris*, which was reclassified as *Cryptosporidium andersoni (C. andersoni)* in 2000 (Lindsay et al., 2000), and *C. parvum* which differed in size, the location of infection and clinical disease.

The first reported cases of *Cryptosporidium* in humans took place in 1976. In one instance, a 39-year-old man presented with severe diarrhoea, mucosal injury in the ileum and jejunum and *Cryptosporidium* oocysts on the epithelium (Meisel, Perera, Meligro & Rubin., 1976). In another, a rectal biopsy of a 3-year-old child found *Cryptosporidium* to be the cause of the child's diarrhoea (Nime, Burek, Page, Holscher & Yardley., 1976).

Today, there are 37 recognised species of this parasite with many more genotypes, each of which has its own host range with different clinical manifestations (Chalmers & Katzer, 2013). Despite *Cryptospordium* being originally thought to be related to coccidia, phylogenetic analysis of the parasite has brought to light that *Cryptosporidium* is actually more similar

to the gregarines (Carreno, Matrin & Barta, 1999; Goater, Goater & Esch, 2014). *Cryptosporidium* has now been formally moved from coccidian to a new subclass called Cryptogregaria (Ryan, Paparini, Monis & Hijjawi., 2016). The most common species of *Cryptosporidium* found worldwide is *C. parvum,* probably due to its zoonotic potential and wide host range (Chalmers & Katzer, 2013).

## 1.2 Lifecycle of *Cryptosporidium*

Hosts become infected with *Cryptosporidium* via faecal-oral transmission, by ingesting sporulated oocysts that are released in the faeces of an infected host (Chalmers et al., 2010). Once ingested, the oocyst will undergo excystation, following triggers such as temperature and pH, where the wall of the *Cryptosporidium* oocyst will open to release four sporozoites (Figure 1 (a)). These sporozoites then go on to infect the epithelial cells of the microvillus border in the gastrointestinal tract (Figure 1 (b)).

Here, sporozoites undergo attachment and invasion before asexual reproduction occurs within a parasitophorous vacuole. This process is called merogony. This process involves the trophozoite nucleus dividing to create type I and type II meronts. Type I meronts release merozoites which have the ability to invade other host cells and continue the asexual reproduction. Type II meronts release merozoites which then invade host cells and undergo sexual reproduction (Figure 1 (c-e)).

Sexual reproduction occurs when meronts differentiate into macrogamonts and microgamonts, which then develop and fuse to form a zygote (Figure 1 (f-i)). Thin-walled oocysts develop (Figure 1 (l)) and these can rupture causing reinfection of the host known as autoinfection. Thick walled oocysts are also produced which are then released in the faeces into the environment, where they are immediately infectious (Figure 1 (j-k)). This thick wall protects the oocysts and makes them very difficult to remove from the environment, where they can survive in for months (King & Monis, 2007). These oocysts are able to cope with a wide range of temperatures, from -22°C to 60°C, and have been reported to survive many commonly used disinfectants and chlorination (Fujino et al., 2002; Goater et al., 2014; Robertson, Campbell & Smith, 1992).

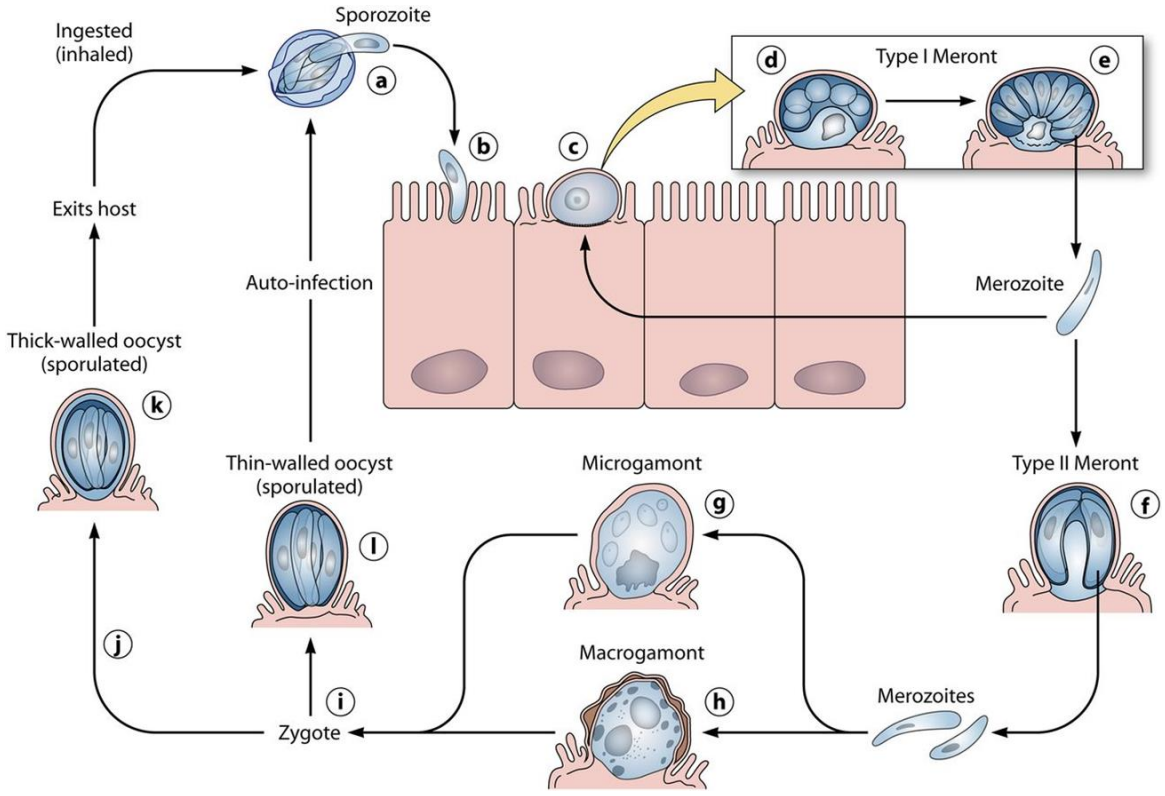


**Figure 1 The lifecycle of *Cryptosporidium parvum* (Bouzid, Hunter, Chalmers, & Tyler, 2013)**

## 1.3 Species of *Cryptosporidium*

**Table 1 Current species list for *Cryptosporidium* as of August 2018.**

| Species Name | Synonym | Host Species | Discovered by/named by |
|---|---|---|---|
| *C. alticolis* | | Voles | Čondlová et al 2018 |
| *C. andersoni* | Mouse genotype | Cattle | Lindsay et al, 2000 |
| *C. apodemi* | | Mice | Čondlová et al 2018 |
| *C. avium* | | Birds | Holubová et al 2016 |
| *C. baileyi* | | Birds | Current et al, 1986 |
| *C. bovis* | Genotype Bovine B | Cattle | Fayer et al, 2005 |
| *C. canis* | Canine genotype | Dogs | Fayer et al, 2001 |
| *C. cuniculus* | Rabbit genotype | Rabbit | Robinson et al, 2010 |
| *C. ditrichi* | | Mice | Čondlová et al 2018 |
| *C. ducismarci* | | Tortoise | Traversa, 2012 |
| *C. erinacei* | | Hedgehog | Kváč et al 2014 |
| *C. fayeri* | Opossum genotype 1 | Marsupials | Ryan et al, 2008 |
| *C. felis* | Cat Genotype | Cats | Iseki, 1979 |
| *C. fragile* | | Toads | Jirku et al, 2008 |
| *C. galli* | *C. blagburni* | Birds | Pavlasek, 1999 |
| *C. homai* | | Guinea Pig | Zahedi et al 2017 |
| *C. hominis* | Genotype 1/genotype H | Humans | Morgan-Ryan et al, 2002 |
| *C. huwi* | *Cryptosporidium piscine genotype 1* | Guppy | Ryan et al 2015 |
| *C. marcopodum* | Marsupial Genotype I | Marsupials | Power & Ryan, 2008 |
| *C. meleagridis* | | Birds | Slavin, 1955 |
| *C. microti* | | Voles | Čondlová et al 2018 |
| *C. molnari* | | Fish | Alvarez-Pellitero et al, 2002 |
| *C. muris* | | Mice | Tyzzer, 1912 |
| *C. parvum* | Genotype 2/genotype C | Mammals including humans | Tyzzer, 1912 |
| *C. proliferans* | *C. muris* strain TS03 | Rodents | Kváč et al 2016 |
| *C. rubeyi* | *Cryptosporidium sp. "c" genotype* | Squirrels | Li et al 2015 |
| *C. ryanae* | Deer-like genotype | Cattle | Fayer et al, 2008 |

| | | | |
|---|---|---|---|
| *C. scophthalmi* | | Fish | Alvarez-Pellitero et al, 2004 |
| *C. scrofarum* | Pig genotype II | Pigs < 4weeks | Kvac et al, 2013 |
| *C. serpentis* | | Snakes | Levine, 1980 |
| *C. suis* | Pig genotype 1 | Pigs | Ryan et al, 2004 |
| *C. testudinis* | | Tortoise | Jezkova et al 2016 |
| *C. tyzzeri* | Mouse genotype 1 | Mice | Xupeng et al, 2011 |
| *C. ubiquitum* | Cervine genotype | Variety of animals | Fayer et al, 2010 |
| *C. varanii* | *C. saurophilum* | Lizards and Snakes | Pavlasek et al, 1995 |
| *C. viatorum* | | Humans | Elwin et al, 2012 |
| *C. wrairi* | | Guinea Pigs | Vetterling et al, 1971 |

# 1.4 *Cryptosporidium*

## 1.4.1 Cryptosporidiosis in Cattle

Cryptosporidiosis was first reported in cattle in a heifer (a young cow which has not yet given birth to a calf) with chronic diarrhoea in 1971 (Panciera et al., 1971) and today it is one of the main causes of gastroenteritis in neonatal calves worldwide (Al Mawly et al., 2015). Cattle can become infected at any age and at any time of the year, although the disease is most common in pre-weaned calves in the spring in both beef and dairy systems (APHA, 2012 - 2017). Studies, which have been carried out longitudinally, provide evidence that all calves in infected herds will shed *Cryptosporidium* oocysts at some point during the first few months of life (Santin, Trout & Fayer., 2008). For pre-weaned calves, the prevalence of cryptosporidiosis is variable. In the UK, the reported prevalence varies from 28 – 80% (Brook, Hart, French & Christley., 2008; Wells et al., 2015) and appears to be dependent on the type of diagnostic tests used. The main clinical signs of cryptosporidiosis are profuse watery diarrhoea, which is often coupled with loss of appetite, abdominal pain and nausea. This watery diarrhoea can result in severe dehydration which, if not properly treated, can lead to the death of the animal. Further complications can

arise such as reduced growth rates if animals are not managed appropriately (Klein, Kleinova, Volek & Simunek., 2008).

The most common species of *Cryptosporidium* which are found in cattle are *C. parvum, C. bovis, C. ryanae and C. andersoni* (Thomson, Innes, Thomson & Katzer,. 2016). *Cryptosporidium parvum*, which has been reported to infect a wide variety of mammals, is the main species responsible for zoonotic human infections. *Cryptosporidium parvum* is the most common species in neonatal calves from as early as 4 days until around 1 month of age (dos Santos Toledo et al., 2017; Santin, Trout & Fayer., 2007; Xiao & Herd, 1994) and is the only species of these four that has been reported to cause clinical disease. Infection with *C. parvum* results in damage to villi of the small intestine, making it very difficult for the animal to absorb water and nutrients. This inability to absorb water results in watery diarrhoea causing the animal to become very dehydrated. *Cryptosporidium parvum* has been found in all ages of cattle, although in much lower concentrations than in pre-weaned calves.

Species more commonly found in older post-weaned calves are *C. bovis* and *C. ryanae*, found from 2 months of age to around 11 months (Santin, Trout & Fayer., 2008; Thomson, 2015). The most common species in pre-weaned calves on some farms in Sweden and Canada is *C. bovis* (Budu-Amoako et al., 2012; Silverlas, Naslund, Björkman & Mattsson., 2010) however it was not associated with clinical disease. *Cryptosporidium ryanae* was originally known as *Cryptosporidium* deer-like genotype, until it was described as a separate species in 2008 (Fayer, Satin & Trout., 2008). There does not appear to be any clinical signs from infection in cattle with these species (Fayer et al., 2008; Fayer et al., 2005; Mirhashemi et al., 2016) however the long-term production effects are currently unknown.

The species *C. andersoni* is most commonly found in adult cattle although has also been reported in calves under 2 months of age (Anderson, 1987;

Enemark et al., 2002). It is different to the other species of *Cryptosporidium* in that it infects the abomasum. This species has not been reported to cause diarrhoea, although it has been associated with production effects such as reduced weight gain and milk yield (Esteban & Anderson, 1995; Lindsay et al., 2000). *Cryptosporidium andersoni* was previously believed to be the only species of *Cryptosporidium* affecting adult cattle; however more recent studies which have used improved oocyst concentration and DNA extraction techniques along with more sensitive molecular detection methods have shown that adult cattle also excrete *C. parvum* (Thomson et al., 2016; Wells et al., 2015; Wells, Thomson, Innes & Katzer., 2016).

### 1.4.2 Cryptosporidiosis in Sheep and Goats

Cryptosporidiosis is less common in sheep and goats compared to cattle, which is likely due to the different management of these animals. Unlike cattle, sheep are more likely to live and have their lambs outdoors rather than indoors. Despite this, some authors have described it as an important pathogen in neonatal lambs and goat kids; causing diarrhoea and mortality (de Graaf et al., 1999). The most common species of *Cryptosporidium* to infect sheep and goats is *C. parvum* (McLauchlin, Amar, Pedraza-Diaz & Nichols., 2000; Mueller-Doblies et al., 2008; Pritchard, Marshall, Giles, Chalmers & Marshall., 2007), although other species *C. ubiquitum, C. bovis* and *C. xiaoi* are also relatively common (Chalmers et al., 2002; Robertson, Björkman, Axen, Fayer., 2014).

Evidence for higher prevalence of *Cryptosporidium* in pre-weaned lambs compared to post-weaned is questionable, as in some studies it would appear there is little difference between the occurrence of *Cryptosporidium* between pre and post-weaned lambs. Work carried out in Australia found the prevalence in pre-weaned lambs to be 24.5%, similar to the 26.2% prevalence found in post-weaned lambs (Ryan et al., 2005; Yang

et al., 2014). However, the pre-weaned lambs were predominantly infected with *C. parvum* whereas the post-weaned lambs were infected with *C. bovis* and a cervine genotype. Therefore, it would appear that, like cattle, *C. parvum* is predominantly found in pre-weaned rather than post-weaned lambs. This has also been shown in a study carried out in lambs in Spain, which showed the prevalence of *C. parvum* to be 66.4% in lambs less than 21 days old and 23% in lambs between 22 and 90 days old (Causape et al., 2002). This study also showed that lambs experienced peak shedding between 8 – 14 days of age, which suggests that this parasite exhibits a similar pattern of infection in lambs as it does in calves. The predominant species in goat kids is *C. parvum*. Goat kids have been reported to shed *C. parvum* genotypes that have also been reported in humans (Quilez et al., 2008).

### 1.4.3 Cryptosporidiosis in Humans

Cryptosporidiosis was first reported in humans in 1976 (Nime et al., 1976) and usually presents as a self-limiting disease. Chronic and potentially life-threatening disease can occur in those with a compromised immune system such as the very young, old and sick (Hunter et al., 2007). *Cryptosporidium* is the second greatest cause of gastroenteritis in children worldwide, although is more commonly seen in developing countries (Ryan & Hijjawi, 2015). As *Cryptosporidium* is a waterborne parasite, the lack of adequate water treatment in these countries explains the increased parasite prevalence. In immunocompetent individuals the prevalence of *Cryptosporidium* infection was estimated as 3-15% in Egypt (Youssef, Adib, Riddle & Schlett., 2008), 13% in Tanzania (Cegielski et al., 1999) and 12.5% in Uganda (Mor et al., 2010). In comparison, it was estimated in 2006 that there are around 8.9 cases per 100,000 people in the UK, based on Health Protection Agency (HPA) reports, most of these being young children (Nichols et al., 2006). Most of these studies used detection methods that were less sensitive than those which are currently available so the true

prevalence was likely to have been even higher. Studies that are more recent include an epidemiological study looking at 22,000 children in four African and three Asian countries. This study aimed to investigate the most common cause of diarrhoeal disease. The most commonly detected pathogen was rotavirus, followed by *Cryptosporidium* (Kotloff et al., 2013). Due to the ubiquitous nature of the parasite, there are a variety of potential transmission routes for human infection through the faecal-oral route such as person to person contact, animal to human contact (zoonotic) or contact with contaminated water or food (Ryan & Xiao, 2014). A risk assessment of waterborne cryptosporidiosis showed that a single oocyst is able to infect 2.79% of immunocompetent people and it is able to amplify relatively quickly through a susceptible population (Pouillot et al., 2004).

Public attention was drawn to this parasite after the major waterborne *Cryptosporidium* outbreak in Milwaukee in 1993. This is estimated to have affected around 403,000 people who presented with various gastrointestinal complaints (MacKenzie et al., 1995). Since then, more work has gone into the development of new research tools in order to source track other outbreaks, which is essential in determining the epidemiology and impact of this parasite.

Cryptosporidiosis is a very under-researched disease despite being one of the main causes of gastrointestinal disease in young children in developing countries (Kotloff et al., 2013). A study carried out by the Global Enteric Multicentre (Levine, Kotloff, Breiman & Zaidi., 2013) found that *Cryptosporidium* was the second most common cause of moderate to severe diarrhoea in children under 2 years old. This was done at seven different sites in sub-Saharan Africa and South Asia. This study also found that the parasite was associated with a higher risk of mortality in children compared to control children that did not have diarrhoea.

In immunocompromised individuals, for example those who have Human Immunodeficiency Virus (HIV), *Cryptosporidium* can spread to other parts of the body such as the gallbladder, pancreas and pulmonary system. A study looking at Acquired Immune Deficiency Syndrome (AIDS) patients with chronic diarrhoea showed that 30% of those suffering from intestinal cryptosporidiosis also had an extra-intestinal infection (Lopez-Velez et al., 1995). This makes the immunocompromised, such as people with HIV, most at risk from *Cryptosporidium* with the disease having the potential to be fatal.

The main species which commonly infect humans are *C. parvum,* which is associated with livestock and tends to spread via livestock to human on farms or at petting zoos, and *C. hominis*, which is considered to be mostly human-specific and outbreaks tend to occur in recreational water and contact with human faeces (Chalmers, 2012). *Cryptosporidium hominis* was originally referred to as *C. parvum* human genotype, however after biological and molecular differences between the two were discovered, it was renamed (Morgan-Ryan et al., 2002). The increase in epidemiological surveys and development of sensitive genotyping techniques has identified both species together to be responsible for over 90% of human cryptosporidiosis (Xiao, 2010).

The options for human treatment of cryptosporidiosis are limited with no licenced treatment in the EU and only one FDA approved drug in the US; nitazoxanide (Chalmers, 2012). This drug has a poor efficacy, especially in people with AIDS. It is essential that more research is done in order to prevent and control the disease more effectively. US spending on research for this parasite is around $4.3 million annually (Ryan & Hijjawi, 2015) but the costs, according to the CDC (Centre for Disease Control and Prevention) in terms of hospitalizations, around $45 million annually. In the UK, it has been estimated that waterborne cryptosporidiosis costs £23 million per year (Pretty et al., 2000). This clearly shows that more money needs to be

put into cryptosporidiosis if we hope to develop effective control strategies.

## 1.4.4 Cryptosporidiosis in Wildlife

*Cryptosporidium* has a wide host range and so there are many *Cryptosporidium* species affecting a wide range of wildlife species including mammals, birds, reptiles, rodents and fish (Appelbee, Thompson & Olson., 2005.). The distribution of species and genotypes of *Cryptosporidium* in wildlife is a relatively unexplored area of research (Zahedi, Paparini, Jian, Robertson & Ryan., 2016).

The general consensus is that wildlife species are likely to become infected with zoonotic *Cryptosporidium* via environmental pollution of faecal waste (Appelbee et al., 2005). Despite oocysts being detected in a variety of wildlife species, modern genotyping has found that most wildlife species are shedding host-adapted genotypes and so they are likely not to be a major risk to public health (Xiao, Fayer, Ryan & Upton., 2004; Zhou et al., 2004). However, there has been one reported human outbreak attributed to wildlife species in the past, where a rabbit that entered the treated water tank caused an outbreak of *C. cuniculus* in Northamptonshire (Puleston et al., 2014). There have also been reports of wildlife contaminating the watershed in New York, which included the human infectious *C. ubiquitum* (Feng et al., 2007). As these are both zoonotic species, there is a potential for wildlife to act as a transmission vector to both humans and livestock.

**Deer**

White-tailed deer, roe deer, red deer, moose and caribou have all been reported to harbour the parasite *Cryptosporidium,* with prevalence ranging from 8% in white-tailed deer, 1.3% in roe deer, 6.2% in red deer and 3.3% in moose (Castro-Hermida, Garcia-Presedo, Gonzalez-Warleta, & Mezo., 2011;

Hamnes et al., 2006; Johnson et al., 2010; Rickard, Siefker, Boyle & Gentz., 1999). Higher prevalence has been found in Scotland where red deer showed a prevalence of 87.5% and roe deer 33%, although sample numbers were much lower than in the previous studies and the area was known to have a problem with *Cryptosporidium* (Wells et al., 2015). It would appear that most of these infections present as asymptomatic, although only adult animals were sampled in all of these studies. At present, very little has been done looking at cryptosporidiosis in neonatal deer species, although the few studies that have been carried out show no symptomatic infection in younger cervids (Fayer et al., 1996; Skerrett & Holland, 2001).

**Rodents**

Wild rodents are widespread throughout the world and are considered a host of *Cryptosporidium* with the common species detected being *C. parvum* and *C. muris* (Lv et al., 2009). In Poland, it was found that prevalence of *Cryptosporidium* is much higher in voles than in mice with a prevalence of 70.6 % in bank voles, 73 % in common voles and 27.8 % in yellow-necked mice (Bajer et al., 2002). In the UK, *C. parvum* was detected in 22-33% of house mice, 21% of wood mice, 9% of bank voles and 63% of brown rats (Chalmers et al., 1995; Chalmers et al., 1997; Chalmers et al., 1994; Webster & Macdonald, 1995)

**Rabbits**

*Cryptosporidium* in rabbits has been previously described to be mostly asymptomatic (Zhang et al., 2012) with a low prevalence; however, there have been reports of gastroenteritis and mortalities on rabbit farms that have been attributed to the parasite (Kaupke, Kwit, Chalmers, Michalski & Rzezutka., 2014; Pavlasek, Lavricka, Tumova & Skrivan., 1996; Shi et al., 2010). It does appear from these studies that clinical symptoms are more

likely to be observed at the time when rabbits are weaned. The symptoms that have been described include anorexia, diarrhoea and death.

*Cryptosporidium* is present in the wild rabbit population, but at a low prevalence with low numbers of oocysts being shed. The most common species that is found in wild rabbits is *C. cuniculus*, however, rabbits have been reported to also become infected with *C. parvum, C. meleagridis* and *C. muris* under experimental conditions (Robinson & Chalmers, 2010).

**Wild Birds**

*Cryptosporidium* has been reported to infect the gastrointestinal tract, renal and respiratory system in birds with the three recognised pathogenic species being *C. meleagridis, C. baileyi* and *C. galli* (Ryan & Xiao, 2014). Other species *C. hominis, C. parvum, C. muris* and *C. andersoni* have also been reported in birds although in much smaller numbers (Jellison, Distel, Hemond & Schauer., 2007; Ng, Pavlasek & Ryan., 2006; Reboredo-Fernandez et al., 2015; Zhou, Kassa, Tischler & Xiao., 2004). Whether these other species were actually infecting the birds has not yet been proven and so it could be that they are purely ingesting and passing them in the faeces without any parasite development occurring. Despite this, they could still pose a risk to animal infection through mechanical transmission. There is some evidence of *C. parvum* resulting in enteritis in captive bred Stone-curlews (Zylan et al., 2008) although further work needs to be done to confirm this. *Cryptosporidium meleagridis* is very similar to *C. parvum* and *C. hominis* at the 18S rRNA region of the genome and has been reported to infect both humans and mammals (Xiao et al., 2002). Therefore, sequencing is essential for the successful *Cryptosporidium* species determination in birds.

*Cryptosporidium baileyi* (*C. baileyi*) is well documented in birds in the UK, especially in the red grouse (*Lagopus lagopus scotica*) (Coldwell et al., 2012). Respiratory infection caused by *C. baileyi* is estimated to affect at

least half the grouse in the moors of North England and around 80% of the grouse in the moors of the Pennine Hills (Baines, Newborn & Richardson., 2014). A study done in the North Pennine moors estimated 39% of birds with *Cryptosporidium* died from the infection (Baines et al., 2018).

*Cryptosporidium* in pheasants has been reported in only a couple of studies, and it is more commonly associated with respiratory disease (Randall, 1986; Whittington & Wilson, 1985). Gastrointestinal disease has been reported in other avian species such as turkeys (Bermudez et al., 1988; Goodwin, Steffens, Russell & Brown., 1988) and quail (Hoerr, Current & Haynes., 1986; Ritter, Ley, Levy, Guy & Barnes., 1986) and these infections tend to be attributed to the species *C. meleagridis*. Pheasants can be in abundance on some farms, especially those that breed and release them for shooting.

Although historically it is not believed that *C. parvum* is commonly found in avian species, a very recent study has found *C. parvum* to be the most prevalent species in broilers, layers and turkeys in Germany (Helmy et al., 2017). This study is one of the few using molecular methods to diagnose cryptosporidiosis in birds and so it could be that, historically, the *C. parvum* prevalence has been underestimated. *C. parvum* has also recently been discovered in captive falcons, where the birds presented with lung adhesions and microcysts (Azmanis et al., 2018).

### 1.4.5 *Cryptosporidium* in the Environment

The environment is thought to be the main reservoir for *Cryptosporidium* oocysts and is thought to be the major source of oocysts for calf infection (Wells, 2015). Most human infections are attributed to water contamination of *Cryptosporidium* oocysts, as oocysts are able to survive routine water treatments such as chlorination (Meinhardt, Casemore & Miller., 1996). This causes issues for the water industry as these oocysts provide a risk to public health. *Cryptosporidium* oocysts enter the environment via livestock and

wildlife faeces (Chalmers et al., 2010). A catchment study in Scotland was conducted following reports of cryptosporidiosis in the local population (Wells et al., 2015). This study found that transmission in the environment is likely to be cyclical with infection occurring on a seasonal basis. This seasonality links with calving seasons and high rainfall washing oocysts from the land into watercourses. It is important that livestock and wildlife are not allowed access to water destined for human consumption and instead are provided with water troughs to reduce this transmission method.

## 1.5 Diagnosis and Identification of *Cryptosporidium* Species

In order to diagnose and treat suspected cases of cryptosporidiosis, it is essential that appropriate and sensitive techniques are used to identify *Cryptosporidium* oocysts. Speciation and genotyping within a species is especially important for epidemiological studies, and source tracking infections from an outbreak in order to improve biosecurity (Chalmers et al., 2009). Today there are many sensitive techniques available for the differentiation of *Cryptosporidium*. The most commonly used techniques are microscopy and molecular methods such as polymerase chain reaction (PCR) and enzyme immunoassays (EIA) (Chalmers & Katzer, 2013; Alexander et al, 2017). At present, the guidelines issued by Public Health England (PHE) state that 'Primary laboratory diagnosis is based on stained microscopy or antigen detection by enzyme immunoassay followed by confirmation using microscopy stains or DNA detection by PCR' (Public Health England, 2017).

### 1.5.1 Microscopy

Traditionally microscopy is the main detection method for *Cryptosporidium* in both human and veterinary diagnostic labs, using auramine phenol (AP) or acid-fast staining such as modified Ziehl-Neelsen (mZN) (Chalmers et al., 2010; Alexander et al., 2017). These two staining techniques were

recommended as the diagnostic tool to use during the first documented major UK waterborne outbreak of *Cryptosporidium* in Oxfordshire, England in 1989 (Richardson et al., 1991). When using mZN staining, *Cryptosporidium* oocysts are stained pink against a blue background and the technique has a sensitivity of around 75.4 %, and a specificity of 100%. Auramine phenol, on the other hand, has a sensitivity of 92.1% and a specificity of 100%, and stains oocysts bright green (Chalmers et al., 2011). Both of these sensitivity studies were directly compared against a gold standard of real time quantitative PCR. Unfortunately, these staining methods have a detection limit of 50,000-500,000 oocysts per gram of human faeces which could be argued to be less sensitive than molecular detection methods (Weber et al., 1991). On the other hand, molecular detection is known to have problems with PCR inhibition (Morgan et al., 1998) and therefore microscopy is valuable in ruling out false negative results. Typically, a combination of both microscopy and further molecular based tests are used such as immunofluorescence microscopy and PCR. (Public Health England, 2017).

Immunofluorescence microscopy such as 4,6' diamidino-2-phenylindole dihydrochloride (DAPI) is commonly used when testing for *Cryptosporidium* oocysts in water and stains the nuclei of sporozoites within oocysts, making them appear blue under UV light, if they are viable (Grimason et al., 1994).

In research or epidemiological outbreak investigations, microscopy tends to be used only to confirm the presence or test the viability of oocysts, and instead, more sensitive molecular techniques such as PCR are being used which are far more informative due to the ability to determine species and genotype (Chalmers & Katzer, 2013).

### 1.5.2 Molecular Detection

New concentration and detection techniques have led to the ability to speciate and genotype *Cryptosporidium*, allowing outbreaks to be tracked

back to their original source (Chalmers & Katzer, 2013). First, DNA needs to be extracted from the oocysts before various genes can be amplified. This amplification allows for analysis of these particular genes, by either their sequence or their size, in order to draw conclusions on the species and genotype that belongs to those oocysts. Molecular techniques allow for the species of *Cryptosporidium* to be determined, which is desirable if looking at zoonotic species. They also allow for parasite genotyping within species to be as discriminatory as possible in epidemiological studies.

**Polymerase Chain Reaction**

Amplification of genes of interest is done using the polymerase chain reaction (PCR). This is either a standard PCR, where only one pair of primers are used to amplify the region of interest, or a nested PCR where there are two pairs of primers. Typically for *Cryptosporidium*, a nested PCR is used which is able to produce more copies of the gene of interest, useful if samples contain small amounts of DNA.

The most common gene which is targeted for *Cryptosporidium* detection is the 18S ribosomal RNA gene. This gene is a multi-copy gene which is therefore more sensitive than other genes commonly amplified: heat shock protein 70 and COWP (Egyed et al., 2003). It can be difficult to differentiate between species using this method, as often the most predominant species tends to be detected and therefore amplified DNA must be sent for sequence analysis. However recently a species specific nested 18S PCR has been developed allowing for the differentiation between the four most common species of *Cryptosporidium* commonly found in cattle (Thomson et al, 2015). Both of these PCR techniques do not provide quantitative results for parasite DNA and can only detect the presence of DNA (Chalmers & Katzer., 2013).

The main problem with molecular detection techniques is the issue with PCR inhibition. Efficient extraction of DNA is key for accurate PCR analysis;

however, oocysts are commonly found in substances such as faeces, slurry, food and water which are likely to contain PCR inhibitors. These inhibitors include carbohydrates, bilirubin and bile salts (Chalmers & Katzer, 2013). The use of PCR for parasite DNA detection in the literature has rarely reported the use of internal controls to rule out false negative results (Schrader, Schielke, Ellerbroek & Johne., 2012). However internal controls do exist which have been used for COWP amplification (Hawash, Ghonaim &l-Hazmi., 2015).

### 1.5.3 Enzyme-Linked Immunosorbent Assay

An enzyme-linked immunosorbent assay (ELISA) or enzyme immunoassay (EIA) is a test which can be used to detect *Cryptosporidium* antibodies in the blood, serum and milk or antigens in faecal material. It is a very useful diagnostic tool due to the fact they are very quick and simple to use, with the ability to deal with large numbers of samples in a short space of time. This makes it a popular tool for diagnosis in human infection (Chalmers et al., 2010). Despite being a costlier technique when compared to acid-fast staining, it has been shown to be more sensitive than the traditional staining technique and is easier to use (Chalmers & Katzer, 2013). Unfortunately, as many available ELISAs are designed for human infections, the sensitivity can be lower than desired when used for animal diagnostics. This technique has shown less than 75% sensitivity when used on animal faecal samples using the ProSpect *Cryptosporidium* Microplate assay (Remel, KA, USA) and so the potential for false negative results are high (Rimhanen-Finne et al., 2007).

### 1.5.4 Lateral Flow Immune Chromatographic Assays

A lateral flow immune chromatographic assay can be used as a diagnostic for *Cryptosporidium* giving a qualitative result. It involves the movement of the sample within a liquid along a strip where molecules from the sample can attach and interact creating a visible positive or negative result

(Posthuma-Trumpie, Korf & van Amerongen., 2009). These assays are ideal for use in the field as no equipment is required and the kits are very easily stored and transported. They have also been shown to have a relatively high sensitivity and specificity ranging between 84.9% -100% and 94% - 100% respectively when compared to real time qPCR (Hameed, Elwakil & Ahmed., 2008; Chalmers et al., 2011; Fleece et al., 2016). Some assays can test for multiple pathogens in the same kit making it very easy to use. Results are often seen within a few minutes allowing for immediate action and treatment (Chalmers & Katzer, 2013).

## 1.6 *Cryptosporidium* Genotyping

### 1.6.1 GP60 Genotypes

The most common method of genotyping *C. parvum* is currently by sequence analysis of the 60 kDa glycoprotein gene, commonly called GP60 gene (Strong, Gut & Nelson., 2000). This technique is widely used for epidemiological studies in both human and animal infections (Leoni,, Mallon, Smith, Tait & McLauchlin., 2007; Wielinga et al., 2008). By analyzing both the differences in non-repeat regions and in the variable number of tandem repeats of TCA, TCG and TCT at the 5' end of the serene coding trinucleotide, a subtype can be determined (Xiao, 2010). The sequence analysis of the non-repeat region splits genotypes into families with the most common family of *C. parvum* found in cattle being IIa and IId, which are zoonotic and are responsible for many human infections. IIc is less common, tends to be associated with human infections and is rarely found in ruminants (Xiao, 2010). The most common GP60 genotype found in human stool samples throughout Scotland was IIa which was found in 94% of 1139 positive faecal samples (Deshpande et al, 2015). The main issue with this type of genotyping is the inability to detect mixed infections due to the use of conventional Sanger sequencing for GP60 analysis. It is therefore essential to genotype based on multiple loci, which do not rely on sequence analysis rather than only use the single GP60 locus.

### 1.6.2 Multilocus Genotyping

Multilocus genotyping addresses the concern of only using one locus to genotype *Cryptosporidium* and so improves the discriminatory ability when compared to using GP60 alone. It allows for the analysis of short tandem repeats at multiple loci using capillary electrophoresis in order to assign a multilocus fragment type (MLFT). A further benefit of using multilocus genotyping is that it allows for the detection of mixed infections, something which is impossible using GP60 alone. A study looking at the common GP60 subtype IIaA15G2R1 showed that, by using multilocus genotyping, there was a high level of heterogeneity within this subtype and so further typing is necessary when looking at the epidemiology of this parasite (Feng et al., 2013). This technique has been determined as the most discriminatory method for looking at *Cryptosporidium* genotypes and so epidemiological studies should now adopt multilocus genotyping. Unfortunately, the use of this technique has not yet been standardised and so different laboratories in different areas of the world rarely use the same loci therefore is very difficult to draw any conclusions on comparisons between studies. Despite this, some progress has been made towards identifying the best candidates for a standardised system (Robinson & Chalmers, 2012).

A study looking at the potential of markers MSA, MSD, MSF, MM5, MM18, MM19, MS9, GP60 and TP14 for use in outbreak investigations for *Cryptosporidium* found that DNA conformation, PCR running conditions and sequence composition could affect the results. However, inter laboratory allele assignation was reproducible and so would be good candidates for outbreak investigations (Chalmers et al., 2017). Despite this, it was concluded that further markers needed to be identified.

## 1.7 Transmission of *Cryptosporidium*

The main route of infection of *Cryptosporidium* to young calves is currently unknown however the main ideas currently being explored include adult cattle, wildlife and the environment. It is clear that the environment would likely be an infection route to young calves, due to the ability of the oocysts to survive for long periods of time in the farm environment; however, the main source of environmental contamination is still unclear. Environmental contamination of *Cryptosporidium* oocysts has been shown to be very high both in areas with cattle access and in calf pens. This includes the pen floor, soil, grass and water (Faubert & Litvinsky, 2000).

For calves, it could be that they are becoming initially infected from *Crptosporidium* oocysts which are coming from their dams. Young calves tend to show clinical signs of infection in the second week of life (Faubert & Litvinsky, 2000; Sanford & Josephson, 1982). This would suggest that infection occurs immediately or very soon after birth. The idea that adult cattle could be the source of this infection is disputed, with the prevalence of *Cryptosporidium* in faeces ranging from 0 to 71.75% (Atwill 2003, Lorenzo Lorenzo 1993).

Another possible infection route could be from wildlife in the vicinity of the farm. Currently research which has been done examining the species and genotypes of *Cryptosporidium* in wildlife species shows that the common calf parasite *C. parvum* can be found in red deer, roe deer, rodents and rabbits (Lv et al., 2009; Robinson & Chalmers, 2010; Smith, Clifton-Hadley, Cheney & Giles., 2014; Wells et al., 2015).

Contaminated drinking and recreational water appear to be the main infection route for humans (Chalmers 2012). Despite it being more common in the developing world, this disease is becoming more of a problem in the developed world (Nasser, 2016). Between the years 2004 and 2010 there have been at least 120 outbreaks of diarrhoea due to waterborne

*Cryptosporidium* worldwide (Baldursson & Karanis, 2011). Outbreaks attributed to contaminated drinking water include an outbreak in Northamptonshire, which was attributed to a rabbit infecting people with the *Cryptosporidium* species *C. cuniculus* (Puleston et al. 2014). More recently, *Cryptosporidium* has been detected in the water that supplies Lancashire resulting in a "boil water" notice (Robins, Burt, Bracken, Boardman & Thompson., 2017). The detection of *Cryptosporidium* oocysts in water suggests that it could be an important infection route and so could also play a role in livestock infection if animals have access to contaminated water. A study which examined farms, wildlife and water within a Scottish catchment found that *Cryptosporidium* oocysts were present at all water sites tested along the river (Wells et al. 2015). This would indicate that water is a possible infection route for livestock, especially if a farm further upstream has an outbreak of cryptosporidiosis. Animals that have access to rivers could be infected via this route and they, in turn, could go on to infect other animals such as neonatal calves on the farm.

A likely route of infection is experiencing contact with other infected hosts. This is true for both neonatal livestock and children. As one infected animal can shed billions of oocysts (Goater et al. 2014) the potential for transmission to other hosts is very high.

## 1.8 Impact of the Disease

### 1.8.1 Impact on Human Growth and Development

Diarrhoeal disease in young children is one of the most important causes of childhood death in developing countries, with most of these diarrhoeal diseases attributed to rotavirus, *Cryptosporidium*, *Shigella*, and *E. coli* (Kotloff et al., 2013; Levine et al., 2013). It is thought that around 700,000 children die every year due to diarrhoea with majority of these occurring in children under the age of 5 years old (Bhutta & Syed, 2015). These

pathogens have been associated with an increased morbidity and a reduction in growth. Children suffering even a single episode of severe diarrhoea in a developing country, suffered a reduction in growth in the following 2 – 3 months (Kotloff et al., 2013).

*Cryptosporidium* has been associated with reduced physical fitness and cognitive function in children (Guerrant et al., 1999) along with impairing the growth rate of children. A study in Peru showed that children which were infected with *C. parvum* had reduced height and weight compared with children of the same age which were not infected in the months following infection, before experiencing catch-up growth (Checkley et al., 1998). The study also showed that children who were infected between 0-5 months of age never caught up in growth with the uninfected children 1-year post infection, unlike children which were infected at a later stage.

Another study conducted in a semi-urban slum in South India described how children which suffered multiple episodes of diarrhoea caused by *C. hominis* had lower height and weight than uninfected children of the same age at 2 years old (Ajjampur et al., 2010). Despite this, the children had caught up by the time they were 3 years of age.

### 1.8.2 Impact on Livestock Growth

Enteritis is the most common cause of mortality in neonatal calves which were submitted to the Agri-Food and Biosciences Institute (AFBI) during the year 2011 (Morrison, Scoley & Barley., 2013). The typical cost associated with diarrheal disease in calves is in the region of £34 per affected calf (Gunn & Stott, 1997). Diarrhoea in pre-weaned calves has been associated with a 12 kg reduction in live weight at 18 months of age and is also responsible for a 3% increase in calf mortality (Morrison et al., 2013). It is important to determine the economic impact of *Cryptosporidium* to cattle farmers as the parasite is endemic on cattle farms in Europe (Ramo et al., 2016) and so could be having a significant economic impact.

The damage which *Cryptosporidium* can cause, such as shortening of gut epithelial cells and severe atrophy of the villi, is highly likely to have a negative impact on livestock growth (Heine et al., 1984). This damage can result in malnutrition, malabsorption and fermentation of undigested milk leading to significant economic losses (Cho & Yoon, 2014).

It has been shown that the parasite *Cryptosporidium* can have an effect on the long-term growth of lambs (Sweeny, Ryan, Robertson & Jacobson., 2011). Lambs positive for *C. parvum* in Australia weighed 1.65 kg less at slaughter age when compared with lambs which were negative for the parasite. A further study found lambs shedding *C. parvum* were associated with lower live weight, ranging from 2.31- 4.52kg (Jacobson et al., 2016) compared with lambs which were not shedding *C. parvum*. These studies show that lambs which were infected with *C. parvum* were shown to have reduced growth rate, live weight and carcase quality when compared with animals which were not infected.

The effect of cryptosporidiosis in calves has not yet been examined long term, although there is evidence of a reduced growth rate in the few weeks post infection (Klein et al., 2008). Lack of research in this area is likely due to the frequent occurrence of concurrent infections of the parasite with rotavirus, coronavirus and *Escherichia coli (E. coli)* making it difficult to pinpoint which is responsible for the reduction in growth rate.

## 1.9 Control of Cryptosporidiosis on Farms

### 1.9.1 Therapeutic

The costs to the producers in the form of vet bills, treatment costs and reduced production efficiency, make *Cryptosporidium* a very important parasite for economic and welfare reasons (Holland, 1990). An infected animal has the potential to shed millions of infective oocysts and so pass the parasite on to other animals and people on the farm (Robertson, 2009).

Management for cryptosporidiosis in calves currently involves rehydration and electrolyte repletion as unfortunately there is a lack of specific treatment options.

One option for treatment is halofuginone lactate which is registered for use in Europe and has been found to lower *Cryptosporidium* oocyst output in the first two weeks after birth (Meganck, Hoflack & Opsomer., 2014; Naciri, Mancassola, Yvore & Peeters., 1993). It can be used both as a treatment and as a preventative although cannot be used in animals which already have diarrhoea. This drug, however, does not completely remove the parasite, and only succeeds in reducing oocyst shedding (Naciri et al., 1993). Therefore, it still renders the calf as an important transmission source to other calves. Halofuginone lactate, when used as a preventative, has to be given every day for the first 7 days of life (Naciri et al., 1993). This is particularly difficult for beef farmers, as removing calves from their dams can be both stressful for the animals and dangerous for the farmer. This treatment can be toxic to dehydrated animals when given at twice the recommended dose (De Waele et al., 2010).

Some research has been carried out looking at the effect of an antibiotic paromomycin on the clinical symptoms and shedding of *Cryptosporidium* oocysts, with positive results. Mice which were immunosuppressed and infected with *C. parvum* oocysts were treated with Paromomycin for 10 days. These mice showed a significant reduction in oocyst shedding, parasite colonization and villus atrophy compared to untreated mice (Healey, Yang, Rasmussen, Jackson & Du., 1995).

This antibiotic has also shown to have good efficacy in livestock. Twelve calves which were fed paromomycin twice a day for 11 days found that this antibiotic reduced the number of oocyst shedding days, the duration of disease and the severity of diarrhoea (Fayer & Ellis, 1993). Another study found similar results, with a reduction in oocyst shedding and clinical

disease, however, diarrhoea did start once the drug was withdrawn (Grinberg et al., 2002).

The treatment which is used for human infection, nitazoxanide, has also been shown to provide some efficacy when used in calves. When calves were treated with 1.5g of nitazoxanide twice daily for 5 days, they showed reduced oocyst shedding and an improved score for faecal consistency compared to a placebo group (Ollivett et al., 2009). However, nitazoxanide is only licenced for use in humans.

### 1.9.2 Vaccines

A vaccine for this disease does not currently exist and this is mainly due to the lack of knowledge about host-pathogen interactions and the immune response to *Cryptosporidium* (Ryan & Hijjawi, 2015). The parasite does not have the plastid-derived apicoplast or processes such as the citrate cycle and cytochrome-based respiratory chain which are classic drug targets (Ryan, Zahedi & Paparini., 2016). Currently, it is very difficult to grow this parasite without an animal host although some progress has been made using a hollow fibre *in vitro* culture (Morada et al., 2016) which is able to generate 1 x $10^8$ oocysts per day.

Many of the vaccine targets tend to focus on proteins present on the surface of the sporozoites (Haserick, Klein, Costello & Samuelson., 2017). However, it has been predicted that immunizing calves is not the solution. Calves are thought to become infected immediately or very soon after they are born and so would not have enough time to mount an immune response to a vaccine before they come into contact with *Cryptosporidium* (Innes et al., 2011). Therefore, it seems the most sensible route would be to immunize the dam, to produce hyperimmune colostrum which is able to protect the calf.

There has been promising work on this approach, with the dams able to pass on protection in their colostrum to the calves. A recombinant protein called rC7 was used to immunize pregnant cows to see if there was any protection against cryptosporidiosis in calves which were given their colostrum. None of the calves which were given the hyperimmune colostrum developed diarrhoea after experimental infection and had a reduced oocyst output compared to the control group (Perryman, Kapil, Jones & Hunt., 1999).

### 1.9.3 Animal Management

Due to the lack of treatment options and a suitable vaccine, it is recommended that this parasite is tackled using appropriate management and biosecurity. The main recommendation is to ensure adequate colostrum uptake by calves which is of the right quality as soon as possible to ensure the best protection. Three litres of colostrum given within the first two hours of life with at least 20g/l IgG will go a long way in providing protection against many pathogens which could infect a young calf and so reduce the severity of cryptosporidiosis (Hotchkiss, Thomson, Wells, Innes & Katzer., 2015). Pre-weaned calves which do not receive sufficient colostrum in the first 24 hours of life have been shown to have a higher risk of disease and mortality, along with 40% higher veterinary costs, 17% lower live weight gains in the first 3 months and take 17 days longer to reach slaughter weight (Morrison et al., 2013).

Cleaning the sheds of faecal material is absolutely essential especially before disinfection as it has been shown that organic material can prevent disinfectants working correctly (Wilson & Margolin, 2003). Cleaning will also reduce pathogen concentrations, such as *Cryptosporidium,* from the calves' environment and so reduce the chances of transmission (Morrison et al., 2013).

Reducing stocking density helps to reduce disease incidence by reducing the environmental load of *Cryptosporidium* oocysts. Reducing age mixing to ensure high shedding animals are kept away from neonates is another management strategy which can be adopted (Hotchkiss et al., 2015; Thomson, 2015). Isolation of infected animals and quarantining new animals is also a reliable way of preventing transmission of diseases around the animals and on the farm.

Appropriate treatment of farm waste is another management method which will reduce the environmental load of pathogens, including *Cryptosporidium*. As *C. parvum* has been found in both the manure and in the pasture samples where the manure was spread (Smith et al., 2014), it is essential that manure is treated properly before being spread, to reduce the risk of parasite transmission. Waste piling of faeces, especially from pre-weaned animals, reduces the infectivity of *Cryptosporidium* oocysts (Jenkins et al., 1999). Another recommendation includes spreading manure on soil before cold weather conditions which will give a period of freeze/thaw cycling as freeze/thaw cycling has been proven to reduce oocyst infectivity (Jenkins et al., 1999).

## 1.10 Disinfectants

### 1.10.1 History

The nature of *Cryptosporidium* oocysts with their robust outer shell, protecting the infective sporozoites, makes removing them from the environment very difficult. They can survive a range of temperatures, for example 775 hours at -22°C (Robertson et al., 1992). They resist many commonly used disinfectants and procedures such as chlorination (Quinn & Betts, 1993; Venczel, Arrowood, Hurd & Sobsey., 1997) and so stronger, more concentrated disinfectants are required. Some studies have been done to look at individual disinfectants to inactivate or destroy these oocysts. However, as the conditions between these studies vary

considerably from the age and condition of the oocysts down to concentration and contact time of the disinfectant it is quite difficult to draw a real conclusion as to which is the most effective.

### 1.10.2 Farm Disinfectants

There are some products on the market which claim to reduce or completely eliminate the viability of *Cryptosporidium* oocysts. Some of these studies have been summarised in Table 2 along with the inclusion of some commonly used disinfectants which have poor efficacy, such as iodine or glutaraldehyde-based disinfectants.

**Table 2. Results of various disinfectants viability on *Cryptosporidium* oocysts**

| Disinfectant | Concentration | Effect on *Cryptosporidium* oocysts | Reference |
|---|---|---|---|
| Neopredisan 135-1 | 3% for 1 hour | No effect on oocyst shedding and diarrhoea unless used in combination with Halocur. When used in combination, delayed shedding occurred. | (Keidel & Daugschies, 2013) |
| | <1% for 1 hour | No inhibitory effect on *Cryptosporidium* oocyst shedding. | (Najdrowski et al., 2007) |
| | 4% 1 hour | Significant inhibition of oocyst shedding, although not as good as heating to 55 degrees Celsius. | |
| | 3% 1 hour | Consistently inactivated over 99.5% of *Cryptosporidium* oocysts. | (Shahiduzzaman et al., 2010) |
| | 3% for 2 hours | Inactivated over 99% of oocysts. | |
| KENO™COX | 2% for 2 hours | Lysed 89% of oocysts and resulted in 97.5% reduction in parasite load when mice were infected with the remaining oocysts. | (Naciri et al., 2011) |
| | 3% for 2 hours | Lysed 91% of oocysts and resulted in 100% reduction in parasite load when mice were infected with the remaining oocysts. | |
| Hydrogen Peroxide | 10% for 2 hours | *Cryptosporidium* oocyst inactivation over 99%. | (Delling et al., 2016) |
| | 3% for 30 mins | 94% inactivation of oocysts. | |
| Hydrogen Peroxide based | 10% Ox-virin for 1 hour | 98.6% inactivation of oocysts based on excystation. | (Quilez et al., 2005) |
| Iodine-based | | (Wescodyne) Failed to reduce infectivity of oocysts after 33 min contact time. | (Weir et al., 2002) |
| | 10% | Decreased excystation but unable to reduce infectivity. | (Wilson & Margolin, 1999) |

| | | | |
|---|---|---|---|
| | 13-18mg for 20 mins | Only 10% oocysts inactivated. | |
| Glutaraldehyde-based | 2.5% for 10 hours | *Cryptosporidium* oocysts still viable and infectious. | (Wilson & Margolin, 1999) |
| | 2% | Unable to inactivate oocysts at greater than three logs. | (Barbee et al., 1999) |

The disinfectant with the most evidence for its efficacy at reducing oocyst viability is known as Neopredisan 135-1© (25% chlorocresol, Menno Chemie, Norderstedt, Germany) (Joachim, Eckert, Petry, Bialek & Daugschies., 2003; Najdrowski, Joachim & Daugschies., 2007; Shahiduzzaman, Dyachenko, Keidel, Schmaschke & Daugschies ., 2010). It is recommended that this product is used at a concentration of 3% for 1-hour contact time as lower concentrations do not have a significant effect on the reduction in oocyst viability (Najdrowski et al., 2007). However, these studies were done under laboratory conditions and this product shows reduced efficacy in the field (Keidel & Daugschies., 2013).

KENO™COX, an amine-based disinfectant has been shown to lyse 89% and 91% of *Cryptosporidium* oocysts at 2% and 3% concentration respectively at a 2-hour contact time, along with reducing the parasite load of mice infected with treated oocysts by 97% (Naciri, Mancassola, Fort, Danneels & Verhaeghe., 2011).

Two other disinfectants on the market Progiene Coxicur ® (Progiene Dairy Hygiene) and Cyclex ® (Kilco International Ltd) claim to be effective against *Cryptosporidium* oocysts. However, so far, no scientific research on this has been published.

Hydrogen Peroxide in concentrations 3-6% has long been recognised for its ability to deactivate *Cryptosporidium* oocysts quickly (Delling, Holzhausen, Daugschies & Lender., 2016). Nowadays it is most commonly used in laboratories for use with cleaning and dealing with spillages as it is non-hazardous in these concentrations (Weir, Pokorny, Carreno, Trevors & Lee.,

2002). It has also been shown to dramatically reduce oocyst viability at 3% concentration for 10 minutes when used to sterilise hospital equipment (Barbee, Weber, Sobsey & Rutala., 1999).

In a farm setting, low concentrations of hydrogen peroxide can be used for cleaning and disinfection of pens and farm equipment (Castro-Hermida et al., 2006; Vassal, Favennec, Ballet & Brasseur., 1998) and can also be used to sterilise animals' drinking water due to its antimicrobial properties (Mohammed., 2016). It degrades rapidly in the environment and so is seen as a safe 'environmentally friendly' product to use.

It is important that disinfection is used as part of a multi-step approach to combatting this parasite. It has been shown that disinfection alone, with Neopredisan 135-1© in this case, does not reduce clinical disease or oocyst shedding. However, when disinfection is coupled with halofuginone lactate treatment, there was the complete eradication of both clinical disease and oocyst shedding in calves for the first two weeks of life (Keidel & Daugschies., 2013).

## 1.11 Summary

*Cryptosporidium* is a protozoan parasite with zoonotic potential and a wide host range affecting in particular neonatal calves with a disease known as cryptosporidiosis. The clinical symptoms of this disease are watery diarrhoea, abdominal pain and loss of appetite, although and the disease is most often self-limiting. Occasionally if dehydration is severe, death can occur.

The main source of infection to neonatal calves is currently unknown due to both the lack of research and lack of sensitive diagnostic measures. Sensitive diagnostics and higher discrimination in genotype designation are required to study the epidemiology and transmission of this parasite to calves.

This disease is self-limiting; however, it is currently unknown whether or not suffering from cryptosporidiosis as a neonatal calf will have a long-term effect on the growth of the calf. It has been shown that growth rate is reduced in human children and lambs and so it could be that there is a longer-term impact caused by this parasite on cattle farms.

A few disinfectants are available to help control *Cryptosporidium* oocysts. However, for some of them, their efficacy in a farm setting or even in the laboratory has yet to be proven. There are currently no published studies to compare commercial farm disinfectants on their ability to inactivate *Cryptosporidium* oocysts, so it is quite difficult to be able to advise farmers on which would be the best to use to help manage *Cryptosporidium* contamination in the farm environment

**Aims of the PhD**

**1. Investigating the transmission routes for *Cryptosporidium* to beef and dairy calves.**

Previous work has found that the genotypes of *C. parvum* differ between calves and adult cattle (Thomson, 2015). This may suggest that calves do not acquire *C. parvum* infections from their mothers and it may be acquired from another source. However, this work has only been completed on one farm and so further farms need to be studied before a conclusion can be drawn. The main aim of this work is to investigate the different potential sources of infection for neonatal calves within a dairy and a beef farm.

- ***Cryptosporidium* species and genotypes in adult cattle:**
    - Investigate the species and genotypes of *Cryptosporidium* which are found in adult cattle. Determine if they carry the zoonotic species *C. parvum* and whether or not they could potentially transmit the parasite to their calves.
- ***Cryptosporidium* species and genotypes in calves:**
    - Compare the data from the adult cattle with the species and genotypes of *C. parvum* which are found in their calves, to see if adult cattle could act as a transmission vector to their calves.
    - Compare species and genotypes of *C. parvum* between dairy and beef cattle and calves to determine how the rearing system could have an effect on the genotypes present.

- ***Cryptosporidium* species and genotypes in wildlife:**

    - Investigate the species and genotypes of *Cryptosporidium* which can be found in wildlife that have access to livestock farms to see if wildlife could act as a potential source of oocyst transmission.

**Hypothesis 1:** Adult cattle and calves will have different *C. parvum* genotypes. This would confirm that adult cattle are unlikely to be a significant source of infection for calves.

**Hypothesis 2:** Wildlife will harbour the same *C. parvum* genotypes as those found in the cattle population making them potential candidates for transmission of the parasite.

**2. Determine the effect of cryptosporidiosis on the long-term health and weight gain of beef calves.**

It has been shown that *Cryptosporidium* impairs the growth of children, mice and lambs although there has been no work looking at the long-term effect that infection with this parasite has on the growth of calves. Therefore, the primary aim of this work was to examine the impact of this parasite on the long-term growth of beef calves.

This was achieved by selecting a farm which has a range of clinical cryptosporidiosis which can be clinically scored on the farm. Calves were scored on the farm as regards their clinical presentation of cryptosporidiosis. The highest scoring animals were compared to the lowest scoring animals with regard to the animals' weight change over a time period spanning birth to 6 months. Faecal samples were taken to confirm that these animals are infected with *C. parvum* and whether or not they are suffering from concurrent enteric infections.

**Hypothesis 3:** Calves which have suffered a severe disease from *Cryptosporidium* infection in early life will have reduced growth and it will have a measurable economic impact.

**3. Compare different disinfectants on their ability to inactivate *Cryptosporidium* oocysts**

*Cryptosporidium* oocysts are very hardy and are resistant to many of the commonly used disinfectants on the farm (King & Monis, 2007). Some disinfectants such as Neopredisan 135 - 1 and KENOCOX have been shown to have a good efficacy against the oocysts (Joachim et al., 2003; Naciri et al., 2011) although some newer disinfectants lack data on their effectiveness. Therefore, the aim of this work was to determine the

efficacy of new disinfectants Cyclex, Progiene Coxicur and Steriplex SD+ by comparing them to proven disinfectants Neopredisan, Kenocox and Hydrogen Peroxide and also to a commonly used farm disinfectant FAM-30.

*Cryptosporidium* oocysts could present a challenge for removal in a farm setting due to the fact the oocysts will be mixed with faeces. To examine the effect this could have, one disinfectant Steriplex SD+ underwent tests to determine its efficacy on oocysts contaminated with faeces (dirty conditions).

There is also very limited data on the reduction in efficacy of disinfectants against *Cryptosporidium* oocysts over time. This would be useful to know so that a farmer is aware whether or not a new batch needs to be made up or whether an older one might still be effective. The efficacy of each disinfectant was be examined 7 days post-dilution to assess this.

- Design an experiment to look at the effect of different disinfectants on the viability of *Cryptosporidium* oocysts using excystation. This study included a commonly used iodine-based disinfectant FAM 30; Proven disinfectants KENO$^{TM}$COX, and Hydrogen Peroxide; and unproven disinfectants Steriplex SD+, Progiene Coxicur and Cyclex.

- Compare these disinfectants against their ability to affect the excystation rate of *Cryptosporidium* oocysts in dirty conditions.

- Compare the disinfectant efficacy 7 days post preparation.

**Hypothesis 4:** Different disinfectants have different efficiencies at inactivating *Cryptosporidium* oocysts.

All of these aims give rise to a set of guidelines which can be communicated to the farming community in order to reduce this disease in

their calves which will include potential transmission routes and guidelines for use of disinfectants.

**Hypothesis 5:** Disinfectants will have a reduced efficacy on dirty oocysts compared to clean oocysts.

**Hypothesis 6:** Disinfectants will have a reduced efficacy 7 days post preparation.

# Chapter 2 The Role of Adult Cattle in the Transmission of *C. parvum* to Calves

## 2.1 Introduction

Transmission of *C. parvum* to calves is an area of research that still remains unresolved to the farming and research community. The parasite is passed between hosts via the faecal-oral route and so naive calves must have come into contact with contaminated faecal material to become infected (Ryan, Fayer & Xiao., 2014). This can occur when a calf suckles a dirty udder or teat from its dam, by licking contaminated pen walls, bedding or other calves. Young calves tend to show clinical signs of cryptosporidiosis in the second week of life (Faubert & Litvinsky, 2000; Sanford & Josephson, 1982). This would suggest that transmission occurs immediately or very soon after birth. The idea that adult cattle could be the source of this infection is contentious, with reported prevalence of *Cryptosporidium* in adult cattle ranging from 0 to 71.75% (Atwill & Pereira 2003, Lorenzo Lorenzo et al., 1993). Due to the very low numbers of oocysts found in adult cattle faeces, it has been suggested that adult cattle do not play a significant role in transmission to their calves for this reason (Atwill & Pereira 2003, De Waele et al., 2012). However, given that as few as 25 oocysts are required to infect a calf (Zambrisky et al., 2013), calves being infected by the low numbers of oocysts shed by adult cattle must remain a possibility. Other possibilities for calf transmission include ingestion of oocysts from environmental contamination, as oocysts are left over following an ineffective cleaning or disinfection procedure. *Cryptosporidium* oocysts may also come from wildlife surrounding the farm or from people such as vets and visitors who have brought it from other contaminated farms. It is important to determine the transmission routes of *C. parvum* to calves in order to come up with preventative measures to reduce calf contact with oocysts and therefore reduce and hopefully eliminate the prevalence of cryptosporidiosis on farm.

A study by Faubert and Litvinsky (2000) showed that the shedding of *Cryptosporidium* oocysts in adult dairy cattle significantly increases from 125 to 500 oocysts/gram at parturition from the periparturient period and then significantly decreases post-parturition to 260 oocysts/gram. From these findings, the authors assumed that adult cattle play a significant role in the transmission of the parasite to calves, as the study also showed that calves become infected immediately after birth (Faubert & Litvinsky, 2000). Unfortunately, no matched genotyping of adult and calf oocysts of the parasite was carried out in order to support this assumption. As the calves were removed from their dams 4 hours after birth into an enclosure outside the barn, the possibility remained that the calves were infected from an alternative source.

The publication of Faubert and Litvinsky (2000) contradicted the results by Atwill in 1998 which found no *C. parvum* in adult cattle (Atwill et al., 1998). Atwill then went on to publish another study (Atwill & Pereira, 2003) which showed again the lack of *C. parvum* shedding by adult dairy cattle on three different farms less than 12 hours prior to calving. In this study Atwill & Pereira (2003) used a more sensitive method than the sucrose flotation and Zielh–Nielsen staining that Faubert & Litvinsky (2000) used. The new technique was able to detect 1 oocyst per gram of faeces, which is more sensitive than the 8 oocysts/g in the study by Faubert & Litvinsky (2000). The new method was the use of immune magnetic separation and direct fluorescent-antibody stain (DFA). However, despite the more sensitive method, only a small amount of faecal material was used by Atwill & Pereira (2003). Only 2g of faecal material was used in comparison to the 500g used by Faubert& Litvinsky (2000), which could result in false negative results.

The underlying reason for the dispute in this area is the difficulty of finding oocysts in adult faecal samples. The larger sample volume, reduced oocyst number and fibrous nature of adult cattle faeces makes detection difficult.

Previous studies relied heavily on microscopy which has been demonstrated to be less sensitive than molecular techniques such as PCR (Chalmers et al., 2011). Recently, a sensitive technique using a larger volume of starting material in an acid flocculation (effectively dealing with reduced oocyst number and fibrous nature of the faeces) has been published which uses 50g of faeces in an acid flocculation along with a salt flotation technique. This has a sensitivity of detection of 5 oocysts per gram and allows for a much larger sample to be analysed rather than the few grams used in previous techniques (Wells et al., 2016).

Despite some studies finding *C. parvum* in adult cattle, in most cases these have not been genotyped in order to determine if the genotype in the adults matches the genotype in the calves. This is an essential next step, particularly using multilocus genotyping, in order to determine whether or not adults really play a role in the transmission of *C. parvum* to their calves. One study used GP60 genotyping and the data suggested that the likelihood for adult cattle playing a major role in transmission is very small (Thomson, 2015).This was due to the fact that multiple genotypes were found in the adults, and in only one adult could the genotype that the calves were infected with be detected. This work only looked at a single dairy farm and so further work is required to confirm this conclusion. This work should include using the more sensitive techniques for testing faecal samples from adult cattle along with more discriminative multilocus genotyping of the *C. parvum* positive samples. This would allow for the study of the dynamics of *C. parvum* transmission between adult cattle and calves.

## 2.2 Aims

1. Assess prevalence of *C. parvum* in adult dairy and beef cattle

2. Determine and compare the species and genotypes of *Cryptosporidium* in adult cattle and their calves

3. Assess how species and genotypes change in pre-weaned dairy calves from birth over a 6-week period

4. Determine the role that adult cattle could play in the transmission of *C. parvum* to their calves

5. Compare dairy and beef rearing systems with regard to species, genotypes and role of adult cattle in *C. parvum* transmission

# 2.3 Materials and Methods

## 2.3.1 Farm and Animal Selection

### Dairy Farm

The participating dairy farm which was chosen had a history of clinical cryptosporidiosis in calves, and is located in Midlothian, Scotland. Male calves were not included in this study. Adult cattle that were due to have a calf within the next 2-3 weeks were housed together indoors. Faecal samples from these cows were taken between 2-3 weeks prior to calving and 1-4 samples were taken per adult during this time period (137 samples from 79 cows). The number and timing of samples per cow can be seen in Appendix 1.

Once a female calf was born, it was removed from the mother within an hour and kept in stone pens (Figure 2). These calves were tube fed pooled colostrum obtained from cows (excluding heifers) on the farm and were not allowed to suckle from their mother. At 2 – 3 days old the calves were moved into a group pen (Figure 2) where they were housed until they were 4 – 6 weeks of age, before moving into a separate group pen.

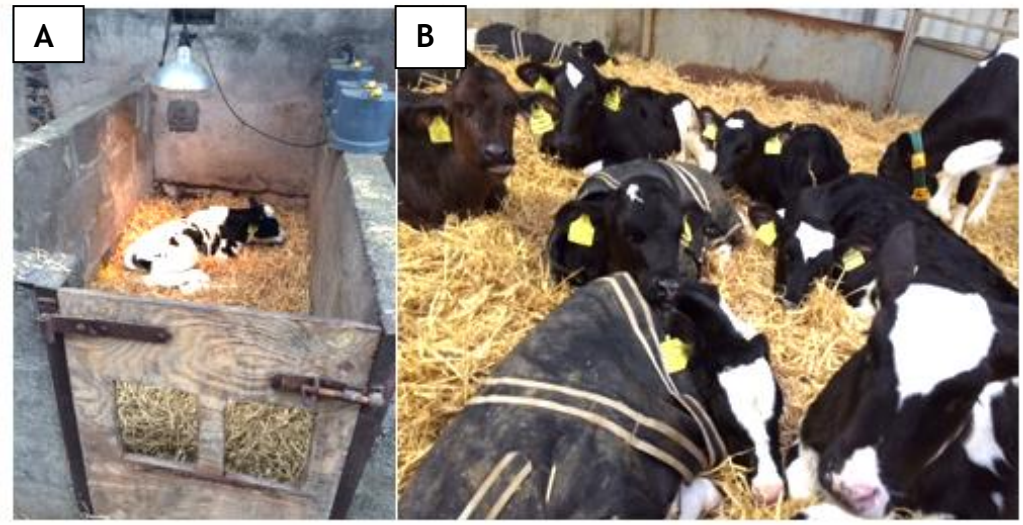


**Figure 2 Calf housing on the dairy farm. Individual calf pen (A) where calves are kept for 2 – 3 days following birth and group calf pen where calves are housed until 4 – 6 weeks of age (B)**

Faecal samples from these calves were collected directly from the rectum using both a gloved finger and a collecting bag. The entire faecal sample was collected and stored at 4 °C until the samples could be processed.

**Beef Farm**

Beef calves were located on a beef suckler herd farm in Perthshire, Scotland. Here the calves were housed individually with their mother until at least 48 hours of age and the calf had been visualised suckling, afterwards, they were housed in group pens with their mothers. Calves included both males and females ranging from 48 hours to 3 months of age. At 3 months, adult cattle and their calves are moved to pasture.

Calves included in this study were sampled by observing the animals and collecting, where possible, the entire faecal sample from the ground. This study included 28 adult Belgian Blue female cattle and 23 of their Belgian Blue x Limousin, mixed gender calves which were housed together.

## 2.3.2 Sample Collection and Processing

**Adult Cattle**

**Dairy Farm**

125g of faecal material was taken from a fresh pat on the ground. One hundred and thirty-seven samples were collected from 79 different cows between 26th October 2015 and 22nd December 2015. Faecal samples from adult cattle only were thoroughly mixed using a wooden spatula and processed using acid flocculation and salt flotation described below before performing DNA extraction.

**Beef Farm**

Samples from 28 cattle, one sample per animal, were collected using 125ml pots from faecal pats on the ground at a single time point, after all of the calves in this study were born. The collection was completed during May 2016. Both adult cattle and calves were housed in the same barn during the time of sampling. The difference in sampling between the dairy and beef farms in this case was due to the decision to compare the two farms being made after the completion of the dairy analysis.

**Calves**

**Dairy Farm**

Faecal samples were collected from the calves (n=38) three times a week for the first 3 weeks of life. Samples were then taken twice a week for weeks 4, 5 and 6 of life. In total this amounted to 329 faecal samples from 38 animals. This was done either from fresh faecal pats on the ground or, if the animal suffered diarrohea, directly from the rectum. Occasionally it was not possible to collect a sample from a particular calf. This is the case for week 2 where three calves could not be sampled, week 3 where one calf could not be sampled and week 4 where three calves could not be sampled. Faecal samples which were collected from the calves were thoroughly mixed inside the plastic bag they were collected in before either 200 µl (watery samples) or the top of an inoculation loop (solid samples) of faeces was removed and added to 1 ml TE buffer (10 mM Tris-HCl, 0.5 mM EDTA). Here the samples went into the DNA extraction step described in 3.3.5.

**Beef Farm**

Faecal samples were collected from pats on the ground from 23 beef calves of mixed gender at the same time as samples from the beef adult cattle

were collected during a single time point in May 2016 (2.3.2 Adult Cattle – Beef). The calves selected for this study ages ranged from 0 – 4 weeks and showed a mixed range of symptoms. These samples were thoroughly mixed inside the plastic bag they were collected in before either 200 µl or a loop of faeces was removed and added to 1 ml TE buffer (10 mM Tris-HCl, 0.5 mM EDTA). Here the samples went into the DNA extraction step described in 3.3.5.

### 2.3.3 Acid Flocculation

The protocol used for processing adult cattle samples for *Cryptosporidium* oocyst concentration was performed as previously described (Wells et al., 2016). This protocol involved weighing 50 g of the faecal sample before adding it to a 1-litre glass cylinder, along with 700 ml $H_20$ and 7ml of 2% $H_2SO_4$ in $dH_20$. The sample was mixed on a magnetic stirrer for 5 minutes before being left to settle for 20-30 minutes until two to three clear layers, the sediment at the bottom, the supernatant in the middle and sometimes a fatty deposit layer at the top, could be seen. Once settled, the middle layer which contained the suspended oocysts was taken with a glass pipette and this was centrifuged at 1100 x g for 20 minutes to create a pellet. Following this, a wash step to transfer the oocysts from a 250 ml container to a 15 ml tube involved washing the container 3 times with 3 ml $H_20$ and moving this to the 15 ml tube. A centrifugation step of 3000 x g for 5 minutes created a pellet containing the oocysts and the supernatant was discarded.

### 2.3.4 Salt Flotation

The pellet obtained from the acid flocculation was used in a salt flotation protocol as previously described (Chalmers et al., 2009a). This involved suspending the pellet in 8 ml of saturated salt followed by trickling 2 ml of $H_20$ on top. The samples were then centrifuged at 1000 x g for 8 minutes and the oocysts were removed by creating a vortex with a pastette and

adding these oocysts to 6 ml $H_20$ in a 15 ml tube. Samples were centrifuged at 1000 x g for 5 minutes. The pellet, containing the purified oocysts, was re-suspended in 1ml TE buffer ready for DNA extraction. Samples were then processed directly for DNA extraction.

### 2.3.5 DNA Extraction

Before DNA was extracted from both the adult cattle and calf samples, they underwent 10 x freeze-thaw cycles in liquid nitrogen in order to break the hard shell of the oocyst and release the parasite DNA. DNA was then extracted according to the manufacturer's protocol for DNA extraction using the NucleoSpin Tissue DNA, RNA and protein purification kit (Macherey-Nagel, NZ740952250).

In short, the sample is centrifuged in TE Buffer at 4,000 x g for 15 minutes and the supernatant discarded. 200 µl Buffer T1 was added and the pellet re-suspended. Samples underwent 10 freeze and thaw cycles in liquid nitrogen (alternating between liquid nitrogen and a 56°C water bath) before 25 µl Proteinase K was added to each sample. Samples were mixed and incubated at 56°C in a water bath overnight.

200 µl Buffer B3 was added and mixed before a further incubation at 70°C for 10 minutes. Samples were centrifuged at 11,000 x g for 5 minutes to remove insoluble particles and the supernatant added to 210 µl of 100% ethanol. The whole sample was then added to each spin column before centrifugation at 11,000 x g for 1 minute. The flow-through in the collection tube was discarded, and the column was replaced back into the collection tube. 500 µl Buffer BW was added and the sample centrifuged at 11,000 x g for 1 minute, discarding the flow-through from collection tubes. 600 µl Buffer B5 was added, samples centrifuged at 11,000 x g for 2 minutes and the flow-through discarded. DNA was then eluted from the membrane by addition of 100 µl $dH_20$, before the final centrifugation at 11,000 x g for 1 minute. DNA was then stored in the freezer at -20°C.

### 2.3.6 18S PCR Amplification

*Cryptosporidium* in cattle was determined using a nested species-specific PCR which amplified the 18S region (Thomson et al., 2016) and allowed the identification of the common cattle species *C. parvum, Cryptosporidium bovis, Cryptosporidium ryanae* and *Cryptosporidium andersoni*. Samples were run in triplicate with a negative control, a DNA extraction control and positive controls for all species in the PCR. The first round reactions were carried out as described previously (Xiao, Alderisio, Limor, Royer & Lal., 2000) using primers in Table 3. Second round reactions were carried out with all four individual species-specific forward primers (Table 3), along with the reverse 18S rRNA primer (AL3032; Table 3). First round PCR products were diluted with 50 µL $dH_2O$ and 1 µL of the product or diluted product was used as a template in a reaction containing 2·5 µL 10× PCR buffer (45 mm Tris–HCL pH 8·8, 11 mm $(NH_4)_2SO_4$, 4·5 mm MgCl2, 4·4 µ m EDTA, 113 µg mL−1 BSA, 1 mm of each of the four deoxyribonucleotide triphosphates (DNTP's), 0·5 unit BioTaq (Bioline, UK) and 10 µm forward and reverse primers. DNA from adult cattle faecal samples was not diluted between first and second round due to the low numbers of oocysts present. The final volume of each reaction was made up to 25 µL using $dH_2O$.

The conditions for the PCR were 3 minutes at 94 ° C, followed by 35 cycles of 45 seconds at 94 °C, 45 seconds at 55 °C and 1 minute at 72 °C. Lastly, there is a 7-minute extension step at 72 °C. The PCR products were then run on a 1.5 % agarose gel, along with the 100bp Promega ladder (Cat No.G210A), using gel electrophoresis and stained with GelRed™ (Biotium, UK). The gel was then examined under UV light using an AlphaImager 2000 and band size estimated.

**Table 3 Forward and reverse PCR primers for 18S, Nested species specific PCR and GP60 PCR. Reverse second round primer AL3032 is used for the reverse primer when nested species specific PCR is being used. Amplicon sizes for *C. hominis* / *C. parvum* (305bp), *C. bovis* (241 bp), *C. ryanae* (415 bp), *C. andersoni* (625 bp).**

| | Primary | Secondary |
|---|---|---|
| 18S | AL1687:<br>TTC TAG AGC TAA TAC ATG CG (20bp) | AL1598:<br>GGA AGG GTT GTA TTT ATT AGA TAA AG (26bp) |
| | AL1691:<br>CCC ATT TCC TTC GAA ACA GGA (21bp) | Primer AL3032:<br>AAG GAG TAA GGA ACA ACC TCC A (22bp) |
| | | 2° Product size 18S: 840bp |
| | Forward Primers Second Round | |
| nssm 18S | *C. hominis* / *C. parvum*:<br>AGAGTGCTTAAAGCAGGCATA (21bp) | *C. bovis* :<br>CTTCTTATTGGTTCTAGAATAAAAATG (27bp) |
| | *C. ryanae*<br>TGTTAATTTTTATATACAATRCTACGG (27bp) | *C. andersoni*<br>GCAAATTACCCAATCCTGAC (20bp) |
| | Primary | Secondary |
| GP60 | Forward:<br>ATAGTCTCCGCTGTATC | Forward:<br>TCCGCTGTATTCTCAGCC |
| | Reverse:<br>GAGATATATCTTGGTGCG | Reverse:<br>CGAACCACATTACAAATGAAG |
| | 1° Product size 18S: 480bp | 2° Product size 18S: 375bp |

### 2.3.7 GP60 PCR amplification

For all *C. parvum* positive samples, the GP60 gene was amplified by PCR and the positive products were submitted for sequencing. This was done by using a nested PCR (Brook et al., 2009). Each well on the PCR plate contained 10 x PCR buffer (45mM Tris-HCl pH8.8, 11 mM (NH4)2SO4, 4.5 mM MgCl2, 4.4 µ EDTA, 113 µg ml-1 BSA and 1mM each of four dNTP's), 0.5 units BioTaq, 10µM each of forward and reverse primers (Table 3). The cycling conditions were the same as for the 18S PCR with a positive (DNA from *C. parvum* oocysts) and negative ($dH_20$) control added. The PCR products were then run on a 1.5 % agarose gel, along with the 100bp Promega ladder (Cat No.G210A), using gel electrophoresis and stained with GelRed™ (Biotium, UK). The gel was then examined under UV light using an AlphaImager 2000 before positive products were submitted for sequencing.

### 2.3.8 Sequencing

Positive PCR amplicons for GP60 PCR (3.3.7) were submitted to MWG Eurofins for sequencing after DNA was purified using the Qiagen PCR purification kit (Qiagen, Cat No28104 (50)). DNA concentration of purified PCR products was quantified using a NanoDrop ND1000 spectrophotometer (Labtech International Ltd, UK).

Sequences were analysed using the SeqManPro program as part of the DNA Star Lasergene 12 core suite (https://www.dnastar.com) and BioEdit Sequence Alignment Software (www.mbio.ncsu.edu/bioedit/bioedit.html).

### 2.3.9 Microsatellite PCR

Microsatellite analysis was carried out on DNA samples which were positive for *C. parvum* by PCR. Four microsatellite markers which were previously described (Brennan, 2009; Mallon, MacLeod, Wastling, Smith & Tait., 2003; Morrison et al., 2008) were used to identify alleles and assign multilocus genotypes to each sample. The reaction included 10 x PCR Buffer (described in 3.3.6), 0.5 units of BioTaq and 10 µM of forward and reverse

primers (Table 4). The primary product was diluted 1:100 before 1µl was added to the second round. A positive control for each microsatellite and negative control was added to each PCR plate.

**Table 4 Forward and reverse primers for microsatellite analysis.**

| | | |
|---|---|---|
| MM5 | Primary Forward<br>TCACAAGTTACCCCTTCTGATGCTG | Primary Reverse<br>TCCACCTCCGGATTGGTTGTG |
| | Secondary Forward<br>CCTGGACTTGGATTTGGACTTACACC | Secondary Reverse<br>GGAGAAGATAAGCTAGCCGAATCT (FAM labelled) |
| MM18 | Primary Forward<br>GTTCAGCTGATACGGGTTTGCAACA | Primary Reverse<br>CATCACCATCTCCTCCGCCAGA |
| | Secondary Forward<br>CTTTCTGGAGGGTTTGTTCCTCC (FAM labelled) | Secondary Reverse<br>CTTCCTGATGATCCAGGCCAAGC |
| MM19 | Primary Forward<br>TGGTTTTAGCTAAGGAAGCGATAG | Primary Reverse<br>CTGCTGCTGCTGTTGCTTTA |
| | Secondary Forward<br>GATTCTGTCAACTTTGAATTCAG (FAM labelled) | Secondary Reverse<br>CCAACCCCGAATTCATTTCCAAC |
| TP14 | Primary Forward<br>GAGAAGGAGCAATGGGAGCA | Primary Reverse<br>TCCTCCTTTTTGCCCTTGAA |
| | Secondary Forward<br>CTAACGTTCACAGCCAACAGTACC (FAM labelled) | Secondary Reverse<br>CAATAAAGACCATTATTACCACC |

**Fragment Analysis**

PCR products underwent fragment analysis (Applied Biosystems; University of Dundee) using Genescan ROX500 as a size standard and the results analysed using STRand (https://www.vgl.ucdavis.edu/informatics/strand.php).

The maximum peak was recorded as the primary fragment size and secondary peaks recorded if there were over one third of the size of the primary peak (Hotchkiss et al., 2015). Only primary peaks are used for the visual representation of the multilocus genotype which was done using Phyloviz (http://www.phyloviz.net).

# 2.4 Results

## 2.4.1 Species of *Cryptosporidium* in Adult Cattle

**Dairy Farm**

Of the 137 adult cattle samples taken from 79 adult cattle prior to their calving, 35.8 % (49/137) were positive for *C. parvum*, 18.2 % (25/137) had a mixed infection of *C. parvum* and *C. andersoni*, 8.0 % (11/137) had *C. andersoni*, 0.7% (1/137) had *C. ryanae*. 37.2 % (51/137) were negative for the four common species of *Cryptosporidium* found in cattle (Figure 3).

Looking at individual animals, 32.9 % (26/79) were positive for *C. parvum*, 32.9 % (26/79) had a mixed infection of *C. parvum* and *C. andersoni*, 8.9 % (7/79) had *C. andersoni*, 1.3 % (1/79) had *C. parvum* and *C. ryanae* mixed infection and 37.2 % (19/79) were negative for the four common species of *Cryptosporidium* found in cattle (Figure 4).

**Beef Farm**

Of the 27 samples collected from 27 cattle, 55.6 % (15/27) had *C. parvum*, 7.4 % (2/27) had *C. andersoni* and one animal had a mix of *C. parvum, C. andersoni, C. bovis* and *C. ryanae*. 33.3 % (9/27) animals were negative for the four common species of *Cryptosporidium* found in cattle (Figure 3).

The most prevalent *Cryptosporidium* species detected in individual adult cattle on both the dairy and the beef farm was *C. parvum*, followed by *C. andersoni* present either on its own or as a mixed infection.

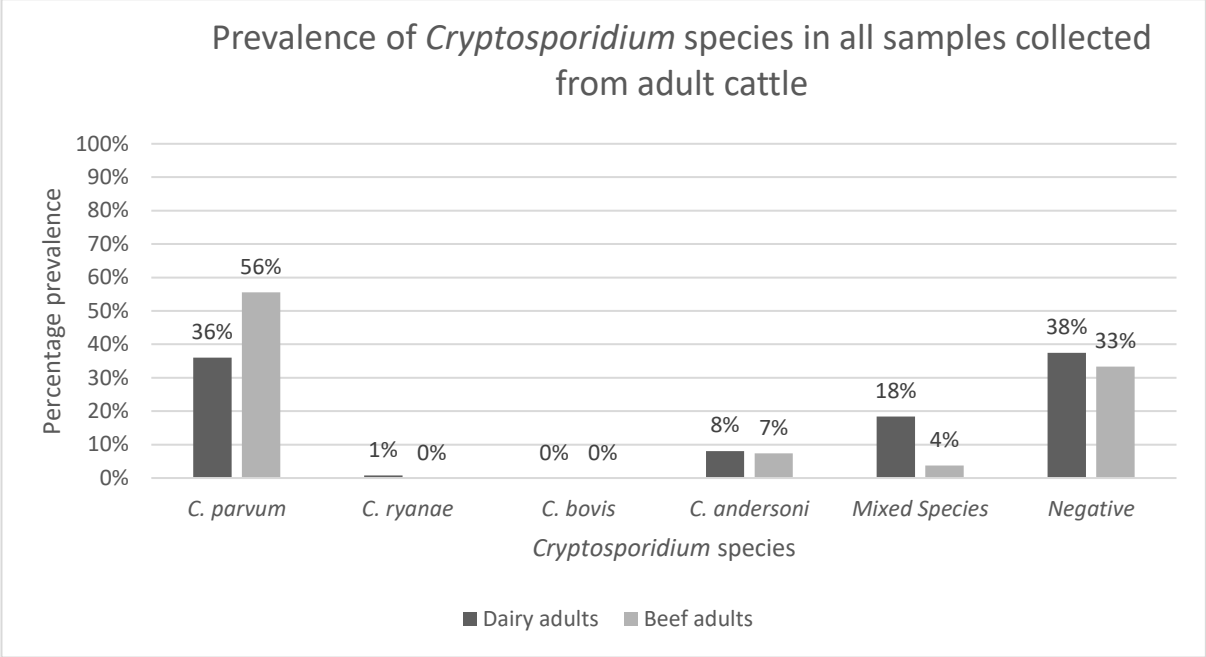


**Figure 3 – Bar chart showing the species of *Cryptosporidium* found in adult cattle from a dairy farm (dark grey) and a beef farm (light grey) where all collected faecal samples (multiple samples per cow) are described in the results.**

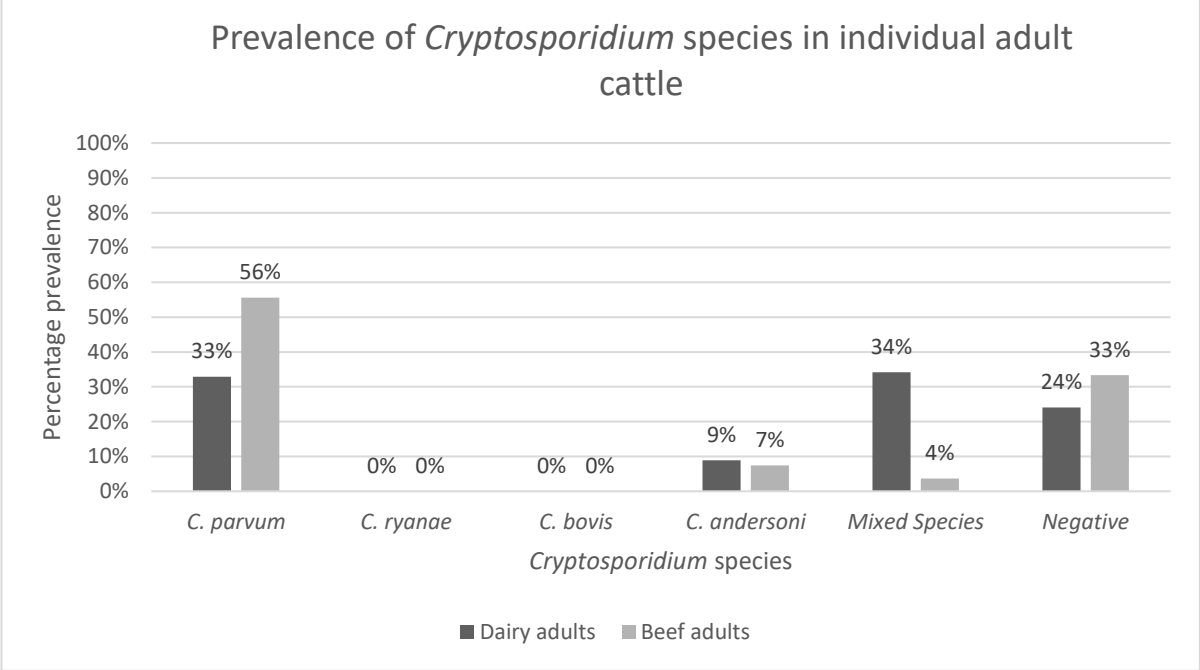


**Figure 4 – Bar chart showing the species of *Cryptosporidium* found in adult cattle from a dairy (dark grey) and a beef (light grey) farm where samples from individual animals only are described in the results.**

## 2.4.2 GP60 Genotypes in Adult Cattle

**Dairy Farm**

Those adult cattle samples that were positive for *C. parvum* (74/137) underwent subtyping using the GP60 gene. Of those 74 adult cattle, 41% (30/74) had the genotype IIaA15R1, 30% (22/74) had the genotype IIaA15G2R1, 3% (2/74) of adult cattle had IIcA5G3 (5/74) animals had IIaA17G1R1. A single animal was positive for IIaA17G2R1 and another for IIaA16G1R1 (Figure 5). Amplification of this gene failed in 13 of the *C. parvum* positive animals.

The most common GP60 genotype on the dairy farm was IIaA15R1 with 41% closely followed by IIaA15G2R1 at 30%.

**Beef Farm**

Seventeen adult cattle were positive by PCR for the species *C. parvum* on the beef farm, and so these underwent GP60 genotyping. The predominant GP60 genotype was IIaA17G1R1 which was present in 69% (11/16) of the adult cattle. IIaA15R1, IIaA19G2R1, IIaA16G3R1 were found in 19% (3/16), 6% (1/16) and 6% (1/16) of the adult cattle respectively (Figure 5).

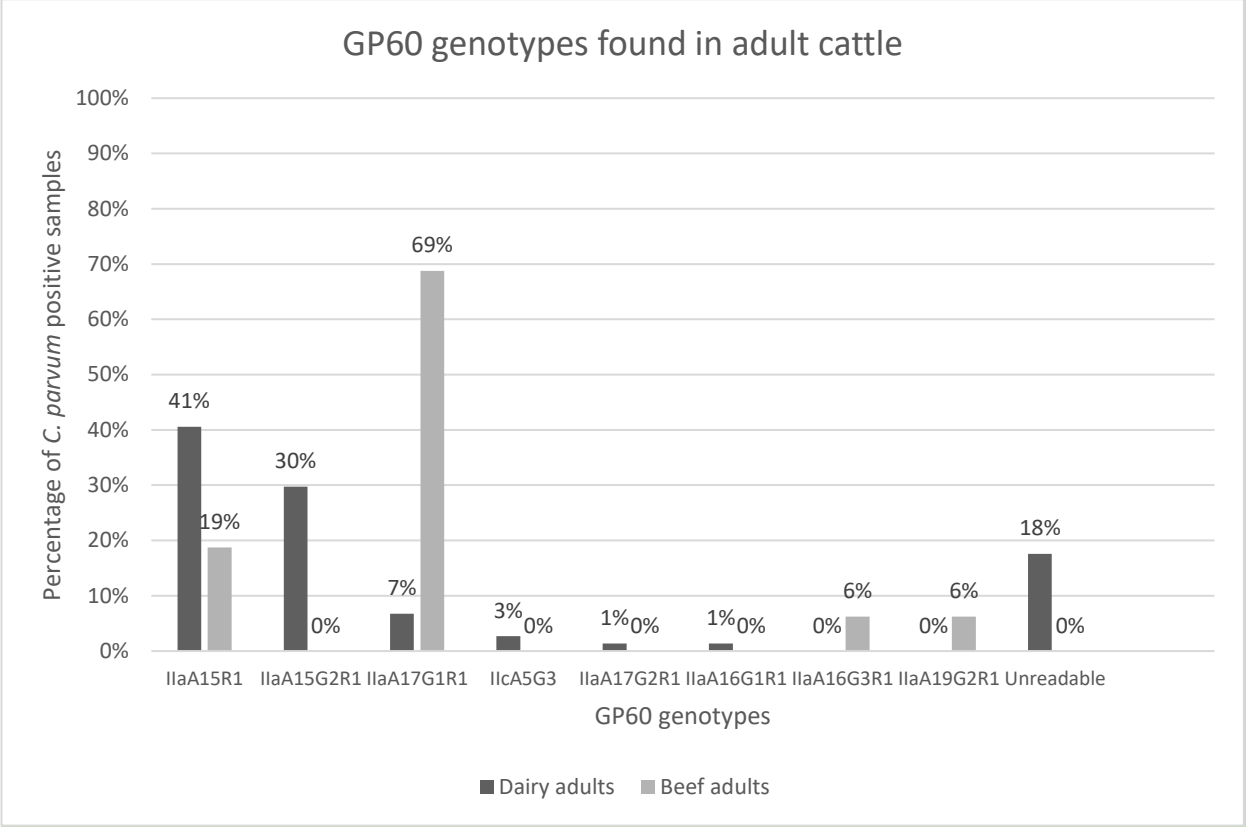


**Figure 5 – Bar chart showing the *C. parvum* GP60 genotypes found in 136 adult cattle faecal samples on a dairy and beef farm.**

## 2.4.3 Species of *Cryptosporidium* in Calves

### Dairy Farm

Calves on the dairy farm were tested for *Cryptosporidium* species from birth until they reached 6 weeks of age (Figure 6 and Figure 7).

In week 1, 25/38 calves were positive for *C. parvum*. In week 2, 23/35 calves were positive for *C. parvum* with one calf showing a *C. parvum* and *C. ryanae* mixed infection. In week 3, 23/37 calves were positive for *C. parvum* with one animal showing a *C. parvum* and *C. bovis* mixed infection and two showing a showing a *C. parvum* and *C. ryanae* mixed infection. In week 4 10/35 calves were positive for *C. parvum*. Two calves were positive for *C. bovis*, 3 calves for *C. ryanae*. One calf was positive for *C. ryanae* and *C. bovis* as a mixed infection, three calves had a *C. parvum* and *C. bovis*

mixed infection and one a *C. parvum* and *C. ryanae* mixed infection. Calves at 5 weeks had 16/38 positive for *C. parvum*, one for *C. bovis* and two calves for *C. ryanae*. Four calves had a *C. parvum* and *C. bovis* mixed infection and two calves a *C. parvum* and *C. ryanae* mixed infection. In week 6, 7/38 calves were positive for *C. parvum*, two for *C. bovis*, and four for *C. ryanae*. There was also one *C. parvum* and *C. bovis* mixed infection. Raw data and individual calf results can be seen in Table 5.

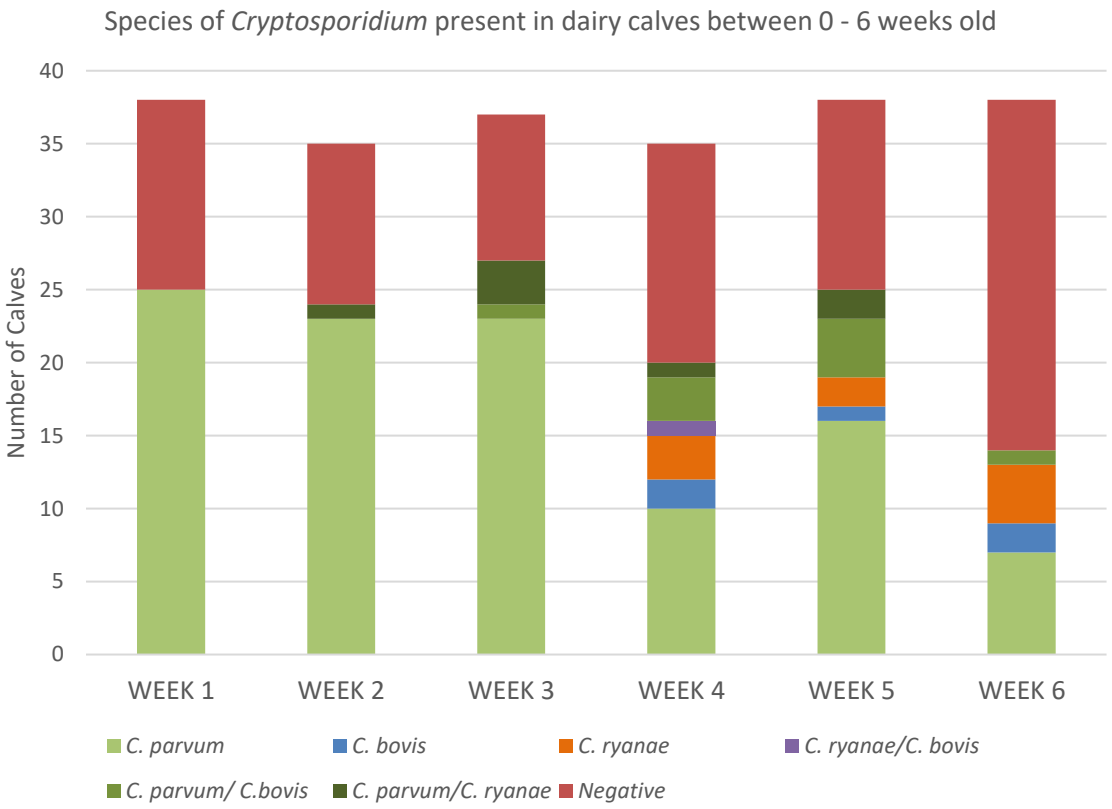


**Figure 6 - Bar chart showing the species of *Cryptosporidium* found in 38 dairy calves from 0 - 6 weeks old, based on sampling 3 times per week for the first three weeks and twice per week for the following three weeks. Results displayed for individual calves.**

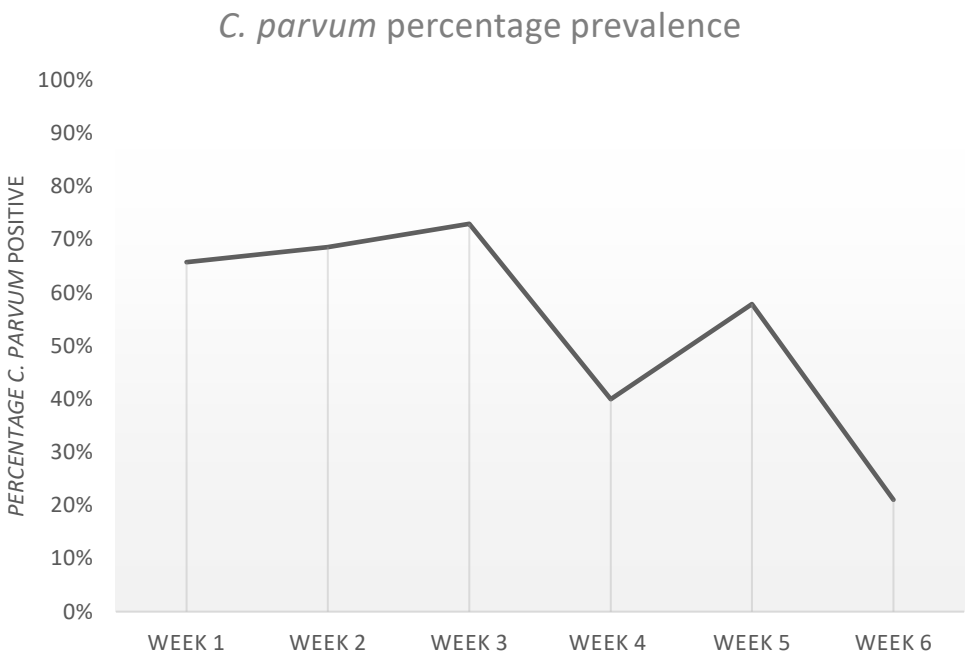


**Figure 7 – Line graph showing the percentage of total calves shedding *C. parvum* in 38 dairy calves from birth to 6 weeks of age.**

**Table 5 Species of *Cryptosporidium* found in calves over 6 weeks of life along with the result obtained from their mother. Symptomatic calves are highlighted in grey.**

| Calf | Mother Result | Born | WEEK 1 | WEEK 2 | WEEK 3 | WEEK 4 | WEEK 5 | WEEK 6 |
|---|---|---|---|---|---|---|---|---|
| **1** | *C. parvum* *C. andersoni* | 30/10/2015 | NEG | NEG | NEG | NEG | NEG | *C. parvum* |
| **2** | *C. parvum* *C. andersoni* | 06/11/2015 | NEG | NEG | **x** | *C. bovis* | *C. parvum C. ryanae* | NEG |
| **3** | *C. parvum* | 06/11/2015 | NEG | NEG | *C. parvum* | *C. parvum* | *C. parvum C. bovis* | NEG |
| **4** | *C. parvum* *C. andersoni* | 06/11/2015 | NEG | NEG | *C. parvum* | NEG | *C. parvum C. bovis* | NEG |
| **5** | NEG | 06/11/2015 | *C. parvum* | **x** | *C. parvum* | NEG | *C. parvum* | NEG |
| **6** | *C. parvum* | 09/11/2015 | NEG | *C. parvum* | *C. parvum* | *C. parvum* | *C. parvum* | NEG |
| **7** | NEG | 09/11/2015 | NEG | *C. parvum* | *C. parvum* | **x** | *C. parvum* | NEG |
| **8** | *C. parvum* | 09/11/2015 | NEG | *C. parvum* | *C. parvum* | NEG | *C. parvum* | *C. parvum* |
| **9** | *C. parvum* | 13/11/2015 | NEG | *C. parvum* | NEG | NEG | NEG | NEG |
| **10** | *C. parvum* *C. andersoni* | 16/11/2015 | *C. parvum* | NEG | NEG | *C. bovis C. ryanae* | *C. parvum* | NEG |
| **11** | *C. parvum* *C. andersoni* | 16/11/2015 | *C. parvum* | NEG | *C. parvum* | NEG | NEG | NEG |
| **12** | *C. andersoni* | 16/11/2015 | NEG | **x** | NEG | NEG | *C. parvum* | NEG |
| **13** | *C. parvum* | 16/11/2015 | *C. parvum* | NEG | *C. parvum C. bovis* | *C. parvum* | *C. parvum* | NEG |
| **14** | *C. parvum* | 20/11/2015 | *C. parvum* | **x** | *C. parvum* | *C. parvum* | *C. parvum* | *C. bovis* |
| **15** | *C. andersoni* | 25/11/2015 | *C. parvum* | *C. parvum* | *C. parvum* | NEG | NEG | NEG |
| **16** | *C. parvum* *C. andersoni* | 27/11/2015 | *C. parvum* | NEG | *C. parvum* | *C. parvum* | NEG | *C. parvum* |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **17** | Andersoni | 27/11/2015 | *C. parvum* | NEG | NEG | NEG | NEG | NEG |
| **18** | *C. parvum* *C. andersoni* | 27/11/2015 | *C. parvum* | *C. parvum* | *C. parvum* | *C. parvum* | *C. parvum* | *C. parvum* |
| **19** | *C. andersoni* | 30/11/2015 | *C. parvum* | *C. parvum* | *C. parvum* | NEG | NEG | NEG |
| **20** | *C. parvum* *C. andersoni* | 09/12/2015 | *C. parvum* | *C. parvum* | *C. parvum* | *C. parvum* | *C. parvum* | *C. ryanae* |
| **21** | NEG | 09/12/2015 | *C. parvum* | *C. parvum* | NEG | NEG | NEG | *C. ryanae* |
| **22** | NEG | 09/12/2015 | NEG | *C. parvum* | NEG | *C. parvum* | *C. parvum* | NEG |
| **23** | *C. parvum* *C. andersoni* | 14/12/2015 | *C. parvum* | *C. parvum* | *C. parvum* | *C. ryanae* | *C. parvum* | *C. bovis* |
| **24** | *C. parvum* *C. andersoni* | 14/12/2015 | *C. parvum* | *C. parvum* | NEG | *C. parvum* | *C. parvum C. bovis* | NEG |
| **25** | *C. parvum* *C. andersoni* | 22/12/2015 | *C. parvum* | NEG | *C. parvum* | NEG | *C. parvum* | NEG |
| **26** | *C. parvum* | 22/12/2015 | *C. parvum* | *C. parvum* | *C. parvum C. ryanae* | *C. parvum C. bovis* | *C. parvum C. bovis* | *C. parvum* |
| **27** | *C. parvum* *C. andersoni* | 30/12/2015 | *C. parvum* | *C. parvum* *C. ryanae* | NEG | Bovis | NEG | NEG |
| **28** | NEG | 30/12/2015 | *C. parvum* | NEG | NEG | *C. parvum* | *C. parvum* | NEG |
| **29** | *C. parvum* *C. andersoni* | 30/12/2015 | *C. parvum* | *C. parvum* | *C. parvum* | **x** | *C. ryanae* | *C. parvum* |
| **30** | *C. parvum* | 02/01/2015 | *C. parvum* | *C. parvum* | *C. parvum* | *C. ryanae* | NEG | *C. ryanae* |
| **31** | *C. parvum* | 03/01/2015 | *C. parvum* | *C. parvum* | *C. parvum* | *C. parvum C. ryanae* | NEG | *C. ryanae* |
| **32** | *C. parvum* | 03/01/2015 | *C. parvum* | *C. parvum* | *C. parvum* | *C. parvum C. bovis* | *C. parvum* | *C. parvum* |
| **33** | *C. parvum* *C. andersoni* | 04/01/2015 | *C. parvum* | *C. parvum* | *C. parvum* | NEG | NEG | NEG |
| **34** | NEG | 05/01/2016 | NEG | *C. parvum* | *C. parvum* | **x** | NEG | NEG |
| **35** | *C. parvum* | 11/01/2016 | *C. parvum* | *C. parvum* | *C. parvum* | NEG | *C. ryanae* | NEG |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **36** | NEG | 11/01/2016 | NEG | *C. parvum* | *C. parvum C. ryanae* | *C. ryanae* | *C. bovis* | NEG |
| **37** | *C. parvum* | 11/01/2016 | *C. parvum* | *C. parvum* | *C. parvum* | NEG | *C. parvum C. ryanae* | NEG |
| **38** | *C. parvum* | 11/01/2016 | NEG | *C. parvum* | *C. parvum C. ryanae* | *C. parvum C. bovis* | *C. parvum* | *C. parvum C. bovis* |

**Beef Farm**

Calves on the beef farm had a single sample tested when the animal was between 0 – 4 weeks of age. Samples from 23 beef calves gave a prevalence of 100% (23/23) for *C. parvum*. No other species of *Cryptosporidium* were found at this age.

## 2.4.4 GP60 Genotypes in Calves

**Dairy Farm**

The first sample which contained *Cryptosporidium* oocysts (if any) from each of the 38 calves within the first two weeks of life was chosen to undergo GP60 subtyping as if calves were infected with *Cryptosporidium* oocysts from their mothers then this is likely to be detected in the first sample with oocysts. If calves were infected by oocysts shed by their mothers it would be expected that they would share the same genotype of *C. parvum*. Of the 34 positive calves (34/38), 100% had the GP60 genotype IIaA15G2R1.

**Beef Farm**

All *C. parvum* positive samples underwent GP60 genotyping and 100% of 23 (23/23) calves on the beef farm had the genotype IIaA17G1R1.

## 2.4.5 Microsatellite Analysis

Amplification of microsatellite loci MM5, MM18, MM19, and TP14 occurred when the sample was both positive for *C. parvum* and the GP60 gene (Table 8).

An allele number was assigned for each locus (Table 6), which was based on previous data (Hotchkiss et al., 2015) and added to as more results were

collected during the course of this PhD. Multilocus genotypes were assigned according to Table 7.

**Table 6 Allele assignment based on fragment size (base pairs (bp)) of each locus.**

| Allele | MM5 | MM18 | MM19 | TP14 |
|---|---|---|---|---|
| 1 | 262 bp | 288 bp | 298 bp | 296 bp |
| 2 | 235 bp | 294 bp | 304 bp | 304 bp |
| 3 | 225 bp | 318 bp | 292 bp | 252 bp |
| 4 | | 412 bp | 316 bp | |
| 5 | | | 270 bp | |
| 6 | | | 253 bp | |
| 7 | | | 281 bp | |
| 8 | | | 285 bp | |
| 9 | | | 495 bp | |
| 10 | | | 222 bp | |

**Table 7 Multilocus genotype assignment based on alleles assigned for each locus and GP60 result.**

| MLG | GP60 | MM5 | MM18 | MM19 | TP14 |
|---|---|---|---|---|---|
| 1 | llaA15G2R1 | 2 | 1 | 1 | 2 |
| 2 | llaA15G2R1 | 2 | 2 | 1 | 2 |
| 3 | llaA15G2R1 | 2 | 1 | 1 | 1 |
| 4 | llaA15G2R1 | 2 | 1 | 3 | 2 |
| 5 | llaA15G2R1 | 2 | 2 | 3 | 2 |
| 6 | IIaA17G2R1 | 2 | 1 | 1 | 1 |
| 7 | IIaA15R1 | 2 | 1 | 1 | 1 |
| 8 | IIaA15R1 | 2 | 1 | 1 | 2 |
| 9 | IIaA15R1 | 2 | 1 | 3 | 1 |
| 10 | IIaA17G1R1 | 2 | 1 | 3 | 1 |
| 11 | IIaA17G1R1 | 2 | 1 | 10 | 1 |
| 12 | IIaA19G2R1 | 2 | 1 | 8 | 1 |
| 13 | IIaA15R1 | 2 | 1 | 8 | 1 |
| 14 | IIaA17G1R1 | 2 | 4 | 3 | 1 |
| 15 | IIaA16G3R1 | 2 | 1 | 3 | 1 |

| | | | | | |
|---|---|---|---|---|---|
| 16 | IIaA17G2R1 | 1 | 1 | 3 | 1 |
| 17 | IIaA15G2R1 | 1 | 1 | 8 | 1 |
| 18 | IIaA17G2R1 | 2 | 1 | 8 | 1 |
| 19 | IIaA15G2R1 | 2 | 1 | 3 | 1 |
| 20 | IIaA15G2R1 | 2 | 1 | 8 | 1 |
| 21 | IIaA17G1R1 | 1 | 1 | 8 | 1 |
| 22 | IIaA15G2R1 | 2 | 1 | 3 | 4 |
| 23 | IIaA17G1R1 | 2 | 1 | 9 | 1 |

**Dairy Farm**

The majority of the calves on the dairy farm (28 out of 35 positive calves) shared a single multilocus genotype (MLG 1), which was detected in only 5.3 % (2/38) of the adult cattle. One calf was positive for MLG 2, one for MLG 3, one for MLG 4 and one for MLG 5. Two calves failed to be fully genotyped at all regions, although one of these was most similar to MLG 1 at 3 out of 3 analysed regions. The majority of the *C. parvum* positive adult cattle, which amplified at all loci, presented with MLG 9 (12/19) which was different to MLG 1 seen in the calves. MLG 9 is different from MLG 1 in 3 out of the 5 loci genotyped. Adult cattle also presented with MLG 3 (4/19), MLG 1 (2/19) and MLG 6 (1/19). The raw data including fragment sizes, MLG numbers and presence of mixed infections can be seen in Table 8 with multilocus genotype assignments summarised in Table 9. These results have been displayed in graphical form in Figure 8.

**Table 8 Allele assignment and multilocus genotype of *C. parvum* positive samples of calves and their mothers on a dairy farm. Adult 18S results are abbreviated as P (*C. parvum*), A (*C. andersoni*) and N (Negative). A 'x' indicates a negative sample. Blank cells indicate that PCR was not done.**

| Animal | GP60 | MM5 | MM18 | MM19 | TP14 | MLG | Mother | 18S | GP60 | MM5 | MM18 | MM19 | TP14 | MLG |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | x | | | | | | 4662 | PA | IIaA15R1/ IIaA15G2R1 | 2 | 1 | 3 | 1 | 9 |
| **2** | x | | | | | | 500 | PA | IIaA15R1 | 2 | 1 | 3 | 1 | 9 |
| **3** | x | | | | | | 4517 | PA | IIaA15R1 | 2 | 1 | 3 | 1 + 2 | 9 |
| **4** | IIaA15G2R1 | 2 | 1 | 3+1 | 2 | 4 | 4687 | PA | IIaA15G2R1 | 2 | 1 | 1 | 2 | 1 |
| **5** | IIaA15G2R1 | 2 | 2 | 3+1 | 2 | 5 | 3292 | N | | | | | | |
| **6** | IIaA15G2R1 | 2 | 1 | 1 | 1 | 3 | 4721 | P | IIaA15R1 | 2 | 1 | 3 | 1 | 9 |
| **7** | IIaA15G2R1 | 2 | 2 | 1 | 2 | 2 | 5174 | N | | | | | | |
| **8** | IIaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 5156 | PA | IIaA15R1 + IIcA5G3 | 2 | 1 | 3 | 1 | 9 |
| **9** | IIaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 4752 | PA | IIaA17G1R1 | 2 | 1 | 1 | 1 | 6 |
| **10** | IIaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 4529 | PA | IIaA15R1 | 2 | 1 | 3 | 1 | 9 |
| **11** | IIaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 5088 | PA | IIaA15R1 | 2 | 1 | 3 | 1 | 9 |
| **12** | x | | | | | | 4673 | A | | | | | | |
| **13** | IIaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 3510 | P | IIaA15G2R1 | 2 | 1 | 1 | 1 | 3 |
| **14** | IIaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 2846 | P | IIaA15R1 | 2 | 1 | 3 | 1 | 9 |
| **15** | IIaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 3345 | A | | | | | | |
| **16** | IIaA15G2R1 | 2 | x | x | 2 | x | 4216 | PA | IIaA15G2R1 | | | | 2 | |
| **17** | llaA15G2R1 | x | x | x | x | x | 2692 | A | | | | | | |
| **18** | llaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 4491 | PA | x | | | | | |
| **19** | llaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 4081 | A | | | | | | |
| **20** | llaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 5117 | PA | IIaA15G2R1 | 2 | 1 | 3 | 2 | 4 |
| **21** | llaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 1460 | N | | | | | | |
| **22** | llaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 3537 | N | | | | | | |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **23** | llaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 5177 | PA | IIaA15R1 | 2 | 1 | 3 | 1 | 9 |
| **24** | llaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 4241 | PA | IIaA15G2R1 | 2 | 1 | 1 | 1 | 3 |
| **25** | llaA15G2R1 | 2 | 1 | 1 | 2+1 | 1 | 5160 | PA | IIaA15R1 | 2 | 1 | 3 | 1 | 9 |
| **26** | llaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 3734 | P | x | | | | 1 | |
| **27** | llaA15G2R1 | 2 | 1 | 1 | 2+1 | 1 | 3939 | PA | IIaA15G2R1 | | | | 1 | |
| **28** | llaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 4739 | N | | | | | | |
| **29** | llaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 2477 | PA | x | | | | 1 | |
| **30** | llaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 4500 | P | llaA15R1 | | | | | |
| **31** | llaA15G2R1 | 2 | 1 | 1 | 2+1 | 1 | 4725 | P | x | | | | 1 | |
| **32** | llaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 4725 | P | x | | | | 1 | |
| **33** | llaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 4775 | PA | IIaA15G2R1 / IIaA15R1 | 2 | 1 | 1 | 1 | 3 |
| **34** | llaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 4249 | N | | | | | | |
| **35** | llaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 4707 | P | IIaA15R1 | 2 | 1 | 3 | 1 | 9 |
| **36** | llaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 4685 | P | IIaA15G2R1 | 2 | 1 | 1 | 1 | 3 |
| **37** | llaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 4678 | P | IIaA15R1 | 2 | 1 | 3 | 1 | 9 |
| **38** | llaA15G2R1 | 2 | 1 | 1 | 2 | 1 | 4803 | P | IIaA15G2R1 | 2 | 1 | 1 | 2 | 1 |

**Table 9 Calves and their multilocus genotypes (MLG) present on a dairy farm along with the MLG present in the mothers.**

| Calf ID | MLG | Mother MLG |
|---|---|---|
| **1** | 18S Negative | MLG 9 |
| **2** | 18S Negative | MLG 9 |
| **3** | 18S Negative | MLG 9 |
| **4** | MLG 4 | MLG 1 |
| **5** | MLG 5 | 18S Negative |
| **6** | MLG 3 | MLG 9 |
| **7** | MLG 2 | 18S Negative |
| **8** | MLG 1 | MLG 9 |
| **9** | MLG 1 | MLG 6 |
| **10** | MLG 1 | MLG 9 |
| **11** | MLG 1 | MLG 9 |
| **12** | 18S Negative | *C. andersoni* positive |
| **13** | MLG 1 | MLG 3 |
| **14** | MLG 1 | MLG 9 |
| **15** | MLG 1 | *C. andersoni* positive |

| | | |
|---|---|---|
| **16** | Similar to MLG 1 | Failed genotyping |
| **17** | Failed genotyping | *C. andersoni* positive |
| **18** | MLG 1 | Failed genotyping |
| **19** | MLG 1 | *C. andersoni* positive |
| **20** | MLG 1 | MLG 4 |
| **21** | MLG 1 | 18S Negative |
| **22** | MLG 1 | 18S Negative |
| **23** | MLG 1 | MLG 9 |
| **24** | MLG 1 | MLG 3 |
| **25** | MLG 1 | MLG 9 |
| **26** | MLG 1 | Failed genotyping |
| **27** | MLG 1 | Failed genotyping |
| **28** | MLG 1 | 18S Negative |
| **29** | MLG 1 | Failed genotyping |
| **30** | MLG 1 | Failed genotyping |
| **31** | MLG 1 | Failed genotyping |
| **32** | MLG 1 | Failed genotyping |
| **33** | MLG 1 | MLG 3 |

| | | |
|---|---|---|
| **34** | MLG 1 | 18S Negative |
| **35** | MLG 1 | MLG 9 |
| **36** | MLG 1 | MLG 3 |
| **37** | MLG 1 | MLG 9 |
| **38** | MLG 1 | MLG 1 |

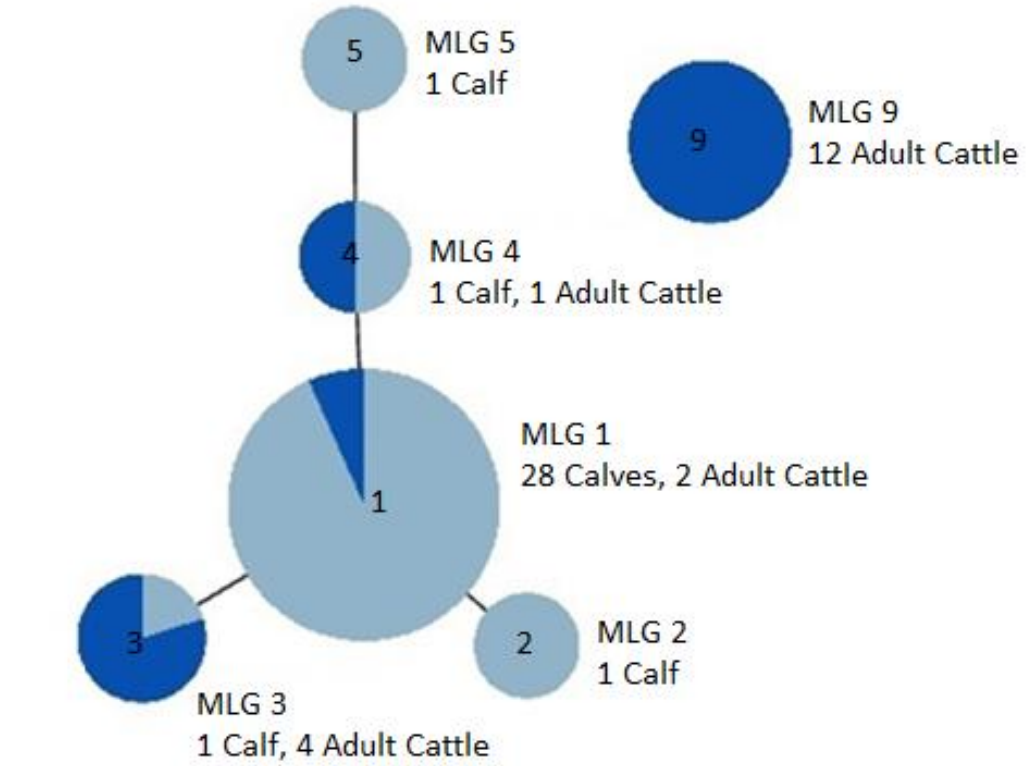


**Figure 8 - Multilocus genotypes of *C. parvum* for paired adult cattle and their female calves on a dairy farm.**

**Each node in Figure 8 represents a single multilocus genotype (MLG) and these are joined by a line if they share 4 out of the 5 alleles. The size of the node represents the number of animals which have that MLG (adult cattle are indicated by dark blue and calves by light blue).**

Prevalence of *C. parvum* peaked at both week 3 and 5 weeks of age (Figure 7). Therefore, calves which were positive for *C. parvum* at weeks 1, 3 and 5 were selected for multilocus genotyping to analyse how the genotypes change over this period. Multilocus genotype results for initial oocyst shedding which occurred in week 1 and both of these peaks, week 3 and week 5, can be seen in Table 10 which shows that the genotype during the 6 week period changes with a predominant genotype present in week 1 (MLG 1along with a single case of MLG 2, MLG 3, MLG 4 and MLG 5) and a different predominant genotype at week 3 (MLG 4 along with four mixed

infections with MLG 22). Week 5 shows calves are infected with MLG 4 although faecal samples were much harder to type at all 5 loci.

**Table 10 Multilocus genotypes (MLG) present in a selection of calves in weeks 1, 3 and 5. The dominant peak in fragment analysis is listed first, followed by the secondary peak in the event of a mixed infection. 'Similar to' is described when full genotyping was unsuccessful but the results available are most similar to a pre-identified MLG. '?' symbolises that the genotyping results were both incomplete and dissimilar to other identified MLGs.**

| Calf ID | Week 1 | Week 3 | Week 5 |
|---|---|---|---|
| 3 | Negative | MLG 4 | ? |
| 4 | Mixed MLG 4 + 1 | MLG 4 | Unknown, similar to MLG 4 |
| 5 | Mixed MLG 5 + 2 | Mixed MLG 4 + 22 | MLG 4 |
| 25 | Mixed MLG 1 + 3 | Mixed MLG 4 + 22 | ? |
| 37 | MLG 1 | Mixed MLG 4 + 22 | ? |
| 23 | MLG 1 | MLG 4 | ? |
| 20 | MLG 1 | Mixed MLG 4 + 22 | ? |
| 18 | MLG 1 | MLG 4 | Unknown, similar to MLG 4 |
| 14 | MLG 1 | Unknown, similar to MLG 4 | MLG 4 |
| 7 | MLG 2 | MLG 4 | Negative |

**Beef Farm**

The majority of calves on the beef farm shared a single genotype, MLG 10. This was shared by 25.0 % (7/28) of the adult cattle. Only one calf presented with a different genotype MLG 11 although this was a mixed genotype with the predominant MLG 10. Eight calves presented with a mixed genotype with MLG 10 as the dominant genotype and MLG 23 as the minor genotype – MLG 23 is different from MLG 10 at only the MM19 locus. Of the adults which amplified at all loci, MLG 10 was the most common genotype (7/13), although MLG 12, MLG 13, MLG 14 and MLG 15 were also present in 2, 2, 1 and 1 out of 13 of the adult cattle respectively. The multilocus genotypes are summarised in Table 11. The raw data including fragment sizes, MLG numbers and presence of mixed infections can be seen in Appendix 2.

**Table 11 Multilocus genotypes (MLG) in beef calves and their dams.**

| Calf | Calf MLG | Dam MLG |
|---|---|---|
| 1 | MLG 10 | Unable to amplify at all loci |
| 2 | MLG 10 | MLG 10 |
| 3 | MLG 11 + 10 | MLG 13 |
| 4 | MLG 10 + 23 | 18S Negative |
| 5 | MLG 10 | Unable to amplify at all loci |
| 6 | MLG 10 | MLG 13 |
| 7 | MLG 10 | 18S Negative |
| 8 | Negative | 18S Negative |
| 9 | MLG 10 | MLG 10 |
| 10 | MLG 10 | Unable to amplify at all loci |
| 11 | MLG 10 + 23 | MLG 10 |
| 12 | MLG 10 + 23 | MLG 14 |
| 13 | MLG 10 | MLG 10 |
| 14 | MLG 10 | MLG 15 |
| 15 | MLG 10 | 18S Negative |
| 16 | MLG 10 + 23 | MLG 12 |
| 17 | MLG 10 + 23 | MLG 12 |
| 18 | MLG 10 + 23 | MLG 10 |
| 19 | MLG 10 | MLG 10 |
| 20 | MLG 10 + 23 | 18S Negative |
| 21 | MLG 10 + 23 | MLG 10 |

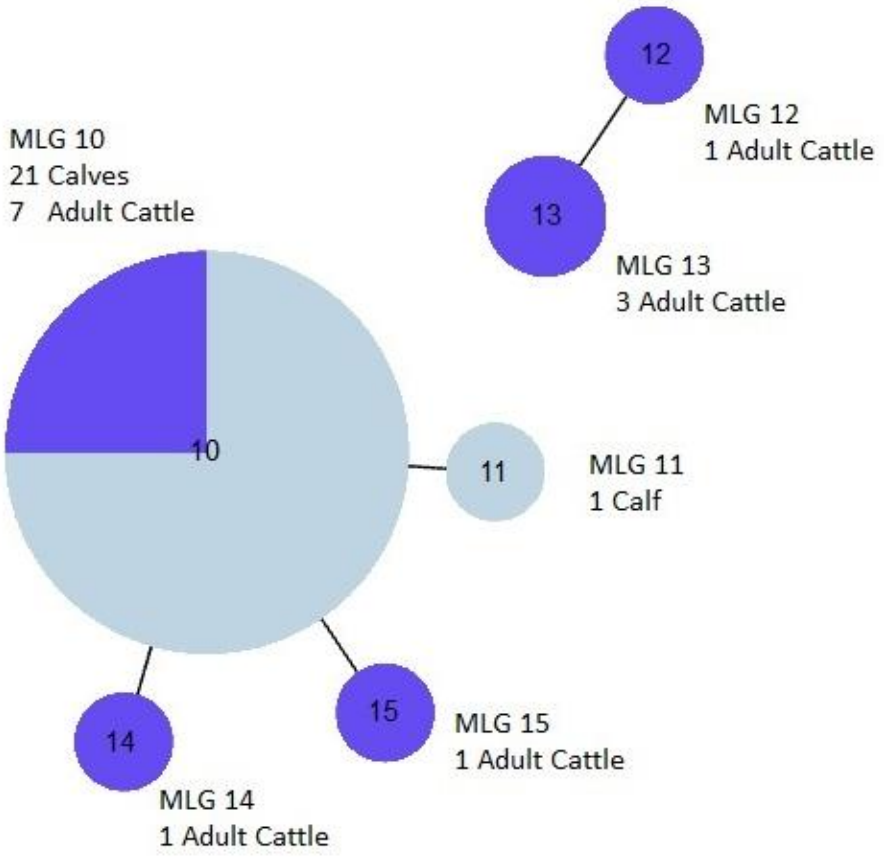


**Figure 9 - Multilocus genotypes for *C. parvum* in paired adult cattle and their calves on a beef farm.**

**Each node in Figure 9 represents a single multilocus genotype (MLG) and these are joined by a line if they share 4 out of the 5 alleles. The size of the node represents the number of animals which have that MLG split into adult cattle (dark blue) and calves (light blue).**

## 2.4.6 Matched Dam-Calf

### Dairy Farm

Results for matching the calves with their dams on the dairy farm gave 2.6 % (1/38) initial matches for multilocus genotype of *C. parvum* (Table 12). Any *C. parvum* sample which tested negative for *C. parvum* or proved unable to amplify at all loci was termed 'unreadable' and so it is unknown if a match occurred between that dam and its calf. This was the case for 16/38 pairs. Some of the adult cattle are labelled as 'not MLG 1' as the regions which did amplify are different from MLG 1 and so can be

concluded as not being a match with the calf. This was the case for 5 pairs. In total, there were 22 interpretable pairs, and of those 22 only one pair matched (4.5%).

**Table 12 Matched adult cattle and calf multilocus genotypes (MLG) on a dairy farm**

| Calf number | Calf MLG for *C. parvum* | Dam MLG for *C. parvum* | Match? |
|---|---|---|---|
| **1** | 18S Negative | 9 | Unknown |
| **2** | 18S Negative | 9 | Unknown |
| **3** | 18S Negative | 9 | Unknown |
| **4** | 4 | 1 | No |
| **5** | 5 | 18S Negative | Unknown |
| **6** | 3 | 9 | No |
| **7** | 2 | 18S Negative | Unknown |
| **8** | 1 | 9 | No |
| **9** | 1 | 6 | No |
| **10** | 1 | 9 | No |
| **11** | 1 | 9 | No |
| **12** | 18S Negative | 18S Negative | Unknown |
| **13** | 1 | 3 | No |
| **14** | 1 | 9 | No |

| | | | |
|---|---|---|---|
| **15** | 1 | 18S Negative | Unknown |
| **16** | 18S Negative | Unreadable | Unknown |
| **17** | 18S Negative | 18S Negative | Unknown |
| **18** | 1 | Unreadable | Unknown |
| **19** | 1 | 18S Negative | Unknown |
| **20** | 1 | 4 | No |
| **21** | 1 | 18S Negative | Unknown |
| **22** | 1 | 18S Negative | Unknown |
| **23** | 1 | 9 | No |
| **24** | 1 | 3 | No |
| **25** | 1 | 9 | No |
| **26** | 1 | Incomplete (not MLG 1) | No |
| **27** | 1 | Unreadable | Unknown |
| **28** | 1 | 18S Negative | Unknown |
| **29** | 1 | Incomplete (not MLG 1) | No |
| **30** | 1 | Incomplete (not MLG 1) | No |

| | | | |
|---|---|---|---|
| **31** | 1 | Incomplete (not MLG 1) | No |
| **32** | 1 | Incomplete (not MLG 1) | No |
| **33** | 1 | 3 | No |
| **34** | 1 | 18S Negative | Unknown |
| **35** | 1 | 9 | No |
| **36** | 1 | 3 | No |
| **37** | 1 | 9 | No |
| **38** | 1 | 1 | Yes |

**Beef Farm**

Results for matching the calves with their dams on the beef farm gave 33.3% (7/21) initial matches for multilocus genotype of *C. parvum*. Matches can be seen in Table 13. Any *C. parvum* sample which tested negative for *C. parvum* or proved unable to amplify at all loci was termed 'unreadable' and so it is unknown if a match occurred between that dam and its calf, this was the case for 8/21 pairs. In total, there were 13 interpretable pairs, and of those 13, 7 pairs matched (53.8 %).

**Table 13 Matched adult cattle and calf multilocus genotypes (MLG) on a beef farm**

| Calf | Calf MLG | Dam MLG | Match? |
|---|---|---|---|
| 1 | 10 | 18S Negative | Unknown |
| 2 | 10 | 10 | Yes |
| 3 | 11 | 13 | No |
| 4 | 10 | 18S Negative | Unknown |
| 5 | 10 | 18S Negative | Unknown |
| 6 | 10 | 13 | No |
| 7 | 10 | 18S Negative | Unknown |
| 8 | 18S Negative | 18S Negative | Unknown |
| 9 | 10 | 10 | Yes |
| 10 | 10 | Unreadable | Unknown |
| 11 | 10 | 10 | Yes |
| 12 | 10 | 14 | No |
| 13 | 10 | 10 | Yes |
| 14 | 10 | 15 | No |
| 15 | 10 | 18S Negative | Unknown |
| 16 | 10 | 12 | No |
| 17 | 10 | 12 | No |
| 18 | 10 | 10 | Yes |
| 19 | 10 | 10 | Yes |
| 20 | 10 | 18S Negative | Unknown |
| 21 | 10 | 10 | Yes |

## 2.5 Discussion

**Prevalence of *C. parvum* in calves.**

The results from this study show that 100% of calves sampled in both the dairy and beef calves tested positive for *C. parvum* on at least one occasion. *C. parvum* was the only species identified on the beef farm, however this was based on single time point sampling rather than looking over a period of time as in the dairy calves. *C. parvum* was the most predominant species over 6 weeks on the dairy farm. *C. bovis* was found as a mixed infection with *C. parvum* at 3 weeks of age but was not found as a single infection until the calves were 4 weeks of age. These data strongly support the consensus that *C. parvum* is the most common species in neonatal calves.

The most common species reported in young calves in numerous countries worldwide is *C. parvum* (Broglia, Reckinger, Cacció & Nöckler., 2008; Geurden et al., 2007; Robertson et al., 2014; Santin et al., 2004; Trotz-Williams et al., 2006), including a large-scale study in the US. Fifteen dairy farms spanning seven states found that *C. parvum* was the predominant species responsible for 85% of infections in pre-weaned calves aged between 5 days and 2 months (Santin et al., 2004). Similarly, neonatal calves aged 7 – 21 days from 16 farms in Ontario were all infected with *C. parvum* and no other species was found (Trotz-Williams et al., 2006). Calves which were younger than 10 weeks old in 100 dairy and 50 beef farms in Belgium were found to predominantly be infected with *C. parvum* (92.1 %), although *C. bovis* was also found in 7.0 % of the calves (Geurden et al., 2007). In the UK specifically, *C. parvum* has been found as the most predominant species in calves with a median age of 26 days sampled from 41 farms around Cheshire (Brook et al., 2009), and calves from 80 dairy farms aged between 0 – 24 months in a study spanning England and Wales (Smith et al., 2014). Therefore, the high prevalence of *C. parvum* in pre-

weaned calves in this study is expected, although 100% of calves on both the dairy and the beef farm testing positive is higher than previous reports of 85% (Santin et al., 2004) and 92.1% (Geurden et al., 2007). This is likely due to an age bias in the results as the calves in this study were tested between 0-6 weeks rather than up to 10 weeks and so were more likely to test positive for *Cryptosporidium*. Additionally, the dairy calves were sampled 3 times per week for the first three weeks of their life. The other two prevalence studies are not longitudinal in this way and instead are based on point sampling (Geurden et al., 2007; Santin et al., 2004), therefore positive results in this study are more likely.

*C. bovis* is reported to be the most common species detected in neonatal calves in other countries such as China and Sweden with even speculation that the species could cause clinical disease (Silverlas, Bosaeus-Reineck, Naslund & Björkman., 2013). In the Henan province of China, a study testing 801 faecal samples from pre-weaned calves on eight farms showed that *C. bovis* was the most prevalent species found in samples from calves between 1 – 8 weeks of age, although it was closely followed by *C. parvum* (Wang et al., 2011). *C. parvum* was the most prevalent in 1 and 3-week-old calves and *C. parvum* and *C. bovis* were equal in prevalence in 2-week-old calves, still indicating that *C. parvum* is the most prevalent in neonatal calves (0-21 days of age). *C. bovis* was identified in 74 % of pre-weaned calves, in 500 herds from five areas of Sweden (Silverlas et al., 2010). However, these calves where anywhere from 1-61 days of age. Calves from these 500 herds that were under 14 days of age were predominantly infected with *C. parvum*.

**Species and genotypes in pre-weaned dairy calves from birth over a 6-week period.**

Very few research groups have conducted longitudinal studies on calves to see how species and genotypes change as the calves age. This study adds further insight into how species and genotypes change over a six-week period. In the longitudinal studies which have been conducted, calves are initially infected with *C. parvum* for the first few weeks of life before species such as *C. bovis* and *C. ryanae* are detected (Rieux, Chartier, Pors & Paraud., 2013; Santin et al., 2008; Thomson, 2015). Peak shedding of *C. parvum* has previously been reported as occurring at 2 weeks of age (Santin et al., 2004). However, the results from this study, in dairy calves, showed a peak at 3 weeks of age (Figure 7). Another peak was detected at 5 weeks of age which could be explained by the animals moving into a new pen at this stage. Using microsatellite analysis, the positive animals in each peak consisted of different genotypes showing that the *C. parvum* genotype changes over this time period. This could be in response to changing pens, mixing with different animals and facing different environmental oocyst contamination.

Species *C. ryanae* and *C. bovis* were found from weeks 2 and 3 respectively on the dairy farm, although both were as a mixed infection with *C. parvum*. This is earlier than many studies report. However, the nested multiplex PCR used made mixed infections easy to identify, which may have gone unnoticed in other studies perhaps using sequencing to speciate. The results are similar to a study done in France which found *C. bovis* as early as 11 days of age and *C. ryanae* from 17 days of age (Rieux et al., 2013). As the described pre-patent period for *C. bovis* and *C. ryanae* is 10 – 12 days, it stands to reason that these species are not detected until the second week of life (Fayer et al., 2008; Fayer et al., 2005). However other studies do show calves shedding *C. bovis* as early as 1 week old (Silverlas et al., 2010; Wang et al., 2011) indicating a shorter pre-patent period than the

previously described 10 – 12 days (Fayer et al., 2005). It does suggest however that these calves likely became infected with these species very shortly after birth. Calves in this study were housed in close proximity to calves aged between 2 and 3 months of age, and so this could be the potential route of infection. In this study, these species did not appear as a single infection until week 4.

*C. bovis,* although detected in pre-weaned calves (Silverlas et al., 2010; Wang et al., 2011), is commonly found in post-weaned calves. One study found this species in only 9% of samples from pre-weaned calves but in 55% of post-weaned calves on 15 dairy farms from the US (Santin et al., 2004). A study in England and Wales found that *C. bovis* was more likely to be shed by older calves and adult cattle than by pre-weaned calves (Smith et al., 2014). This work stands in agreement with this hypothesis with only 6.8% of a total 221 samples taken over 6 weeks on the dairy farm testing positive for *C. bovis* and thus showing a small percentage of both species in pre-weaned calves. *C. ryanae* is also most commonly found in post-weaned cattle around the world, including the US, UK, India, and Japan (Amer et al., 2009; Brook et al., 2009; Santin et al., 2008; Venu et al., 2012). Pre-weaned calves in this study had a 7.7% prevalence of *C. ryanae*, showing that although present in pre-weaned calves, its prevalence is low.

The most common GP60 genotype found on the dairy farm during this study was IIaA15G2R1 which is one of the most common genotypes of *C. parvum* found in both livestock and humans (Brook et al., 2009; Feng et al., 2013; Geurden et al., 2007). This GP60 genotype was present as the sole GP60 genotype in the calves and in 30% of the adult cattle on the dairy farm. However, the results in this present study suggest that when other loci are amplified there are different genotypes within the IIaA15G2R1 GP60 subtype assignment and so it is essential that we do not rely on a single marker when trying to understand genotype diversity.

**Dairy and beef rearing systems**

Previous work has reported a higher prevalence of *Cryptosporidium* in dairy calves compared to beef calves. Prevalence of *Cryptosporidium* across 100 dairy and 50 beef farms in Belgium has shown this difference; an average of 37 % in dairy calves compared to 12 % in beef calves (Geurden et al., 2007). According to the Animal and Plant Health Agency (APHA), 61 % of *Cryptosporidium* diagnoses made on submissions to the GB veterinary diagnostic network in neonatal calves are from the dairy industry, compared to 30.6 % from the beef industry (APHA, 2012 - 2017) although more samples are submitted from the dairy industry than the beef. The artificial rearing system of dairy calves, as calves are removed from the mother, could cause more stress than the more natural system in beef calves which could explain the increase in *Cryptosporidium* prevalence in dairy calves (Garro, Morici, Utges, Tomazic & Schnittger., 2016).

The results of this study do not agree with the hypothesis that dairy calves have a higher prevalence of *Cryptosporidium* compared to beef calves, although this is only based on one dairy and one beef farm. Not only this, but the sample number between the dairy and beef farm was quite different due to the number of animals available to sample on that farm. This differed as the dairy sampling was done longitudinally whereas the beef farm was done as a point sample. Therefore, it is difficult to directly compare the results on these two farms. In this study, all of the beef calves (100 %) tested positive for *Cryptosporidium* which was identical to the 100% of calves on the dairy farm which tested positive on at least one occasion. This difference is likely due to farm selection bias, as farms were chosen because they have had a historical problem with the parasite in order to determine potential transmission routes.

**The role that adult cattle could play in the transmission of *C. parvum* to their calves.**

As concentration methods for oocysts in faecal samples have improved, it has become clear that adult cattle do carry *Cryptosporidium,* with the most common species in the UK being *C. parvum*. Although contesting studies do exist stating that adult cattle do not shed *C. parvum* (Atwill & Pereira, 2003), it is clear that as these do not use the most sensitive concentration techniques available along with a large volume of starting material (Wells et al., 2016). Therefore, they are not comparable with more recent studies. Also, this work was carried out in Tulare, California which is a hotter and drier climate than Scotland and so the prevalence of *Cryptosporidium* is likely to be less based on previous work which found a higher prevalence of *Cryptosporidium* during the rainy season compared to the dry season (Gonzalez-Moreno et al., 2013). The results of this present study show *Cryptosporidium* to be present in the adult cattle in both a dairy farm (36 %) and a beef farm (56%). *C. parvum* is the most prevalent species in adult cattle on two separate farms, one dairy and one beef, in two separate locations in Scotland. This result confirms results found in adult cattle in Scotland using the newer concentration techniques (Wells et al., 2015). Adult cattle shedding *C. parvum* would be prime candidates for *C. parvum* transmission to calves.

Two different farms, with different practices, were analysed; a dairy farm, where calves were removed within a few hours from their dams, and a beef farm where calves and dams were housed together. On both farms the calves presented with a single dominant multilocus genotype, MLG 1 on the dairy farm and MLG 10 on the beef farm. On the other hand, a higher variety of genotypes were seen in the adult cattle, along with an increased prevalence of mixed infections. This pattern of more variety being present in older animals has been shown before in a study done in sheep in Australia, where more species and genotypes were found in post-weaned

sheep compared to pre-weaned (Yang, Jacobson, Gordon & Ryan., 2009). On the dairy farm, only two adults shared the same multilocus genotype which was predominant in the calves, which can be seen in Figure 8. This could suggest that the adult cattle on the dairy farm are unlikely to contribute to the transmission of *C. parvum* to their calves. This has been indicated in previous work which genotyped using GP60 where only one adult cow shared the same genotype with the calves (Thomson, 2015).

On the other hand, 7/13 of the adult beef cattle shared the predominant genotype which was found in the calves MLG 10 which can be seen in Figure 9. This could indicate that adults on the beef farm are more likely to be a source of infection to their calves. However, the adults still showed a much higher variety of genotypes and were housed together with their calves, which only presented with a single multilocus genotype. Therefore, it could be hypothesised that transmission of *C. parvum* is more likely to occur the other way around; the calves are actually a source of *C. parvum* transmission to the adults. Some *C. parvum* genotypes have been found to be more virulent than others (Bouzid, Hunter, Chalmers & Tyler., 2013; Cama et al., 2007) and so would be more efficient at causing infection in very young calves with an immune system which is still developing. Calves could be amplifying this more virulent genotype and causing mass environmental contamination, infecting subsequent calves which are then born. Based on these results, separating cow and calf would unlikely resolve any issues with *Cryptosporidium* on the farm and calves are clearly becoming infected from an alternative source.

**Future work**

Some considerations for the future of this work include the sampling methodology. Faecal samples were collected from the ground for the most part, and so there is a potential for environmental contamination of samples. This was reduced by observation before collection, however contamination does still remain a possibility. Ethical approval is required for direct rectal sampling and therefore, should this work be repeated it would be useful to use this direct method of faecal collection.

The comparison of the dairy and beef systems is difficult due to the difference in sample collection methodology. In future, it would be beneficial to test adult beef cattle before calves are born to determine the risk adult beef cattle pose to the transmission of *Cryptosporidium* oocysts to their calves.

Future work should also include incorporating microscopy to rule out false negative results due to PCR inhibition. This could explain why a high proportion of adult cattle (74% on the dairy farm and 44% on the beef farm) were negative for *Cryptosporidium*.

## 2.6 Conclusion

In conclusion, the most predominant species found in both adult and pre-weaned dairy and beef cattle is *C. parvum*. Other species *C. bovis* and *C. ryanae* were not present in beef calves, and present after 3 weeks of age in dairy calves. The most common GP60 genotype in the dairy calves was IIaA15G2R1, which is the most common GP60 genotype found in livestock. In the beef calves, however, IIaA17G1R1 was the sole genotype found with no animals harbouring the common IIaA15G2R1. It was found that within a GP60 genotype, there are differences at other loci, therefore microsatellite analysis is essential for epidemiological studies of *C. parvum*. No difference was seen in the prevalence of *C. parvum* between the two calf rearing systems of beef and dairy.

When comparing genotypes found in the adult cattle to their calves, only two dairy adults shared the same genotype as the dairy calves. However, in the beef system, adult cattle which were *C. parvum* positive mainly did have the same genotype as the calves. In both cases, adults were both much harder to amplify at all 5 genotyping regions and were more likely to present with mixed infections than the calves. Due to the low infectious dose (Zambrisky et al., 2013), adult dairy cattle could infect their calves via the faecal-oral route on contaminated udders and teats although they are unlikely to play a significant role in the transmission of *C. parvum* to their calves and so control measures to minimise this transmission route is likely to be unnecessary. Further work is required to confirm the role that beef cattle may play in the transmission of *C. parvum* to their calves.

# Chapter 3 The Effect of Cryptosporidiosis on the Growth of Beef Calves

## 3.1 Introduction

Cryptosporidiosis tends to manifest by profuse and watery diarrhoea, dehydration, lethargy and occasionally death in neonatal calves. Diarrhoea occurs as parasites attach and invade gut epithelial cells at the ileocecal junction (in the case of *C. parvum*) (Thomson et al., 2017). This causes villous atrophy, villous fusion and inflammation within the intestine, reducing absorptive surface area and therefore the calf's ability to absorb water and nutrients from the gut (Dinler & Ulutas, 2017; Thompson, Palmer & O'Handley., 2008). The recovery rate for the villi in the gut once the infection has passed is currently unknown. It is therefore unclear whether or not an infection at a young age could reduce the growth rate of calves over time, resulting in production and economic losses. There are very few studies looking at the effect cryptosporidiosis can have on calves long-term, probably because these studies would be very difficult to do outside of a controlled laboratory environment. Many *Cryptosporidium* positive calves also suffer co-infections with other gastrointestinal pathogens such as rotavirus, coronavirus and *E. coli* (Blanchard, 2012; Brar et al., 2017) and so it would be difficult to directly attribute any production losses due to enteric disease to *Cryptosporidium*. Performing this sort of research under controlled conditions would likely be too expensive, as animals would need to remain free of other diseases and kept for a long period of time.

Of the limited work which has been done, it is clear that infection with *Cryptosporidium* at a young age does result in a reduction in short term growth. A reduction in growth has been seen in humans, lambs and mice infected with *C. parvum* (Ajjampur et al., 2010; Lacroix, Mancassola, Naciri & Laurent., 2001; Sweeny et al., 2011) and so it stands to reason that

calves are likely to experience a reduction in growth following cryptosporidiosis in the same way.

A Global Enteric Multicentre study examining diarrhoeal disease in 9439 children younger than 5 years old (and 13129 controls) in sub-Saharan Africa and south Asia found that children with moderate to severe diarrhoea suffered significant growth faltering in the first 60 days following onset of symptoms compared to their matched controls (Kotloff et al., 2013). Not only this, but moderate to severe diarrhoea increased the risk of dying during the 60 day follow up by 8.5 times compared to matched controls. This study shows the real burden diarrhoeal disease has in developing countries and the likelihood for cryptosporidiosis to reduce growth rate in calves.

A review of the Global Burden of Diseases, Injuries and Risk Factors study (GBD) along with previously published and unpublished data found that diarrhoea caused by cryptosporidiosis was associated with a decrease in height-for-age Z score, weight for age Z score and weight for height Z score in children younger than 5 years old (Khalil et al., 2018). This review also estimated that cryptosporidiosis was responsible for an additional 7.85 million disability adjusted life years (DALY). A DALY is the number of years lost due to ill-health, disability or early death.

Rats experimentally infected with *Cryptosporidium* oocysts displayed a reduction in nutrient intake and body weight at peak infection. At day 17 post infection, the rats still remained at the reduced body weight despite nutrient intake increasing to match the control rats (Topouchian et al., 2005). The rats in this study clearly were unable to catch up with the control group and this could be the same with calves. *C. parvum* infection in neonatal rats resulted in hypersensitivity in the jejunum 120 days post-infection (Topouchian et al., 2005). This hypersensitivity is linked to many chronic inflammatory gut conditions (Marion et al., 2006). People who had

suffered with cryptosporidiosis following an outbreak of *C. parvum* attributed to contaminated salad leaves, reported irritable bowel syndrome (IBS) symptoms (27.9 %), weight loss (30.8 %), abdominal pain (37.8%) and joint pain (32.6%) in a questionnaire examining long-term health effects sent out 6 and 12 months post-diagnosis (Stiff, Davies, Mason, Hutchings & Chalmers., 2017). It has been reported that changes in the microbiota as a result of parasitic infection results in an alteration of the metagenomic, transcriptomic, proteomic, and metabolomic structure of the gut, and so the microbiota plays an essential part in gut homeostasis (Partida-Rodríguez et al., 2017). The reported symptoms from those infected with *Cryptosporidium* from salad leaves could be attributed perhaps to an alteration of gut homeostasis by a change in gut microbiota, as many gut disorders are associated with dysbiosis (Certad, Viscogliosi, Chabé & Cacciò., 2017).

Research examining the losses associated with calf enteritis in Scotland found that the average loss per affected calf was £32.92. This considers veterinary costs, calf mortality, costs of labour and loss in calf value. Although this figure is for calf enteritis as a whole, it shows that cryptosporidiosis is likely to result in similar losses (Gunn & Stott, 1997). As this study was conducted over 20 years ago, the likelihood is that this cost has risen.

Scientific studies on experimental lambs in Spain has shown that feed restriction at the pre-weaning stage results in a reduced weight gain long-term and the inability to catch up with the control group (Frutos et al., 2018). The authors of this work suggest that restriction of feed intake results in an alteration of commensal bacteria colonization of the gastrointestinal tract, which occurs during the post-natal phase of life (Taschuk & Griebel, 2012). This alteration can then affect nutrient processing and lifetime feed efficiency. Infection with *C. parvum* is known to both result in a reduced appetite, and damage the epithelial cells in the

gut. It is likely therefore that both of these factors will alter the gut microbiota and therefore the feed efficiency of the animals.

One study exists examining the effect of cryptosporidiosis on the absorptive capacity and permeability of the small intestine in neonatal calves. This study showed that experimentally infected calves had reduced intestinal function up to 14 days post infection (Klein et al., 2008). This was also coupled with a reduction in daily weight gain over a 21-day period post infection from 204 grams per day to 107.

It is clear that a knowledge gap exists on the long-term effects of cryptosporidiosis in cattle. A farm in Perthshire, Scotland was found to have calves suffering from a range of severity levels of clinical cryptosporidiosis. Those calves were also negative for rotavirus, coronavirus and *E. coli* on the first inspection and so this farm was chosen for a longitudinal study examining the effect cryptosporidiosis had on the calves' growth over a 6-month period.

## 3.2 Aims

- Develop a scoring system for the severity of cryptosporidiosis in beef calves.

- Determine the effect that cryptosporidiosis has on the growth of beef calves over a 6-month period.

- Estimate a cost for the reduction in weight gain caused by cryptosporidiosis in beef calves.

## 3.3 Methods

### 3.3.1 Scoring for Cryptosporidiosis

A farm in Perthshire, whose calves suffered a history of clinical cryptosporidiosis, was chosen for participation in this study. Implemented farm management methods such as altering the diet of the pregnant cattle to improve colostrum quality had resulted in calves suffering a wide range of clinical manifestations of cryptosporidiosis. Therefore, the ability to examine how the different severities of cryptosporidiosis affect calves' long term could be assessed in a natural farm setting. This farm also had no historical reports of other gastrointestinal pathogens (Rotavirus, coronavirus, *E. coli* and *Salmonella*) in the calves making it a suitable candidate for examining *Cryptosporidium* as the sole cause of enteritis. This is the same farm which was used for the adult cattle transmission study in Chapter 2 and further details on the farm can be found in 2.3.1.

Limousin x Belgian Blue calves were scored for severity of cryptosporidiosis every second day from their birth until they reached 16 days of age. It has been reported that diarrhoea due to cryptosporidiosis is typically seen in calves in the first 15 days of life (Glombowsky et al., 2017). Preliminary visits and an interview with the farmer confirmed that calves often showed signs of cryptosporidiosis between 6-10 days old. These calves were naturally infected and so infectious dose is unknown. This study was carried out in two batches of calves during the spring calving on two consecutive years 2016 and 2017. The number of calves included in the study was 34 in first year, with one excluded due to a *Salmonella* infection and 37 in the second year, with 10 excluded due to death from cryptosporidiosis (4), infection with *Mycoplasma* (3), lost and presumed stolen (1), leg amputated (1) and orphaned (1). Determining the severity of cryptosporidiosis in calves infected with *C. parvum* on a beef farm was done by developing a clinical scoring system, incorporating scores for both the faecal consistency and the demeanour of the animal. The scoring system that was used for the calves is described in Table 14. Calves were scored using this system by

observing each animal for at least 10 minutes, or until both faecal and demeanour scores were obtained, every second day between birth and 16 days of age. If animals still suffered from diarrhoea at 16 days, they were scored for a further two sessions (4 days). All animals were recorded on video on each day, and the scores were assigned by a single assessor to reduce scoring bias in both years. This was done either in person or based on the video footage. Indicators of disease were determined through experimentally infected calves as part of a different trial and through initial pilot observations and confirmation using PCR. Calves with a high score typically appeared hunched over, dehydrated and lethargic whereas animals with a low score appeared bright and energetic, both of which can be visualised in Figure 10.

**Table 14 Scoring system for cryptosporidiosis**

| | | |
|---|---|---|
| Faecal consistency | Firm | 0 |
| | Semi-formed | 1 |
| | Loose but stays on top of the bedding | 2 |
| | Loose and sifts through the bedding | 3 |
| Demeanour | Standing, happy to rise, ears and eyes normal | 0 |
| | Standing, happy to rise<br>Suffering one or more of lethargic, ear droop, licked back | 1 |
| | As above including hunched over, head down | 2 |
| | Reluctant to rise with one or more of lethargic, ear droop, licked back | 3 |
| | As above including hunched over, head down | 4 |
| | Unable to rise, lethargic, sunken eyes, ear droop | 5 |

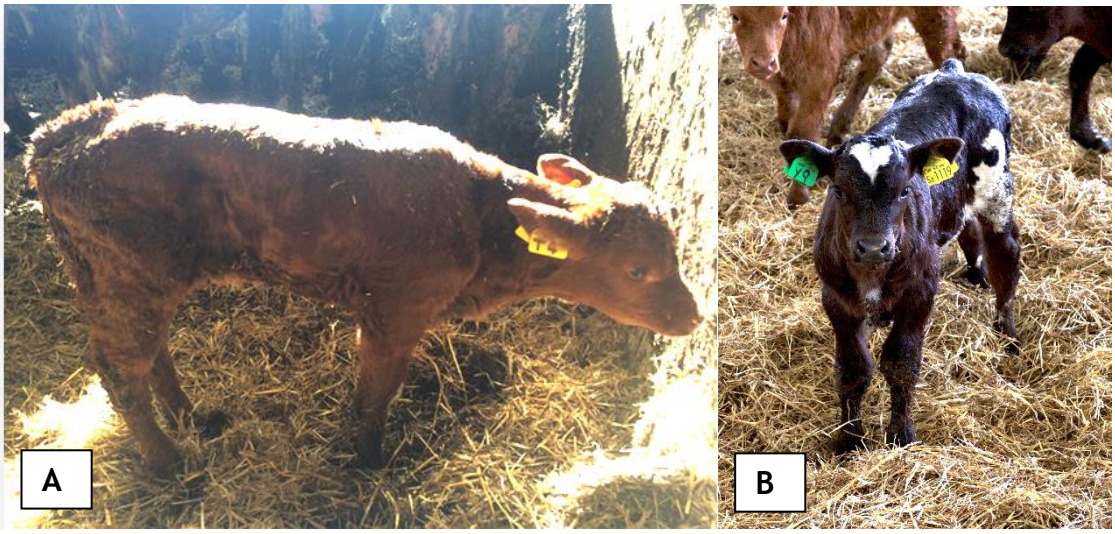


**Figure 10 Image of a high scoring calf (A) and a low scoring calf (B) for severity of cryptosporidiosis.**

Daily scores were attributed to each animal by multiplying the faecal consistency score by a factor of 2 and adding the demeanour score.

Daily score = (Faecal score x 2) + Demeanour

An overall score was assigned after the 16-day period by taking an average of the worst daily score, the preceding score, and the following score, giving an indication of disease duration. If an animal had two high scores of an equal value, the earliest score was chosen. If an animal's highest score was on scoring occasion one, then the first three scores were used to calculate the total score.

Total score = Average (worst score + preceding score + following score)

Overall scores were used to assign each animal into one of three groups: Severe infection (High) described animals which suffered severe diarrhea for three or more days with a poor demeanour, Mid-range disease (Medium) covered animals which suffered severe diarrhea for less than three days and no clinical signs of infection (Low) included animals with no diarrhea, although may have scored a demeanour score of 1 on two occasions or less.

### 3.3.2 *Cryptosporidium* PCR

All calves were tested for *Cryptosporidium* between 3-6 days of age which is the reported age when calves typically start to shed *C. parvum* (Dinler & Ulutas, 2017). The protocol for the 18S nested species-specific multiplex (nssm) PCR can be found in 3.3.6 which allowed for species determination. Any *C. parvum* positive samples underwent genotyping at the GP60 gene and loci MM5, MM18, MM19 and TP14 to determine the multilocus genotypes of the affected calves. Protocols for GP60 sequencing and multilocus genotyping can be found in 3.3.7 – 3.3.9.

### 3.3.3 Test for other Gastrointestinal Pathogens

In order to determine that cryptosporidiosis was the sole cause of the symptoms observed in the calves, faecal samples were collected, where possible, for all animals with at least one sample for every calf. These samples were tested for rotavirus, coronavirus and *E. coli*. This test was done using the MSD Animal Health Expertis Scourcheck kit which involved diluting a small amount of faeces into the provided sample tube using a provided spoon. The sample was shaken and a strip inserted, before being allowed to sit for 10 minutes while the liquid migrates up the strip. Results can then be visualised in the form of red lines indicating a positive or negative result, where a single line indicates a negative and a double, a positive. Any suspected *Salmonella* cases were examined and tested by the local veterinarian.

Animal health records were monitored over a 6-month period to ensure all animals were treated the same for gastrointestinal nematodes, and not suffering from anything which may have affected the growth rates. Any animal which suffered other health conditions, or was exposed to circumstances which may negatively affect its weight gain (events such as but not limited to: death of a calf mother or a broken leg.) during the 6-month period was excluded from the study.

### 3.3.4 Weighing Calves

Calves were weighed at 3, 4 and 6 months of age in the first year using an aluminium cattle platform (Allied Weighing. http://www.alliedweighing.co.uk). The birth weights of calves in the first year were estimated by the farmer due to the lack of weighing facilities. As the lack of weighing facilities at calf birth in 2016 resulted in the use of estimated birth weights, the study was repeated for the second year where animals were weighed at birth and then again at 4, 5 and 6 months of age. Calves on the farm were weighed at birth using a small scale designed for sheep (IAE Ltd) and then afterwards using the cattle platform incorporated into a cattle race.

### 3.3.5 Statistical Analysis

Statistical analysis was performed using Minitab using a Kruskal-Wallis and subsequent Mann-Whitney tests for the second year due to unequal variances between the groups in year 2. Analysis of the effect of gender was analysed using a one-way ANOVA as the ANOVA assumptions were met.

## 3.4 Results

### 3.4.1 Scores to Determine the Severity of Cryptosporidiosis in Calves

All calves were assigned a faecal score and a demeanour score every second day until they reached 16 days of age, or 20 days of age if diarrhoea was present at day 16. Therefore, each animal was scored between 8 – 10 times. In 2016, all calves which were included were successfully scored for the full described period. In 2017, 4 calves died during the scoring period. The daily overall score, animals' final score and severity classification are described in Table 17 for year 1 (2016) and in Table 18 for year 2 (2017).

**Scoring for 2016**

Calves in the first scoring year of 2016 typically began showing signs of clinical cryptosporidiosis between 4-6 days of age (scoring occasion 2 and 3). However, some calves received high scores even in the first couple of days of life (scoring occasion 1). The results are shown in Table 17. All calves had finished exhibiting clinical signs of cryptosporidiosis by 14-16 days of age (scoring occasion 7 and 8).

For the year 2016, animals were placed into one of three groups based on the scoring system described in 3.3.1. The number of animals in each group were 17 severely infected individuals which suffered diarrhoea for an extended period, 5 calves with a mid-range disease and 12 calves with no signs of clinical disease. This is summarised in Table 15 below. The average score for severe disease, mid-range disease and no clinical disease was 6, 3 and 0 respectively

**Table 15 - Number of animals in each severity group 2016 along with the group's average score.**

| | Number of calves | Average score |
|---|---|---|
| Severe disease | 17 | 6 |
| Mid-range disease | 5 | 3 |
| No clinical disease | 12 | 0 |

## Scoring for 2017

Calves in the second scoring year of 2017 typically began showing signs of clinical cryptosporidiosis at the same time as the previous year between 4-6 days of age (scoring occasion 2 and 3). By day 6, 84.2 % (16/19) of medium and high scoring calves had diarrhoea. This can be seen in Table 18. All calves had finished exhibiting clinical signs of cryptosporidiosis by 14-16 days of age (scoring occasion 7 and 8). Four calves died during the scoring process of this study, three died due to cryptosporidiosis and one due to a heart condition as confirmed by the farm vet.

In 2017, the split between the three groups was 11 calves with a severe disease, 14 with a mid-range disease and 8 with no signs of clinical cryptosporidiosis. This is summarised in Table 16 below. The average score for severe disease, mid-range disease and no clinical disease was 6, 3 and 0 respectively which is identical to the average scores in 2016.

**Table 16 - Number of animals in each severity group 2017 along with the group's average score.**

| | Number of calves | Average score |
|---|---|---|
| Severe disease | 11 | 6 |
| Mid-range disease | 14 | 3 |
| No clinical disease | 8 | 0 |

**Table 17 Daily joint calf scores for faecal consistency and demeanour for year 1 (2016) on each scoring occasion along with their overall score and severity group to which they were assigned.**

| | Scoring Occasion (Every second day following birth) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Calf ID | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | Overall Score | Severity Group |
| 16-28 | 4 | 0 | 9 | 8 | 10 | 8 | 4 | 4 | 0 | 0 | 9 | High |
| 16-10 | 9 | 7 | 6 | 5 | 0 | 0 | 0 | 0 | | | 7 | High |
| 16-29 | 0 | 0 | 4 | 10 | 7 | 2 | 2 | 1 | | | 7 | High |
| 16-31 | 0 | 5 | 8 | 10 | 3 | 3 | 0 | 0 | | | 7 | High |
| 16-11 | 8 | 7 | 2 | 0 | 0 | 0 | 0 | 0 | | | 6 | High |
| 16-13 | 0 | 6 | 8 | 5 | 5 | 0 | 0 | 0 | | | 6 | High |
| 16-15 | 0 | 3 | 4 | 7 | 6 | 6 | 0 | 0 | | | 6 | High |
| 16-18 | 0 | 1 | 0 | 6 | 6 | 9 | 4 | 0 | | | 6 | High |
| 16-19 | 2 | 9 | 7 | 7 | 6 | 0 | 0 | 0 | | | 6 | High |
| 16-32 | 0 | 0 | 10 | 9 | 4 | 4 | 4 | 0 | | | 6 | High |
| 16-33 | 0 | 0 | 6 | 6 | 5 | 4 | 0 | 0 | | | 6 | High |
| 16-16 | 2 | 0 | 3 | 7 | 4 | 6 | 0 | 0 | | | 5 | High |
| 16-17 | 0 | 0 | 10 | 5 | 7 | 6 | 0 | 0 | | | 5 | High |
| 16-20 | 2 | 9 | 4 | 3 | 4 | 1 | 0 | 0 | | | 5 | High |
| 16-23 | 7 | 3 | 5 | 4 | 4 | 2 | 6 | 0 | 0 | | 5 | High |
| 16-25 | 10 | 6 | 0 | 10 | 10 | 7 | 6 | 3 | 0 | 0 | 5 | High |
| 16-26 | 0 | 0 | 8 | 7 | 2 | 0 | 0 | 0 | | | 5 | High |
| 16-21 | 2 | 5 | 7 | 1 | 0 | 0 | 0 | 0 | | | 4 | Medium |
| 16-22 | 0 | 8 | 0 | 0 | 0 | 0 | 0 | 0 | | | 3 | Medium |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 16-27 | 2 | 3 | 0 | 8 | 2 | 0 | 0 | 0 | | | 3 | Medium |
| 16-34 | 0 | 0 | 0 | 0 | 2 | 6 | 0 | 0 | | | 3 | Medium |
| 16-30 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 0 | | | 2 | Medium |
| 16-8 | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | | | 1 | Low |
| 16-14 | 0 | 2 | 1 | 0 | 4 | 0 | 0 | 0 | | | 1 | Low |
| 16-24 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | | | 1 | Low |
| 16-1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | 0 | Low |
| 16-2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | 0 | Low |
| 16-3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | 0 | Low |
| 16-4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | 0 | Low |
| 16-5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | 0 | Low |
| 16-6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | 0 | Low |
| 16-7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | 0 | Low |
| 16-9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | 0 | Low |
| 16-12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | 0 | Low |

**Table 18 Daily joint calf scores for faecal consistency and demeanour for year 2 (2017) on each scoring occasion along with their overall score and severity group to which they were assigned.**

| | Scoring Occasion (Every second day following birth) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Calf ID | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | Overall Score | Severity Group |
| 17-8 | 8 | 7 | 5 | 3 | 0 | 0 | 0 | 0 | | | 7 | High |
| 17-9 | 4 | 4 | 6 | 8 | 6 | 2 | 0 | 0 | | | 7 | High |
| 17-27 | 0 | 3 | 9 | 9 | 4 | 3 | 0 | 0 | | | 7 | High |
| 17-31 | 0 | 2 | 6 | 5 | 5 | 8 | 8 | 3 | 0 | 0 | 7 | High |
| 17-15 | 0 | 4 | 0 | 7 | 8 | 4 | 0 | 0 | | | 6 | High |
| 17-19 | 0 | 0 | 6 | 7 | 9 | 3 | 0 | 0 | | | 6 | High |
| 17-20 | 0 | 3 | 8 | 7 | 1 | 4 | 7 | 2 | 0 | 0 | 6 | High |
| 17-23 | 4 | 1 | 9 | 8 | 4 | 4 | 3 | 0 | 0 | | 6 | High |
| 17-26 | 0 | 0 | 7 | 6 | 5 | 5 | 5 | 0 | 0 | | 6 | High |
| 17-12 | 0 | 2 | 0 | 6 | 7 | 2 | 4 | 6 | 0 | 0 | 5 | High |
| 17-24 | 1 | 0 | 8 | 8 | 0 | 0 | 0 | 0 | | | 5 | High |
| 17-13 | 0 | 2 | 0 | 6 | 5 | 0 | 0 | 0 | | | 4 | Medium |
| 17-16 | 0 | 3 | 7 | 2 | 3 | 0 | 0 | 0 | | | 4 | Medium |
| 17-17 | 0 | 0 | 2 | 7 | 4 | 4 | 6 | 0 | 0 | | 4 | Medium |
| 17-21 | 0 | 0 | 0 | 6 | 6 | 7 | 0 | 0 | | | 4 | Medium |
| 17-22 | 3 | 0 | 7 | 4 | 4 | 8 | 1 | 0 | 0 | | 4 | Medium |
| 17-25 | 0 | 1 | 0 | 5 | 5 | 6 | 0 | 0 | | | 4 | Medium |
| 17-32 | 0 | 3 | 2 | 7 | 4 | 4 | 2 | 0 | 0 | | 4 | Medium |
| 17-7 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | | | 3 | Medium |
| 17-14 | 0 | 0 | 0 | 5 | 5 | 0 | 2 | 2 | 0 | 0 | 3 | Medium |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 17-28 | 0 | 0 | 6 | 2 | 3 | 0 | 0 | 0 | | | 3 | Medium |
| 17-29 | 0 | 2 | 6 | 1 | 1 | 0 | 0 | 0 | | | 3 | Medium |
| 17-33 | 0 | 2 | 0 | 6 | 2 | 2 | 2 | 0 | 0 | | 3 | Medium |
| 17-36 | 0 | 2 | 2 | 0 | 7 | 2 | 0 | 0 | | | 3 | Medium |
| 17-11 | 0 | 0 | 2 | 0 | 6 | 0 | 6 | 0 | | | 2 | Medium |
| 17-34 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 0 | | | 1 | Low |
| 17-1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | 0 | Low |
| 17-2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | 0 | Low |
| 17-3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | 0 | Low |
| 17-4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | 0 | Low |
| 17-5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | 0 | Low |
| 17-6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | 0 | Low |
| 17-10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | 0 | Low |
| 17-18 | 0 | 0 | 9 | 10 | Died due to cryptosporidiosis | | | | | | | |
| 17-30 | 0 | 2 | 6 | Died due to heart condition | | | | | | | | |
| 17-35 | 1 | 11 | Died due to cryptosporidiosis | | | | | | | | | |
| 17-37 | 0 | 1 | 11 | 10 | Died due to cryptosporidiosis | | | | | | | |

## 3.4.2 Weights of Calves Collected over 6 months

**Table 19 Weights of calves at birth, 3, 4 and 6 months in year 1, 2016. *The birth weight was estimated by the farmer.**

| Calf ID | Crypto Severity | Birth Weight* | 3 months | 4 months | 6 months |
|---|---|---|---|---|---|
| 16-28 | High | 45 kg | 115 kg | 145.5 kg | 200 kg |
| 16-10 | High | 55 kg | 170 kg | 220.5 kg | 289.5 kg |
| 16-29 | High | 40 kg | 138.5 kg | 188 kg | 259 kg |
| 16-31 | High | 65 kg | 163 kg | 219.5 kg | 272.5 kg |
| 16-11 | High | 65 kg | 157.5 kg | 206.5 kg | 290.5 kg |
| 16-13 | High | 50 kg | 146.5 kg | 190 kg | 246 kg |
| 16-15 | High | 55 kg | 139 kg | 193 kg | 264 kg |
| 16-18 | High | 55 kg | 146 kg | 190 kg | 252 kg |
| 16-19 | High | 55 kg | 189.5 kg | 239.5 kg | 300.5 kg |
| 16-32 | High | 55 kg | 145.5 kg | 192.5 kg | 250 kg |
| 16-33 | High | 40 kg | 151.5 kg | 202.5 kg | 270 kg |
| 16-16 | High | 50 kg | 158 kg | 205 kg | 259.5 kg |
| 16-17 | High | 50 kg | 170 kg | 214.5 kg | 285.5 kg |
| 16-20 | High | 55 kg | 139 kg | 183.5 kg | 233 kg |
| 16-23 | High | 45 kg | 136 kg | 186 kg | Excluded – Salmonella |
| 16-25 | High | 60 kg | 145.5 kg | 188 kg | 244 kg |
| 16-26 | High | 50 kg | 122 kg | 162 kg | 220.5 kg |
| 16-21 | Medium | 55 kg | 164 kg | 217 kg | 296 kg |
| 16-22 | Medium | 50 kg | 171 kg | 215 kg | 279 kg |
| 16-27 | Medium | 45 kg | 149 kg | 192 kg | 250 kg |
| 16-34 | Medium | 50 kg | 151 kg | 196 kg | 247.5 kg |
| 16-30 | Medium | 50 kg | 148.5 kg | 198.5 kg | 280 kg |
| 16-8 | Low | 55 kg | 188.5 kg | 244 kg | 302 kg |
| 16-14 | Low | 60 kg | 183 kg | 228 kg | 286.5 kg |
| 16-24 | Low | 65 kg | 138 kg | 189 kg | 259 kg |
| 16-1 | Low | 55 kg | 185 kg | 225.5 kg | 296.5 kg |
| 16-2 | Low | 55 kg | 187 kg | 239 kg | 300 kg |

| | | | | | |
|---|---|---|---|---|---|
| 16-3 | Low | 55 kg | 177.5 kg | 222 kg | 286 kg |
| 16-4 | Low | 55 kg | 203 kg | 260.5 kg | 330 kg |
| 16-5 | Low | 55 kg | 179 kg | 218 kg | 270.5 kg |
| 16-6 | Low | 55 kg | 193 kg | 249 kg | 301 kg |
| 16-7 | Low | 55 kg | 181 kg | 230 kg | 298.5 kg |
| 16-9 | Low | 55 kg | 188.5 kg | 249 kg | 319 kg |
| 16-12 | Low | 65 kg | 156 kg | 202 kg | 264 kg |

Table 19 shows the weights collected at 3, 4 and 6 months of age for calves in year 1 (2016). The mean weight of the calves at birth was 52.81 kg for the high scoring group, 49.16 kg for the medium scoring group and 57.08 kg for the low scoring group with standard deviations of 7.30 kg, 3.76 kg and 3.96 kg respectively. The mean weight of the calves at three months of age was 149.78 kg for the high scoring group, 153.25 kg for the medium scoring group and 179.96 kg for the low scoring group with standard deviations of 18.44 kg, 12.44 kg and 17.25 kg respectively. The mean weight of the calves at 4 months of age was 196.28 kg for the high scoring group, 200.75 kg for the medium scoring group and 229.67 kg for the low scoring group with standard deviations of 22.66 kg, 12.56 kg and 20.51 kg respectively. The mean weight of the calves at their final 6-month weighing was 258.53 kg for the high scoring group, 270.50 kg for the medium scoring group and 292.75 kg for the low scoring group with standard deviations of 26.88 kg, 20.99 kg and 21.05 kg respectively. This 6-month data has one animal excluded (16-23. Table 19) due to being diagnosed with a *Salmonella* infection.

The weight of the animals over the three weighing periods after birth is summarised in Figure 11. This figure shows that the gap in weight between the cryptosporidiosis severity groups occurs within the first three months and is maintained until the animal is 6 months old. It also shows that even animals with a mid-range severity score suffer a drop in weight compared to the animals with no signs of clinical disease.

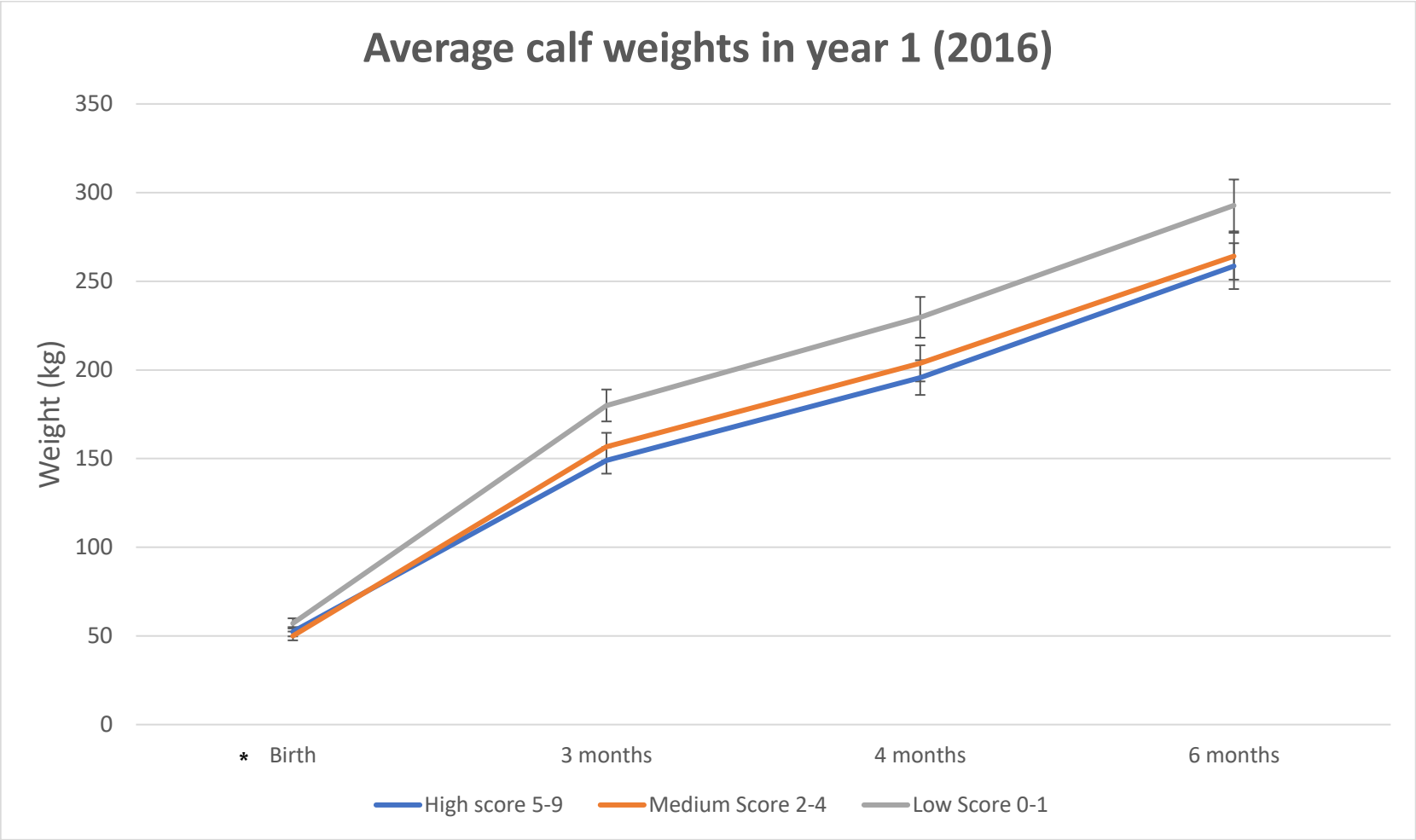


**Figure 11 Average weights of calves over a 6-month period based on their cryptosporidiosis severity level. Error bars represent 95% confidence interval of the mean. *Birth weight is estimated by the farmer.**

**Table 20 Weights of calves at birth, 4, 5 and 6 months in year 2, 2017.**

| Calf ID | Severity | Birth Weight | 4 months | 5 months | 6 months |
|---|---|---|---|---|---|
| 17-8 | High | 34 kg | Excluded *Mycoplasma* | | |
| 17-9 | High | 42 kg | 165 kg | 227 kg | 268 kg |
| 17-27 | High | 55 kg | 223 kg | 289 kg | 347 kg |
| 17-31 | High | 46 kg | 108 kg | 135 kg | Excluded lost mother |
| 17-15 | High | 48 kg | 177 kg | 227 kg | 272 kg |
| 17-19 | High | 44 kg | 174 kg | 237 kg | 281.5 kg |
| 17-20 | High | 40 kg | 171.5 kg | 218 kg | 242.5 kg |
| 17-23 | High | 53 kg | 159.5 kg | 222 kg | 265 kg |
| 17-26 | High | 55 kg | 177 kg | 227 kg | Lost/Stolen |
| 17-12 | High | 60 kg | 203 kg | 262 kg | 318.5 kg |
| 17-24 | High | 54 kg | 184.5 kg | 233 kg | 280 kg |
| 17-13 | Medium | 41 kg | 183 kg | 238 kg | 281.5 kg |
| 17-16 | Medium | 44 kg | Excluded amputated leg | | |
| 17-17 | Medium | 41 kg | 173 kg | 226 kg | 263 kg |
| 17-21 | Medium | 42 kg | 218 kg | 275 kg | 341 kg |
| 17-22 | Medium | 47 kg | 171 kg | 231.5 kg | 257 kg |
| 17-25 | Medium | 45 kg | 182.5 kg | 249 kg | 314.5 kg |
| 17-32 | Medium | 41 kg | 116.5 kg | 156.5 kg | 199.5 kg |
| 17-7 | Medium | 44 kg | 213.5 kg | 263 kg | 333 kg |
| 17-14 | Medium | 50 kg | 207 kg | 265 kg | 331 kg |
| 17-28 | Medium | 49 kg | 185 kg | 246 kg | 288 kg |
| 17-29 | Medium | 49 kg | 160 kg | 211.5 kg | 251 kg |
| 17-33 | Medium | 46 kg | 129.5 kg | 172 kg | 204 kg |
| 17-36 | Medium | 45 kg | Excluded *Mycoplasma* | | |
| 17-11 | Medium | 44 kg | 178 kg | 218 kg | 250 kg |
| 17-34 | Low | 48 kg | 174 kg | 241 kg | 300 kg |
| 17-1 | Low | 39 kg | 241 kg | 283.5 kg | 318 kg |
| 17-2 | Low | 50 kg | Excluded *Mycoplasma* | | |
| 17-3 | Low | 53 kg | 235 kg | 279 kg | 310 kg |
| 17-4 | Low | 54 kg | 209 kg | 265 kg | 304 kg |
| 17-5 | Low | 44 kg | 198 kg | 259 kg | 294 kg |
| 17-6 | Low | 41 kg | 183.5 kg | 244.5 kg | 285 kg |
| 17-10 | Low | 39 kg | 194 kg | 244 kg | 292 kg |

Table 20 shows the weights collected at birth, 4, 5 and 6 months of age for calves in year 2 (2017). The mean weight of the calves at birth was 46.25 kg for the high scoring group, 46.5 kg for the medium scoring group and 46 kg for the low scoring group with standard deviations of 6.58 kg, 5.14 kg and 6.09 kg respectively. The mean weight of the calves at 4 months of age was 175.12 kg for the high scoring group, 175.8 kg for the medium scoring group and 204.93 kg for the low scoring group with standard deviations of 20.48 kg, 5.14 kg and 6.09 kg respectively. The mean weight of the calves at 5 months of age was 228.96 kg for the high scoring group, 228.1 kg for the medium scoring group and 259.43 kg for the low scoring group with standard deviations of 36.72 kg, 38.34 kg and 17.29 kg respectively. The mean weight of the calves at their final weighing at six months of age was 281.7 kg for the high scoring group, 277.1 kg for the medium scoring group and 300.43 kg for the low scoring group with standard deviations of 34.71 kg, 49.51 kg and 11.28 kg respectively. The weight of the animals over the three weighing periods after birth is summarised in Figure 12. This figure supports the results shown in the first year in that that the gap in weight between the cryptosporidiosis severity groups occurs within the first four months and is maintained until the animal is 6 months old.

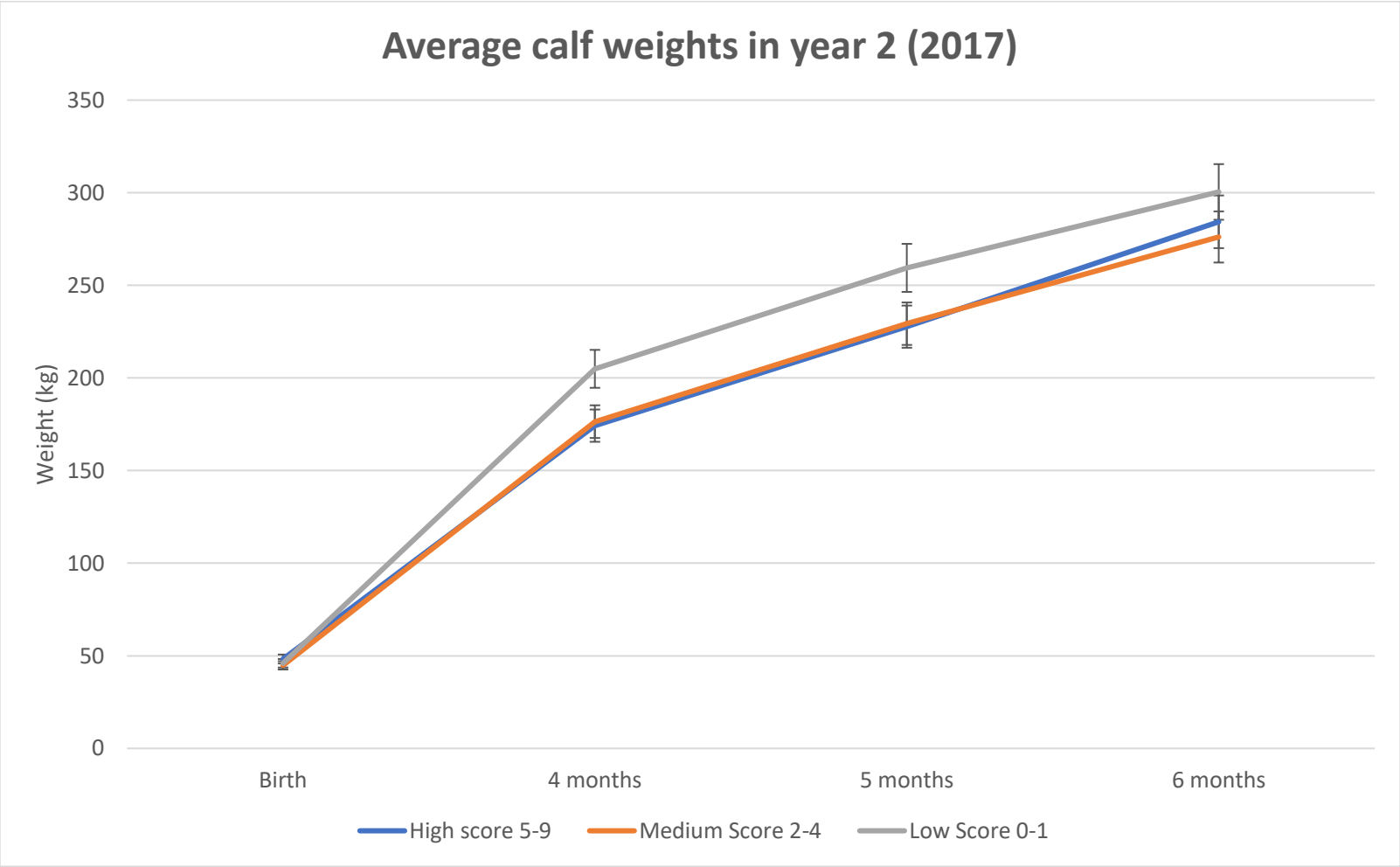


**Figure 12 Average weights of calves over a 6-month period based on their cryptosporidiosis severity level. Error bars represent 95% confidence interval of the mean.**

### 3.4.3 Effect on Calf Growth Results for Year 1

In year 1, results indicate that animals with severe cryptosporidiosis have reduced growth over a 6-month period when compared to animals with no clinical signs of the disease using estimated birth weights. A boxplot for the weight gain over a 6-month period in the three groups of animals can be seen in Figure 13. Preliminary results suggest that the likely weight difference would be around 29.95 kg between severely infected and calves with no clinical signs of disease.

In year 1 there was no significant association between the gender of the calf and the severity of cryptosporidiosis which occurred (p= 0.386).

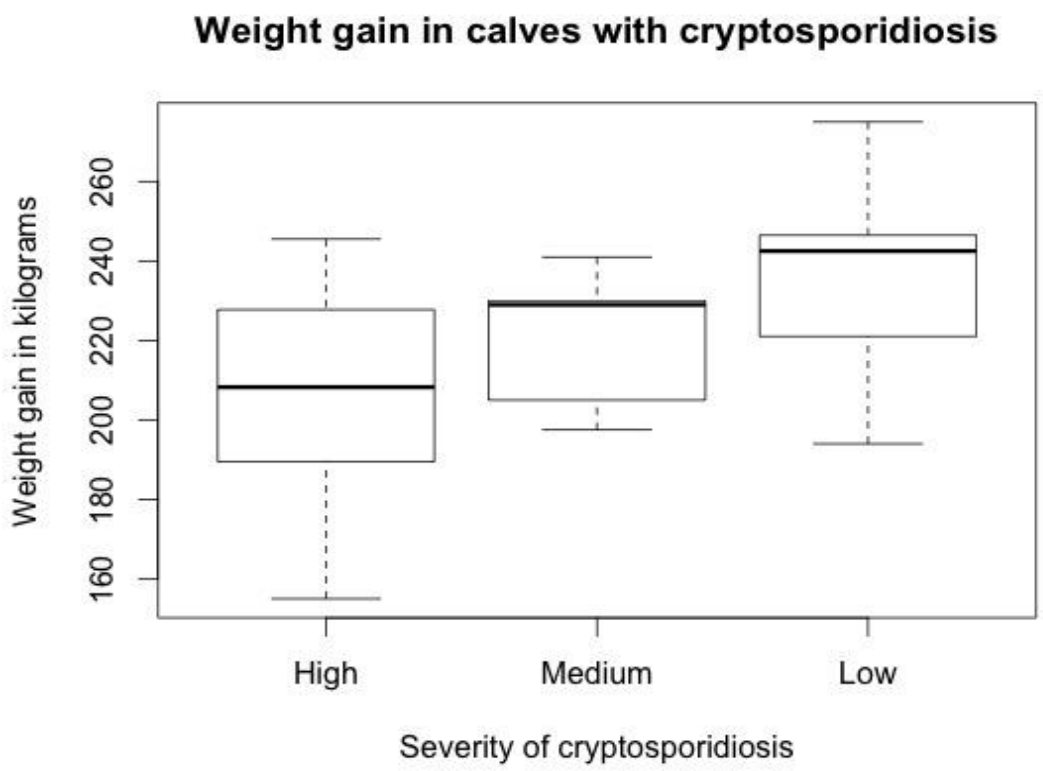


**Figure 13 Comparison of weight gain from birth to 6 months in calves with different severity of cryptosporidiosis for year 1 (2016) 1. Severe clinical disease 2. Mid-range disease 3. No clinical disease. The rectangle represents the second and third quartiles, the horizontal line inside indicates the median value and the lower and upper quartiles are shown as vertical lines either side of the rectangle. Note: Birth weights are an estimation in this year.**

Figure 13 displays a boxplot where the central line illustrates the median weight gain for each group. This is 208.25 kg for severely affected calves, 229 kg for mid-range disease and 242.5 kg for calves with no clinical disease. The minimum and maximum values are represented by the ends of the box whiskers. This is 155 kg – 245.5 kg for severely affected calves, 197.5 kg -241 kg for mid-range disease and 194 kg – 275 kg for calves with no clinical disease.

### 3.4.4 Effect on Calf Growth Results for Year 2

In 2017, calves in the severely infected group were 39.7 kg lighter on average than calves with no clinical signs of disease. Figure 14 shows the median weight gain for each group is 223 kg for severely affected calves, 239 kg for mid-range disease and 255 kg for calves with no clinical disease. The minimum and maximum values are represented by the ends of the box whiskers. This is 202.5 kg – 237.5 kg for severely affected calves, 158 kg - 292 kg for mid-range disease and 244 kg – 299 kg for calves with no clinical disease.

Animals with severe cryptosporidiosis had a significantly reduced growth over a 6-month period ($p=0.008$) when compared to those animals with no clinical disease. Again, those animals which were in the mid-range group showed no statistically significant difference to the other two groups ($p = 0.466$ between severe and mid-range groups) and ($p = 0.474$ between mid-range and no clinical disease groups). The results for the weight gain in the three groups can be seen in Figure 14. In 2017, there was no significant association between the gender of the calf and the severity of cryptosporidiosis which occurred ($p = 0.880$).

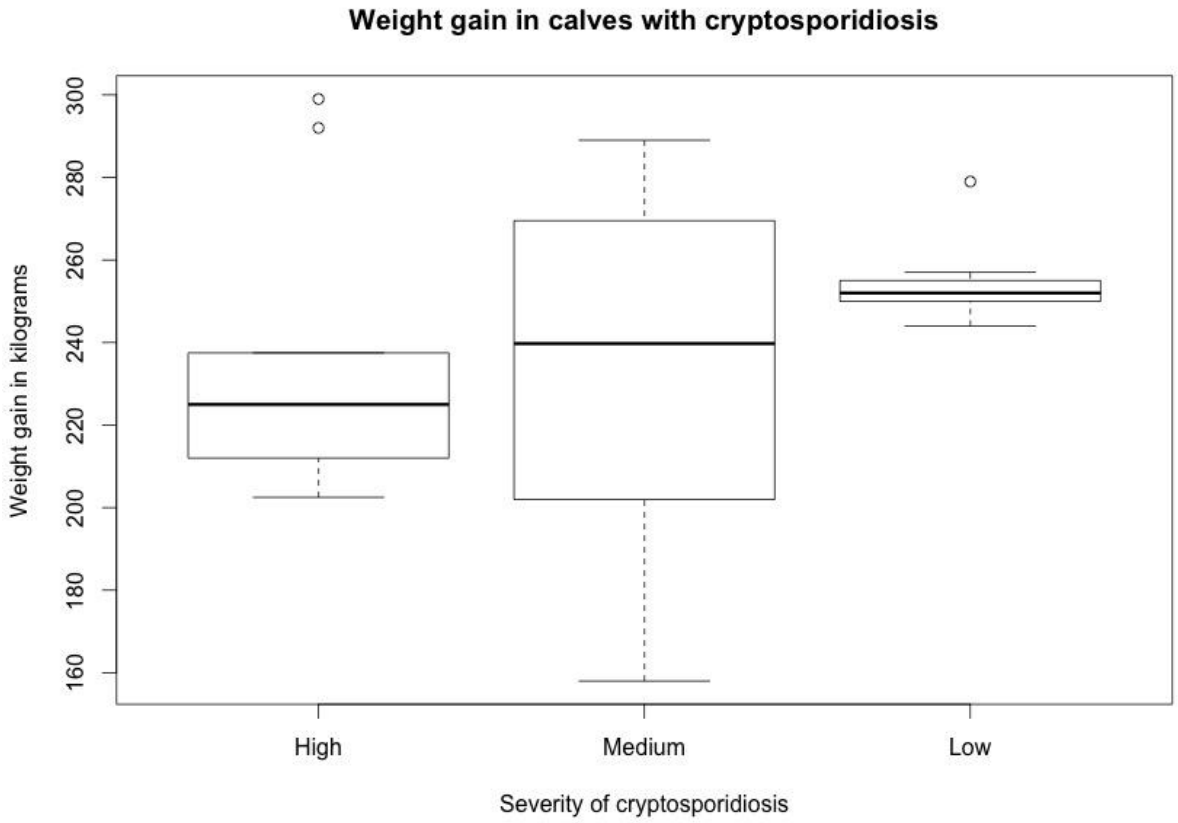


**Figure 14 Comparison of weight gain from birth to 6 months in calves with different severity of cryptosporidiosis for year 2 (2017) 1. Severe clinical disease 2. Mid-range disease 3. No clinical disease. The rectangle represents the second and third quartiles, the horizontal line inside indicates the median value and the lower and upper quartiles are shown as vertical lines either side of the rectangle.**

### 3.4.5 Health monitoring

All animals in this study tested negative for coronavirus, rotavirus and *E. coli* F5 (K99) and all animals included in this study tested positive for *C. parvum* using 18S nested species-specific PCR. One animal was diagnosed with *Salmonella* in 2016 and was excluded from the study. Three animals in 2017 fell sick with *Mycoplasma* infections and these were excluded from the study. One animal in 2017 suffered a broken leg which was amputated and another went missing and was presumed stolen and so these were also excluded.

### 3.4.6 Genotyping Results

Twenty-six calves (76.5%) which were scored in the first 16-20 days of life tested positive for *C. parvum* in the year 2016 and 26 (92.9%) positive in 2017. Of those, 22 and 25 were successfully genotyped in 2016 and 2017 respectively using GP60, MM5, MM18, MM19 and TP14. The genotyping results from calf samples can be seen in Table 21. All calves over the two years were infected with multilocus genotype MLG 10 with mixed infections present in 9 calves in 2016 (Table 21) and 1 calf in 2017 (Table 22). MLG 10 dominated all but one of these mixed infections (16-15, Table 21). All mixed infections where MLG 10 was dominant were mixed with the minor MLG (IIaA17G1R1, 2, 1, 9, 1).

**Table 21 Genotyping results for year 1 (2016) calves.**

| CALF | GP60 | MM5 | MM18 | MM19 | TP14 | MLG |
|---|---|---|---|---|---|---|
| 16-11 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 16-13 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 16-18 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 16-15 | IIaA17G1R1 | 2 | 1 | 10 + 3 | 1 | 11 |
| 16-20 | IIaA17G1R1 | 2 | 1 | 3 + 9 | 1 | 10 |
| 16-19 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 16-22 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 16-25 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 16-24 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 16-21 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 16-14 | IIaA17G1R1 | 2 | 1 | 3 + 9 | 1 | 10 |
| 16-12 | IIaA17G1R1 | 2 | 1 | 3 + 9 | 1 | 10 |
| 16-10 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 16-16 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 16-17 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 16-27 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 16-30 | IIaA17G1R1 | 2 | 1 | 3 + 9 | 1 | 10 |
| 16-29 | IIaA17G1R1 | 2 | 1 | 3 + 9 | 1 | 10 |
| 16-31 | IIaA17G1R1 | 2 | 1 | 3 + 9 | 1 | 10 |
| 16-28 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 16-34 | IIaA17G1R1 | 2 | 1 | 3 + 9 | 1 | 10 |
| 16-32 | IIaA17G1R1 | 2 | 1 | 3 + 9 | 1 | 10 |

**Table 22 Genotyping results for year 2 (2017) calves.**

| Calf | GP60 | MM5 | MM18 | MM19 | TP14 | MLG |
|---|---|---|---|---|---|---|
| 17-25 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 17-27 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 17-32 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 17-5 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 17-15 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 17-19 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 17-26 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 17-34 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 17-20 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 17-18 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 17-14 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 17-36 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 17-11 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 17-33 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 17-17 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 17-22 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 17-23 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 17-31 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 17-9 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 17-12 | IIaA17G1R1 | 2 + 3 | 1 | 3 | 1 | 10 |
| 17-7 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 17-37 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 17-29 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 17-30 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |

## 3.5 Discussion

**Determine the effect that cryptosporidiosis has on the growth of beef calves over a 6-month period.**

The long-term effect in calves following neonatal cryptosporidiosis is currently unknown. However, these findings indicate that severe disease at a young age does significantly reduce the growth rate of calves over a 6-month period. Diarrhoea does reduce the growth of calves (Pardon et al., 2013; Postema & Mol, 1984) however data directly attributing reduced growth rate to *Cryptosporidium* is limited to a single study which only examined the calves for 21 days (Klein et al., 2008).

The results of this study address this knowledge gap and showed a significant difference in weight gain between the severely affected calves and those with no clinical signs of disease. This was suggested in the preliminary data collected in the first year, although without birth weights for the calves this cannot be assumed. This was the reason for the study being repeated in the second year which confirmed the first year's preliminary results. The results of this study are in contrast to a study done in New York where growth rate in dairy calves with diarrhoea were examined (Virtala et al., 1996). That study found no significant difference in growth rate between calves with diarrhoea and those without in the first three months of life. They were unable to attribute *Cryptosporidium* as the cause of the diarrhoea however, and so it could be that it was not the causative agent. The measurements were also estimated using a commercial tape around the heart girth rather than using scales. In this study, *Cryptosporidium* was identified as being the major cause of the diarrhoea observed in both experiments and weigh scales were used to provide more accurate readings.

There was no significant difference between calves in the mid-range disease level compared to the severe disease and 'no clinical disease'

group. This is likely to be attributed to the scoring system used. 'Mid-range disease' covered a wide range of disease manifestations from a single episode of diarrhoea to those which suffered for three days. Many calves suffer what is known as 'white scours' or 'milk scours' which occurs when either the calf drinks more milk than it can absorb, or if there is a high level of long chain unsaturated fatty acids in the milk (Okada, Goto, Furukawa, Ikuta & Yasuda., 2009). This type of scour is not considered problematic and tends to clear up very quickly. As calves in the no clinical disease group still tested positive for *C. parvum* using the 18S PCR, it could be that some of the mid-range disease animals were placed there due to misidentified white scour rather than scour caused by *C. parvum*. Despite this, the results show an increase in median weight for each disease group as the clinical severity of cryptosporidiosis decreases.

It would have been desirable to have weighed the calves for longer than 6 months. However, due to this study being completed on a working farm which sold the animals at 6 months of age to a variety of different buyers, the study had to adapt to the farmer and farm management practices. Weighing calves for longer would have given a better indication as to whether or not the animals eventually catch up in their growth. Research on children with cryptosporidiosis between 0 – 5 months of age in Peru were found never to catch up with children post infection with the control group (Checkley et al., 1998). Therefore, it is likely that calves would not have caught up to the group with no clinical disease. With the results that are available on growth rates from this study, it is indicated that the damage in weight gain occurs very early in life, with the animals keeping that gap between severity groups over the coming months, this can be seen in Figure 11 and Figure 12.

New research published after the completion of this study from Estonia has shown that calves infected with *Cryptosporidium* and treated with halofuginone lactate had a lower weight gain than those calves infected

with *Cryptosporidium* that were not treated (Niine, Dorbek-Kolin, Lassen & Orro., 2018). Calves on the farm in this study in Perthshire were treated with halofuginone lactate when diarrhoea was observed and so this research from Estonia could indicate that it is actually the treatment that is causing the reduction in weight gain. However, the calves in the 'not treated group' in the Estonia study were on average a week older than the calves in the treated groups. The calves in the treated group were also, on average, 16.8 kg lighter than the untreated animals. Therefore, as calves averaged a weight gain in the region of 750-860 g per day in that study, then it could be that the difference between these groups is not as large as its authors concluded and that time was a confounding factor. This study also reported that 14/21 animals that were not treated died and 71% of the deaths were caused by diarrhoea. Animals which died during that study were not submitted for necropsy, and instead the cause of death was attributed to what was most likely based on the calves' symptoms. Therefore, even if in the unlikely event that halofuginone lactate does result in a weight gain reduction, not treating infected calves would result in a higher economic loss overall. The cause of death for six calves was listed as 'respiratory infection' and so it could be that this infection may have also had an effect on the weight gain in these groups.

Gastrointestinal pathogens such as rotavirus, coronavirus, *Salmonella* and *E. coli* are considered to be widespread in calves (Blanchard, 2012) with an estimated prevalence of rotavirus, the next most important pathogen responsible for diarrhoea in cattle, being 33.3% based on published literature over the last three decades (Papp et al., 2013). There is evidence that infection with some of these pathogens also results in growth stunting. In experimental mice, *E. coli* infection was associated with growth impairment compared to control animals (Roche, Cabel, Sevileja, Nataro & Guerrant., 2010). It was fortunate that none of the tested calves tested positive for these pathogens. This means that the reduced weight gain between the groups of calves could be more accurately attributed to

*C. parvum*. It would be impossible to rule out all potential pathogenic causes of reduced weight gain in calves and so it is possible that these animals also suffered from alternative infections. All animals were housed and grazed together under the same management, along with having their health records monitored, and so the chances of this are small.

The multilocus genotype of *C. parvum* was the same in all calves over both years with little evidence of mixed infections (MLG 10) which can be seen in Table 21 and Table 22 in 3.4.6. This single genotype present suggests that instead of some calves being affected with a more virulent genotype, some of the calves must have had a better immune response, and so have an increased ability at dealing with the parasite. There is no association between *C. parvum* shedding and diarrhoea in calves, and therefore there are more factors at play which affect the severity of disease rather than oocyst shedding alone (Glombowsky et al., 2017; Silverlås, de Verdier et al., 2010). The infectious dose that each calf received was unknown as calves were examined in a natural farm setting. The use of individual pens for calving on beef farms could lead to a build-up of *Cryptosporidium* oocysts and other pathogens which are shed by cows and their calves over the duration of the calving season, thereby exposing calves born later in the season to a higher infectious dose. This higher infectious dose would increase the likelihood of the calf developing diarrhoea (Blanchard, 2012). Work looking at growth impairment caused by *E.coli* in mice found that growth impairment was dependant on micro-organism burden and infectious dose (Roche et al., 2010) and so the same is likely to be true for *Cryptosporidium*. This is likely one of the most likely reasons for the difference in disease severity.

Calf housing, the frequency of cleaning and the disinfectant used all have an effect on the prevalence of *Cryptosporidium* on the farm (Castro-Hermida, González-Losada, & Ares-Mazá., 2002) along with feed source, water source and contact with other domestic animals (Ayele, Seyoum &

Leta., 2018). However, as this study was done on one farm with a single management regime, these are not the likely reason for the difference in severity of cryptosporidiosis. This difference in severity of cryptosporidiosis could instead be colostrum quality and quantity that the calf received at birth, the presence of an unidentified pathogen or potentially the animal's genetics (Silverlås et al., 2010). Previous work has found that first calving heifers are likely to have reduced colostrum quality and quantity and so that could have an effect on the severity of cryptosporidiosis seen (Blanchard, 2012). Infection with *Cryptosporidium* has resulted in malnutrition. However, undernutrition also makes the host more susceptible to *Cryptosporidium* (Coutinho et al., 2008). Undernourished children in Kenya aged between 6 – 59 months were more susceptible and more likely to suffer from severe diarrhoeal disease than children without malnutrition (Tickell et al., 2017). Nutritional status, therefore, has an effect on disease susceptibility, and this is further supported by this work, where two calves in the year 2017 both died from cryptosporidiosis in the first week of life. Both of these animals lost their dam shortly after birth and were being hand-reared. It is likely that both of these calves were undernourished, making them more susceptible to the parasite.

**Estimate a cost for the reduction in weight gain caused by cryptosporidiosis in beef calves.**

On average, the farmer for this study received £2.80 per kg when these animals went to market (Farmer – Personal communication). An average difference of 39.7 kg in animal weight at 6 months of age in the second year, which had birth weight measurements, means an animal with severe disease could result in an average loss of £111.16 to the farmer. Once this parasite is on farm, it tends to affect all susceptible animals, neonatal and pre-weaned calves, due to its ability to survive many disinfectants and temperature extremes (Blanchard, 2012; Fujino et al., 2002) and so it is likely that a high proportion of animals would be exposed if the parasite is

already present on the farm. This is the likely explanation for most of the calves (76.5% in 2016 and 92.9 % in 2017) in this study testing positive for *C. parvum* on the 18S multiplex PCR, despite suffering a range of clinical disease.

Further work is required to determine animal susceptibility to *Cryptosporidium* infection in order to identify options to reduce the parasite burden on farms.

## 3.6 Conclusion

This work shows that severe disease with *C. parvum* in neonatal calves significantly reduces their growth rate over a 6-month period. Results suggest that this reduction in growth rate occurs in the first month of life, and these animals fail to catch up over the 6-month period. Based on a specific cost analysis for this particular farm, a severely infected calf could be worth on average £111.16 less when it goes to market when compared with a calf with no clinical signs of the disease. Results from this study indicate that even calves with a mid-range severity of disease suffer from a decrease in weight gain, and so control of this parasite is paramount in reducing the economic impact it presents.

# Chapter 4 The Role of Wildlife in *Cryptosporidium* Transmission to Calves

## 4.1 Introduction

*Cryptosporidium* species are well documented in a wide range of different wildlife species from all over the world. The wide host range of these parasites (Goater et al., 2014) makes this unsurprising. *Cryptosporidium* species similar to *C. parvum* in the rabbit *(Oryctolagus cuniculus)* intestine was first described by Tyzzer in 1912 (Tyzzer, 1912) and was first documented as occurring naturally in rabbits in 1979 (Inman & Takeuchi, 1979). The species attributed to observed oocysts in early studies was assumed to be *C. parvum* due to similarities in oocyst size with the species *Cryptosporidium cuniculus*, although it is assumed that many of these oocysts were misidentified and they were in fact *C. cuniculus* (Appelbee et al., 2005). *C. cuniculus* is the most commonly documented species in the rabbit and was formerly known as the rabbit genotype (Robinson et al., 2010). *Cryptosporidium cuniculus* is the most commonly identified species of *Cryptosporidium* in almost every documented rabbit species in the literature so far, wild and domesticated (Robinson & Chalmers, 2010), and *C. cuniculus* is also reported to infect humans (Puleston et al., 2014). Although rabbits are able to become infected with other *Cryptosporidium* species such as *Cryptosporidium parvum* (*C. parvum*) under laboratory conditions (Mosier, Cimon, Kuhls, Oberst & Simons., 1997), it is not commonly found in the domestic and wild rabbit population (Robinson & Chalmers, 2010).

*Cryptosporidium parvum* was identified using molecular techniques in a wild rabbit in New Zealand (Learmonth, Ionas, Ebbett & Kwan., 2004) by sequencing ITS-1, COWP, ß-tubulin, PolyT, RNR-R1 and ssu rRNA genes. It was also identified in farmed rabbits in China (Xiao et al., 2002), however the sequences used for *C. parvum* identification in both of these studies

were using partial ssu rRNA sequences, and further sequence analysis using phylogenetic analysis and sequence alignment showed that these were actually rabbit genotype, now known as *C. cuniculus* (Robinson & Chalmers, 2010). Many of the existing rabbit studies had no speciation performed at all and so it is unclear what the true prevalence of *C. parvum* in rabbits actually is (Robinson & Chalmers, 2010).

Rabbits tested all over the world have been found to harbour *Cryptosporidium cuniculus*, which could not only be an issue for the rabbits themselves if they suffer clinical disease, but also pose a risk to public health (Robinson & Chalmers, 2010). Clinical disease due to cryptosporidiosis has been reported in farmed rabbits (Kaupke et al., 2014) and neonatal laboratory rabbits (Mosier et al., 1997). Domesticated pet rabbits also show clinical disease (Shiibashi et al., 2006) and could potentially infect humans directly through physical contact via the faecal-oral route, or wild rabbits could infect humans indirectly by contaminating public water courses with zoonotic *Cryptosporidium* species oocysts. However, studies which have been conducted examining the risk that rabbits could play in the transmission of oocysts of zoonotic *Cryptosporidium* species to humans show no evidence to support this. There was no difference between the prevalence of *Cryptosporidium* in companion animals including rabbits in owners with and without cryptosporidiosis (Bern et al., 2002; Smith et al., 2009). These studies conclude that humans are unlikely to be a risk for their pets. Since these studies were conducted, oocysts were passed from rabbit to human following a waterborne outbreak of cryptosporidiosis in Northamptonshire, England which was attributed to the parasite *C. cuniculus*. This occurred when a rabbit gained access and drowned in a treated water tank (Chalmers et al., 2009).

The prevalence of all *Cryptosporidium* species in rabbits, demonstrated by two large-scale studies of 109 wild rabbit samples in Warwickshire

(Chalmers, 1996) and 28 wild rabbit samples from Norfolk (Sturdee, Chalmers & Bull., 1999), is between 0 – 0.9% (Robinson & Chalmers, 2010). Reports have indicated that only low levels of *Cryptosporidium* oocyst (unknown species) shedding are detected in older rabbits (Inman & Takeuchi, 1979; Pavlasek et al., 1996; Shiibashi et al., 2006) The prevalence and oocyst shedding may be low, however very few *C. parvum* oocysts (as low as 25 in some cases) are required to cause infection in calves (Teunis, Chappell & Okhuysen., 2002; Zambrisky et al., 2013) and therefore this low-level shedding, if it was *C. parvum*, could still pose a risk to both the human and livestock population. There is a distinct lack of information on the species and genotypes harboured by the Scottish rabbit population and so this is an area of further interest, especially when *Cryptosporidium parvum* is such a problem on many Scottish cattle farms (Gunn & Stott, 1997).

*Cryptosporidium* in pheasants *(Phasianus colchicus)* has not been reported in the literature so far, except in a single study from the Czech Republic which reported *Cryptosporidium baileyi* and *Cryptosporidium meleagridis* oocyst detection in farmed pheasants (Maca & Pavlasek, 2016). In other avian species, *Cryptosporidium* is more commonly associated with respiratory infections (Randall, 1986; Whittington & Wilson, 1985). Gastrointestinal infections have been reported in other avian species such as turkeys (Bermudez et al., 1988; Goodwin et al., 1988) and quail (Hoerr et al., 1986; Ritter et al., 1986) and these infections tend to be attributed to the species *C. meleagridis*. Pheasants can be in abundance on some farms, especially as 20 million pheasants are released in Britain every year to supplement shooting (Draycott, Parish, Woodburn & Carroll., 2000). Although historically it is not believed that *C. parvum* is commonly found in avian species, a very recent study has found *C. parvum* as the most prevalent species in broilers, layers and turkeys in Germany (Helmy et al., 2017). There is currently no study which reports *C. parvum* in birds coupled with clinical disease and therefore there is no evidence that *C. parvum*

undergoes its lifecycle in birds. Birds could still act as mechanical vectors however and could concentrate oocysts in areas where wild birds congregate, which is very often on farms making them important candidates for further study.

The level of infection in wildlife species is likely to be underestimated. Very little is known about the distribution of *Cryptosporidium* species in wildlife as most research focus is put into human infections and livestock disease with *Cryptosporidium* (Appelbee et al., 2005; Zahedi et al., 2016). In the search for potential transmission routes of *Cryptosporidium* to both humans and livestock, greater emphasis and more time should be spent on researching cryptosporidiosis in wildlife, as evidence linking human outbreaks from wildlife sources is scarce (Zahedi et al., 2016). It is therefore important to define if this scarcity is because wildlife to human transmission of zoonotic *Cryptosporidium* does not happen, or if it does happen but it is not reported because there is a lack of research into it. Focus should be put on researching *Cryptosporidium* species and genotypes in wildlife living in close contact with livestock and water source inlets which should include species such as deer, rabbits, rodents and birds. The main focus for many large-scale wildlife *Cryptosporidium* studies is to understand potential risk to human public health (Appelbee et al., 2005) with many of these studies being in the form of a water catchment study; researching the species and genotypes present at a catchment level which may include livestock, wildlife and the environment (Feng et al., 2007; Koompapong & Sukthana, 2012; Robinson et al., 2011; Wells et al., 2015; Zahedi et al., 2016).

Currently, the primary source of *C. parvum* to calves is unknown and is likely to come from many different sources. With an underdeveloped and naïve immune system, even a low-level infection could very quickly spread throughout a group of naïve calves (Zambrisky et al., 2013). Therefore, the risk of oocyst transmission to calves from wildlife is certainly a possibility.

The population of wild rabbits in Scotland and pheasants in the UK is estimated to be 9.5 million and 35 million, respectively (Harris, Morris, Wray & Yalden.,1995; Musgrove et al., 2013) and with very little information available on the *Cryptosporidium* species and genotypes that these host species harbor, it is important to assess rabbits and pheasants as a potential as a transmission vector of *Cryptosporidium,* with particular focus on *C. parvum*.

## 4.2 Aims

- Determine the prevalence of *C. parvum* in wild rabbits and pheasants.

- Analyse the genotypes shed in the faeces of rabbits and pheasants.

- Assess the risk that these wildlife species pose in the transmission of *C. parvum* to calves.

## 4.3 Methods

### 4.3.1 Sample Collection

Faecal samples from 359 rabbits (*Oryctolagus cuniculus)* collected from 18 different farms in Scotland were used for this study. All of these farms were selected for inclusion by identifying farms from which samples were available from other studies. A proportion of these samples (16 farms) were collected as part of a previous study testing for *Mycobacterium avium subspecies paratuberculosis* (*Map*) (Fox et al., 2018) and were collected over a period of 3 years from rabbits which were euthanised. The other two farms were included as samples were also available from previous studies. These two farms were located in Scotland and 10 rabbit faecal samples were collected from each farm on one occasion in December 2017. Once transported to the laboratory, these rabbit samples were stored in the fridge at 4°C until they could be processed.

Faecal samples from 50 pheasants were collected a single day during spring calving during the years 2016 (n= 30) and 2017 (n=20) from a beef suckler farm in Perthshire which was the same farm used as part of the adult cattle to calf study in Chapter 2 and the growth study in Chapter 3. This would allow for direct comparison of species and genotypes of *Cryptosporidium* between the pheasants and the cattle on that farm. Material from pheasants was collected from the ground by observing the birds and collecting whole faecal material from individuals in a bijoux. Once transported to the laboratory, these pheasant samples were stored in the fridge at 4°C until they could be processed.

### 4.3.2 Sample Processing

Each faecal sample from rabbits and pheasants were soaked in 3 ml $H_20$ overnight to break up the pellets and then vortexed to mix. The faecal samples then underwent a salt flotation as described in 2.3.4 (Chalmers et al., 2009). This involved 8 ml of saturated salt solution $NaCl_2$ being added to each sample and mixed thoroughly. 2 ml of $H_20$ was trickled down the

side of the tube to create a layer of water on top of the salt solution. After centrifugation, a vortex was created to lift the oocysts into the water layer which could then be removed with a pipette.

Extraction of parasite DNA was done according to the manufacturer's protocol for DNA extraction and is described in full in 2.3.5, using the NucleoSpin Tissue DNA, RNA and protein purification kit (Macherey-Nagel, NZ740952250) which included an added step of 10 x freeze/thaw cycles in liquid nitrogen (Thomson et al., 2016).

### 4.3.3 Molecular Characterisation

Species of *Cryptosporidium* were determined by 18S PCR. All DNA samples were run in triplicate with a negative control, a DNA extraction control and positive controls. The oligonucleotide primer sequences and PCR conditions were as described previously 18S PCR (Xiao et al., 1999). Each well on the PCR plate contained 10 x PCR buffer (45mM Tris-HCl pH8.8, 11 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 4.4 µl EDTA, 113 µg ml-1 BSA and 1mM each of four dNTP's) (Burrells et al., 2013), 0.5 units BioTaq (Bioline, UK), 10µM each of forward and reverse primers (primers are described in Table 1 in 2.3.6). The conditions were 3 minutes at 94°C, followed by 35 cycles of 45 seconds at 94°C, 45 seconds at 55°C and 1 minute at 72°C. Lastly, there is a 7 minute extension step at 72°C. The PCR amplicons were then run on a 1.5 % agarose gel using gel electrophoresis and stained with GelRedTM (Biotium, UK). The gel was then examined under UV light to look for a DNA band. Any positive 18S PCR amplicons were sent for sequencing using Eurofins Genomics Tubeseq service (described in full in 2.3.8) after DNA was purified using the Qiagen PCR purification kit (Qiagen, Cat No28104). (50)).

The GP60 gene was sequenced in all *C. parvum* positive samples, the protocol is described in full in 2.3.7 along with primers. This PCR is done by using a nested PCR (Brook et al., 2009). Each well on the PCR plate

contained 10 x PCR buffer (45mM Tris-HCl pH8.8, 11 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 4.4 µl EDTA, 113 µg ml-1 BSA and 1mM each of four dNTP's) (Burrells et al., 2013), 0.5 units BioTaq (Bioline, UK), 10µM each of forward and reverse primers. The program was the same as listed for the 18S PCR with a positive and negative control added. The PCR products were then run on 1.5 % agarose gels, stained with GelRedTM (Biotium, UK), using gel electrophoresis and then examined under UV light before being sent for sequencing (2.3.8).

Microsatellite analysis was carried out on a selection of samples from rabbits and all the pheasants which tested positive for *C. parvum* using the 18S PCR and confirmed by sequence analysis. The selection of rabbits was chosen due to the difficulties in PCR amplification and so only rabbits with positive results in all triplicate PCR reactions for both the 18S and GP60 PCR were chosen for microsatellite analysis (n=37). The protocol for microsatellite analysis can be found in 2.3.9, which includes the PCR protocol for the loci MM5, MM18, MM19 and TP14 along with primers used and fragment analysis software.

## 4.4 Results

### 4.4.1 Species of *Cryptosporidium* in Rabbits

The species of *Cryptosporidium* found in 359 wild rabbits located on 18 different farms in Scotland are shown in Table 23 with the frequency of each species shown in Figure 15. The overall prevalence of *Cryptosporidium* species in rabbits was 37.33%. The most common species identified was *C. parvum*, which was present in the faeces of 78 rabbits (21.73 %), one of which was a mixed infection with *C. cuniculus*. *Cryptosporidium parvum* was located on 15 out of the 18 tested farms. *Cryptosporidium cuniculus* was the second most commonly found species, being detected in 21 rabbit samples (5.85 %), one of which was a mixed infection with *C. parvum*, and this species was found on 11 out of 18 farms. *Cryptosporidium andersoni* was the third most common species found, being detected in the faeces of 17 rabbits (4.74 %) across 7 out of 18 farms. A small number of rabbit samples had alternative species; one rabbit sample was positive for *C. ubiquitum,* two for *Cryptosporidium* skunk genotype and one for *Cryptosporidium* deer genotype. Only one rabbit showed signs of mixed species infections, which was a mix of *C. parvum* with *C. cuniculus*. 15 of the rabbits tested positive on the 18S PCR, however, failed sequencing and so the species of *Cryptosporidium* present is unknown. The majority of the tested rabbits (225/359 – 62.67 %) were negative for *Cryptosporidium* based on the 18S PCR.

**Table 23 Species of *Cryptosporidium* found in 359 rabbits over 18 farms in Scotland.**

| Farm ID | Sample no | *C. parvum* | *C. cuniculus* | *C. andersoni* | *C. ubiquitum* | Skunk genotype | Deer genotype | *C. parvum*, *C. cuniculus* mix | Failed sequencing | Negative |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 54 | 12 | 3 | 4 | 0 | 0 | 0 | 0 | 0 | 35 |
| 2 | 26 | 7 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 17 |
| 3 | 9 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 |
| 4 | 28 | 12 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 11 |
| 5 | 19 | 6 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 11 |
| 6 | 16 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 |
| 7 | 35 | 6 | 1 | 2 | 0 | 0 | 0 | 0 | 1 | 25 |
| 8 | 27 | 0 | 1 | 3 | 0 | 1 | 0 | 0 | 8 | 14 |
| 9 | 25 | 1 | 2 | 5 | 0 | 0 | 0 | 0 | 3 | 14 |
| 10 | 27 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 22 |
| 11 | 7 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| 12 | 9 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 7 |
| 13 | 20 | 2 | 2 | 0 | 1 | 0 | 0 | 0 | 0 | 15 |
| 14 | 4 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 3 |
| 15 | 24 | 3 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 18 |
| 16 | 9 | 4 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| 17 | 10 | 4 | 2 | 1 | 0 | 0 | 1 | 0 | 0 | 2 |
| 18 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 |
| **Total** | **359** | **77** | **20** | **17** | **1** | **2** | **1** | **1** | **15** | **225** |

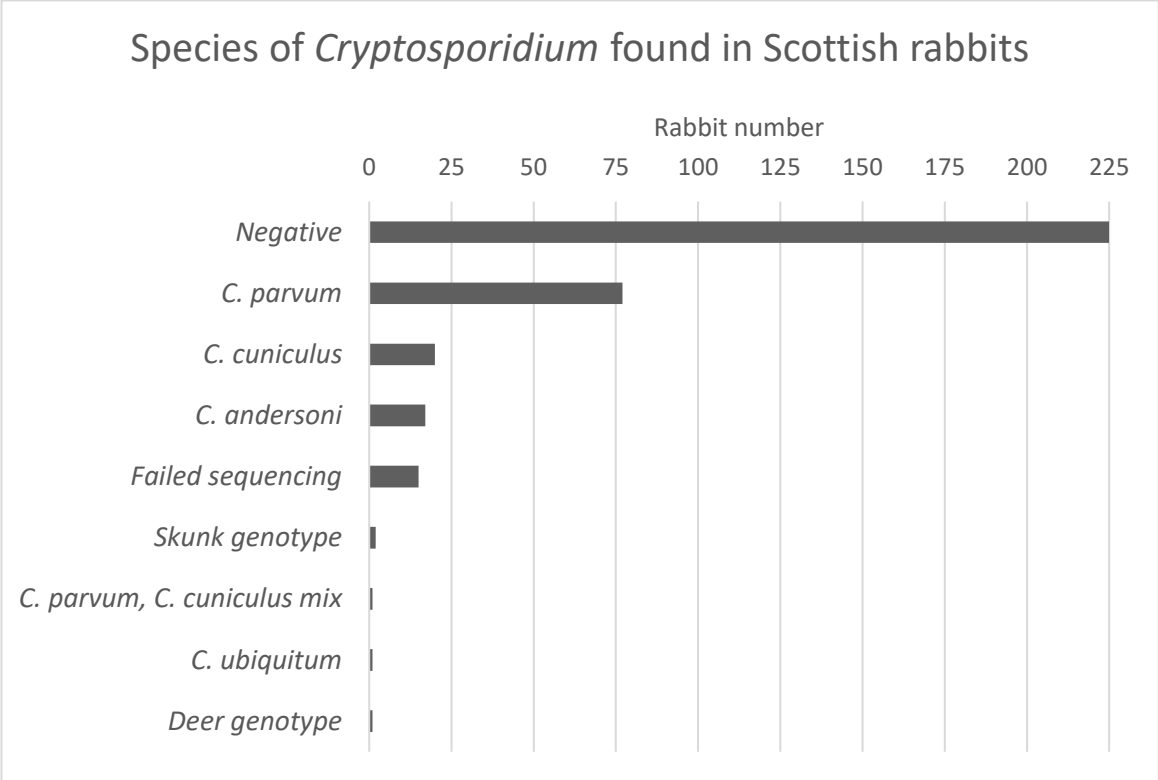


**Figure 15 Species of *Cryptosporidium* detected in 359 Scottish wild rabbits from 18 separate farms collected between 2008 – 2017.**

## 4.4.2 Genotypes of *C. parvum* in Rabbits

Any *C. parvum* positive rabbits underwent GP60 genotyping in order to determine what genotypes are present in wild rabbits in Scotland. Many of the rabbits proved difficult to genotype with 25/77 (32.47 %) proving impossible to genotype at the GP60 locus. Of those that did amplify, 25/77 had IIaA15R1 (32.47 %), 22/77 had IIcA5G3 (28.57 %), 3/77 had IIaA15G2R1 (3.90 %) and 2/77 had IIaA19G2R1 (2.60 %) (Figure 16).

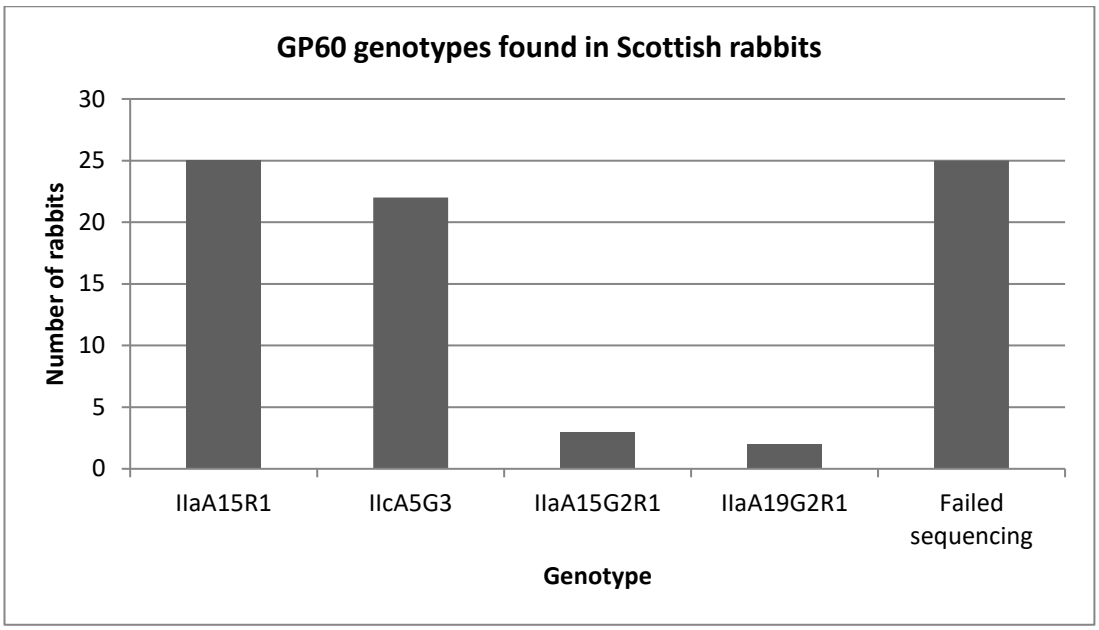


**Figure 16 GP60 genotypes of *C. parvum* present in 77 wild rabbits in Scotland.**

## Microsatellite analysis

Rabbit samples which tested positive in triplicate on the GP60 PCR underwent microsatellite analysis (n= 47) where loci MM5, MM18, MM19 and TP14 were amplified. Of those 47, 29 were successfully genotyped at all 4 of the loci. Genotyping PCR was repeated until no template DNA remained. The full table of fragment size results for 47 rabbit samples along with farm number can be seen in Appendix 3. In brief, 11 different genotypes were identified in 29 rabbit samples (Table 24). There were two multi-locus genotypes (MLG) which were the most prevalent and they were MLG 24 and MLG 27 which were present in 9 and 5 rabbit samples and spread across 5 and 3 farms respectively. Three rabbit samples from 2 farms had MLG 30. Two rabbit samples from one farm had MLG 34, two rabbit samples from two farms had MLG 28 and the other multi-locus genotypes MLG 12, MLG 25, MLG 26, MLG 31, MLG 32, MLG 33, MLG 35 and MLG 36 were only found in a single rabbit each. It is clear that the most common genotypes are present on multiple farms (Figure 17). Genotypes do not seem to be

isolated by farm and instead rabbits on the same farm present with many different *C. parvum* genotypes.

**Table 24 Allele assignment for each locus along with GP60 sequence and multilocus genotype assignment for *C. parvum* positive rabbits.**

| Rabbit ID | Farm no. | MM5 | MM18 | MM19 | TP14 | GP60 | MLG |
|---|---|---|---|---|---|---|---|
| 1 | 1 | 2 | 1 | 3 | 1 | 8 | 24 |
| 2 | 1 | 2 | 1 | 3 | 1 | 8 | 24 |
| 5 | 1 | 2 | 1 | 3 | 1 | 7 | 30 |
| 12 | 1 | 2 | 1 | 3 | 1 | 7 | 30 |
| 16 | 1 | 2 | 1 | 3 | 2 | 7 | 25 |
| 19 | 1 | 1 | 1 | 3 | 1 | 8 | 27 |
| 26 | 2 | 2 | 1 | 1 | 2 | 7 | 34 |
| 28 | 2 | 2 | 2 | 3 | 1 | 8 | 26 |
| 147 | 2 | 2 | 1 | 1 | 2 | 1 | 35 |
| 148 | 2 | 2 | 1 | 3 | 1 | 8 | 24 |
| 149 | 2 | 2 | 1 | 4 | 1 | 8 | 31 |
| 150 | 2 | 2 | 1 | 1 | 2 | 7 | 34 |
| 29 | 3 | 1 | 1 | 1 | 1 | 1 | 12 |
| 30 | 3 | 2 | 1 | 3 | 1 | 8 | 24 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 31 | 3 | 2 | 1 | 3 | 1 | 7 | 30 |
| 33 | 3 | 2 | 1 | 1 | 1 | 8 | 32 |
| 39 | 4 | 2 | 1 | 3 | 1 | 8 | 24 |
| 40 | 4 | 1 | 1 | 3 | 1 | 8 | 27 |
| 125 | 4 | 1+2 | 1 | 1+3 | 1 | 8 | 27 |
| 126 | 4 | 1 | 1 | 3 | 1 | 8 | 27 |
| 49 | 5 | 2 | 1 | 3+1 | 1 | 8 | 24 |
| 50 | 5 | 2 | 1+2 | 3 | 1 | 8 | 24 |
| 51 | 5 | 2 | 1+2 | 3+1 | 1 | 8 | 24 |
| 52 | 5 | 2 | 1 | 3 | 1 | 8 | 24 |
| 68 | 6 | 2 | 2 | 1 | 1 | 8 | 28 |
| 70 | 6 | 1 | 1 | 3 | 1 | 8 | 27 |
| 71 | 6 | 1 | 1 | 3 | 1 | 7 | 33 |
| 79 | 7 | 2 | 2 | 1 | 1 | 8 | 28 |
| 152 | 7 | 1+2 | 1 | 1 | 1 | 7 | 36 |

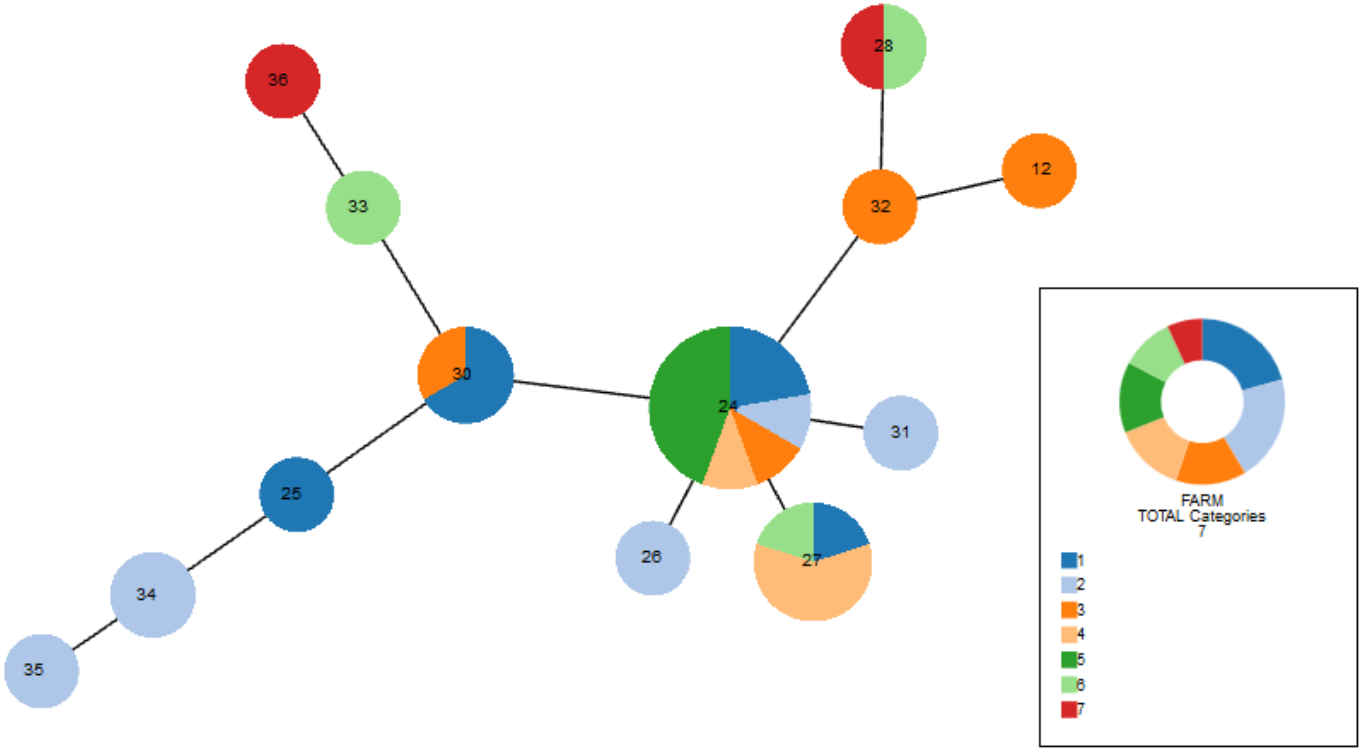


**Figure 17 Multilocus genotypes of *C. parvum* present in a selection of rabbits collected from 359 rabbits on 18 different farms. Each node represents a different multilocus genotype with the MLG number written inside, the size of the node represents rabbit number which possess that genotype and the colour represents the farm number that MLG was found on. Nodes are joined by a line if they share 4 out of 5 alleles of GP60, MM5, MM18, MM19 and TP14. Where mixed infections occurred, the dominant peak was used for analysis.**

### 4.4.3 Species of *Cryptosporidium* in Pheasants

Samples from 30 individual pheasants in 2016 and 20 individual pheasants in 2017 gave a 40 % (11/30) and a 65 % (13/20) prevalence of *Cryptosporidium* in 2016 and 2017 respectively. The total prevalence of *Cryptosporidium* found in faeces of pheasants over the two years was 48 % (24/50).

The species identified in the pheasants was primarily *C. parvum*. In 2016, 8/11 *Cryptosporidium* positive pheasants were shedding *C. parvum* and 3/11 were positive for both *C. bovis* and *C. parvum*. In 2017, 12/13 *Cryptosporidium* positive pheasants were shedding *C. parvum* and the other pheasant *Cryptosporidium* positive PCR product failed sequencing.

### 4.4.4 Genotypes of *C. parvum* in Pheasants

*Cryptosporidium parvum* positive faecal samples from pheasants over both sampling years were analysed at the GP60 gene. The genotype IIaA15G2R1 was found in 5 pheasants and IIaA17G2R1 in another 5 pheasants were present in *C. parvum* positive pheasants in 2016. A single pheasant was carrying the genotype IIaA15R1.

In the pheasant faecal samples collected in 2017, the genotype present was primarily IIaA17G1R1 which was identified in 9/13 pheasants. One pheasant had a mixed infection which was likely to be with IIaA15G2R1 based on the reverse primer sequence read. PCR products from three pheasants, unfortunately, failed to sequence at the GP60 gene.

The overall genotypes which were found in the pheasant faecal samples can be seen in Figure 18.

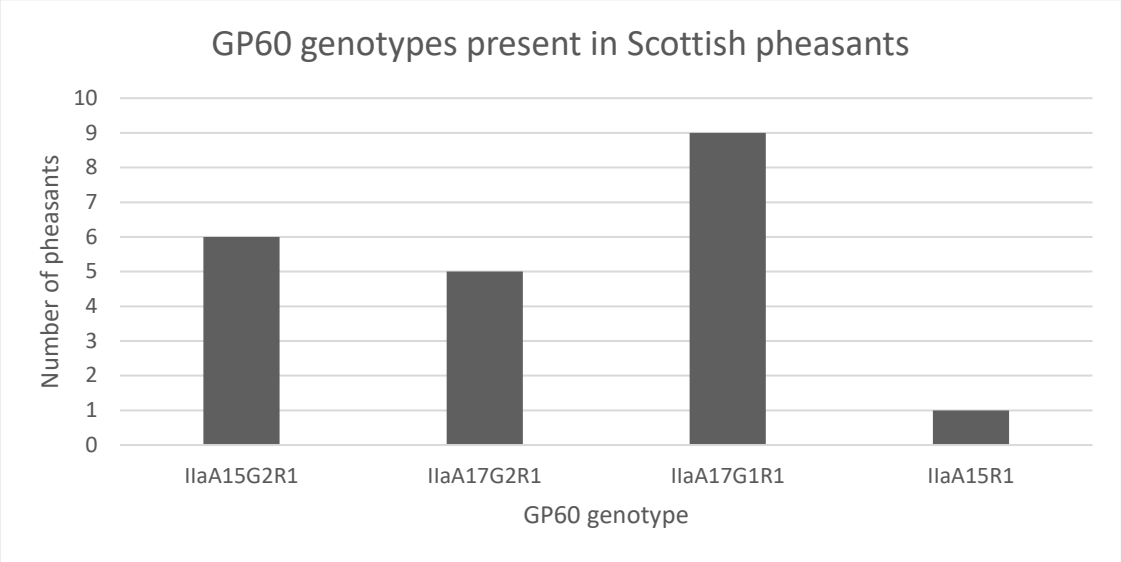


**Figure 18 The GP60 genotypes present in *C. parvum* positive pheasants living wild in Perthshire, Scotland.**

## Microsatellite Analysis

The *C. parvum* positive pheasant samples also underwent microsatellite analysis by amplifying the MM5, MM18, MM19 and TP14 regions. The full results for microsatellite analysis, along with mixed infections can be seen in Appendix 4 (Allele assignment description and associated fragment sizes can be seen in 2.3.9). A summary of the multilocus genotypes present in the pheasant samples can be seen in Table 25. Three pheasant samples had the species *C. parvum* which was MLG 10, two had MLG 16, two had MLG 19, a single pheasant was shedding MLG 17, one pheasant was shedding MLG 18 and another was positive for MLG 20. Both pheasant samples with MLG 18 and MLG 20 had mixed infections present at the MM5 locus.

**Table 25 Multilocus genotypes of *C. parvum* in Scottish pheasants.**

| Multilocus genotype | Number of pheasants | Notes |
|---|---|---|
| 10 | 3 | |
| 16 | 2 | |
| 17 | 1 | |
| 18 | 1 | Mixed infection |
| 19 | 2 | |
| 20 | 1 | Mixed infection |

### 4.4.5 Comparison of Pheasant *C. parvum* Genotypes with Calves

The genotyping results for the cattle and calves present on the same farm analysed over the same time period in which the pheasants were collected can be seen in full in the results section of Chapter 3. The results are summarised in Figure 19.

In 2016, the multilocus genotypes present in the calves was MLG 10 in 21 calves and MLG 11 present in one calf which was present as a mixed infection with MLG10. In 2017, all 24 calves had MLG 10 present in their faecal sample. Overall, only three of the 50 tested pheasants over the two years had MLG 10 (6 %) and all three of those pheasants were sampled in 2017. Other MLG's in the pheasants included MLG 16, MLG 17, MLG 18, MLG 19 and MLG 20. None of which were found in adult cattle or calves. Adult cattle in 2016 were shedding MLG 10, MLG 12, MLG 13, MLG 14 and MLG 15.

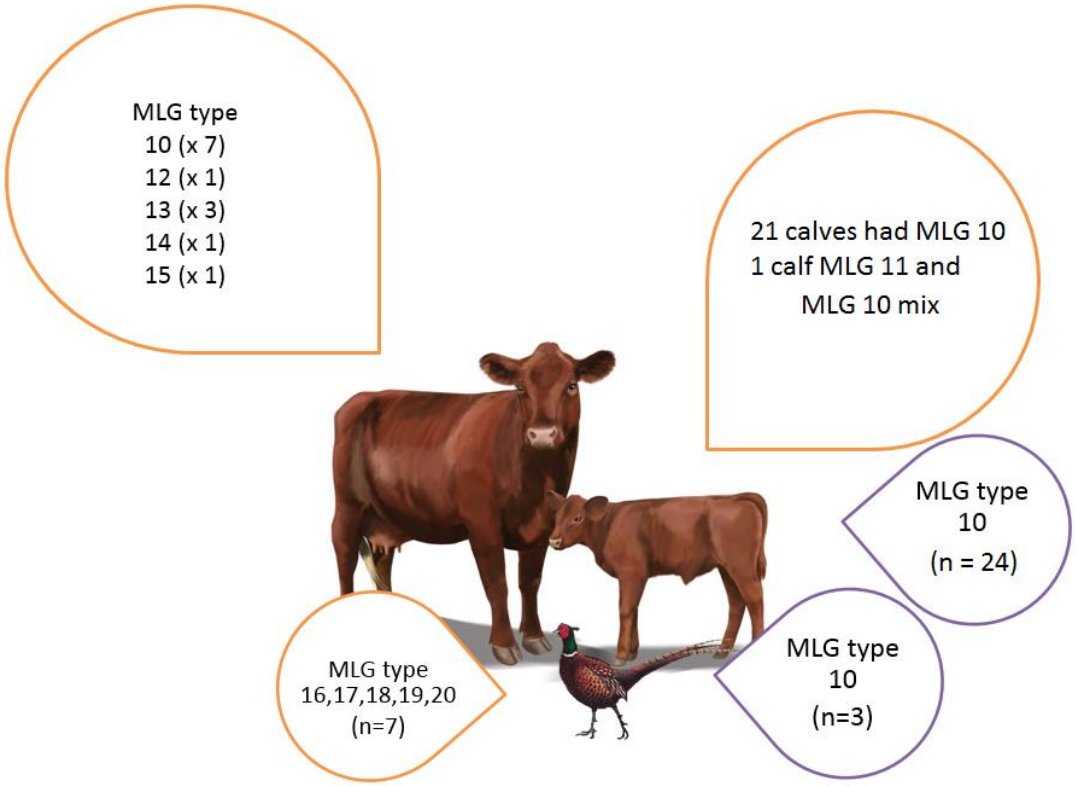


**Figure 19 Multilocus genotypes of *Cryptosporidium parvum* in cattle, calves and pheasants on a single farm in Perthshire, Scotland. Samples collected in 2016 circled in orange and samples collected in 2017 circled in purple.**

## 4.5 Discussion

**Determine whether or not wild rabbits and pheasants shed *C. parvum*.**

Cryptosporidiosis is poorly documented in wildlife despite identifying the presence of *Cryptosporidium* oocysts in their faeces. This is down to the difficulties with analysing clinical disease in a wild animal population without culling the animals and examining their digestive tracts. Therefore, the incidence of cryptosporidiosis, along with its severity in many wildlife species, is unknown. Cryptosporidiosis does occur in farmed rabbits (Kaupke et al., 2014) and also rabbits that have been experimentally infected with *Cryptosporidium* oocysts (Mosier et al., 1997). However, the concentration of oocysts ingested in a laboratory setting or a farm setting where animals are kept in close proximity is unlikely to occur in a natural setting and so the rabbits may not suffer clinical disease. This could be the reason for the difficulties in genotyping positive samples which occurred in this study. Twenty-five out of 77 (32.47 %) *C. parvum* positive rabbit samples failed to amplify at the GP60 locus. Due to the difficulties in amplification, only a selection of rabbit samples which amplified in all three replicates for GP60 PCR were selected for further typing using microsatellite analysis. Despite this, only 29/47 (61.70 %) rabbit samples were successfully typed at all four loci, and this required many repeats of the PCR (until all template DNA was gone) to achieve. The infection level could be very low with the rabbits suffering no clinical disease, picked up only by the highly sensitive molecular techniques employed in the analysis of the faecal samples. Alternatively, it could also be that negative results are due to PCR inhibition, and the only way to rule this out would either be to incorporate a positive internal control or perform microscopy on the negative samples.

The rabbits from farms around Scotland had *C. parvum* as the most common species of *Cryptosporidium* in their faeces, as identified by 18S PCR and sequence analysis. This is an unusual finding based on previous

studies done in Warwickshire and Norfolk which predominantly found *C. cuniculus* in the wild rabbit population in the UK (Robinson & Chalmers, 2010). The prevalence found in these other studies was between 0.9 - 7.1 % which is much lower than the 37.33 % found in this present study in Scotland. The rabbits in the study in Norfolk were located on or near farmland and shared their habitat with grazing livestock (Sturdee et al., 1999), similar to the wild rabbits used for this present study. The rabbit samples from Norfolk and Warwickshire were analysed using only microscopy (Chalmers, 1996; Sturdee et al., 1999) and as PCR was used for this present study that may explain the higher prevalence. A study undertaken in Australia looked at the species of *Cryptosporidium* in wild rabbits from four separate locations and found *C. cuniculus* to be the only species present using PCR and phylogenetic analysis (Nolan, Jex, Haydon, Stevens & Gasser., 2010). Very little information about the locations used for the Australian study is provided and so it is unknown whether or not these rabbits were located nearby livestock farmland. All rabbits sampled for this present study were collected on or very close to farms with livestock and so this could be the reason for the high prevalence of *C. parvum* which is not seen in these other studies. A study undertaken in an Australian catchment found *C. cuniculus* as the only *Cryptosporidium* species in rabbit faecal samples using quantitative PCR (qPCR) despite there being livestock in the same catchment (Zahedi et al., 2016). However, the levels of *Cryptosporidium* in the livestock was very low, with only 7 % (10/142) of cattle and 2.3 % (3/128) of sheep testing positive. Of the positive cattle, only six animals were positive for *C. parvum* and no sheep tested positive for that species (Zahedi et al., 2016). Therefore, the environmental load of *C. parvum* was likely to be very low in this area and this could explain why it was not detected in the rabbits.

The results from the pheasants support the hypothesis that wildlife is more likely to have low level infections with *Cryptosporidium* picked up from the farm environment. Pheasants in this area did have a high prevalence of *C.*

parvum, which was detected in 48% of the tested pheasants over the two years. There are few reports in the literature on *Cryptosporidium* in pheasants. Pheasants, which were collected from markets in Brazil, were tested using PCR for *Cryptosporidium* along with many other avian species such as turkey, chicken, ostrich, quail and guinea fowl. None of the pheasants tested positive for *Cryptosporidium,* however it was found in chicken, quail and turkey, although *C. parvum* was not detected (da Cunha, Cury & Santin., 2018). Ring necked pheasants *(Phasianus colchicus)* from the Czech Republic tested positive by PCR for *Cryptosporidium* in 14.1 % of tested individuals. However again *C. parvum* was not detected (Maca & Pavlasek, 2016).

*Cryptosporidium parvum* mouse genotype was detected in a white eared pheasant (*Crossoptilon crossoptilon*) using PCR in the Qinghai province of China although this was an isolated case (Karanis et al., 2007). Therefore this is the first time that *C. parvum* has been detected in a larger study focussing on pheasants and this is valuable knowledge with regards to the transmission and epidemiology of *C. parvum*.

Despite the high prevalence of *C. parvum* in pheasants on this particular farm in Perthshire, it was once again very difficult to genotype the positive faecal samples which could indicate a low infection level. Alternatively, it could be down to PCR inhibition, and continuing this work by performing microscopy on all negative samples would help to eliminate this possibility. Only three of the 50 pheasants had the same genotype which was present in the calves on the same farm, and all three of those pheasants were tested in 2017. In 2016, the pheasants were free roaming and so could have picked up the oocysts from another farm in the area. However, in 2017 the pheasants were being trapped on the farm, where they were collected every couple of days for relocation. Therefore, these pheasants would be spending more time on this particular farm and could be more likely to pick up this particular genotype and pass oocysts in their faeces. There is also a

possibility that as samples were collected from the ground, that environmental contamination may have occurred.

**Assess the risk that these wildlife species pose in the transmission of *C. parvum* to calves.**

Rabbits and pheasants could be a potential transmission vector for *C. parvum* to calves. As little as 25 oocysts are required to infect a calf (Zambrisky et al., 2013) and many infected calves will shed billions of oocysts so even low-level oocyst shedding has the ability to cause disease in a calf if the calf comes into contact with infectious rabbit or pheasant faeces.

Given the fact the pheasants were located in a farm environment with calves which had a known history of clinical cryptosporidiosis, it is perhaps unsurprising that *C. parvum* oocysts were detected in the pheasant faeces. The likelihood is that the pheasants were not clinically infected and the oocysts were instead just passing through the pheasants' digestive systems. Examining the guts of the pheasants at post-mortem would confirm if infection and parasite multiplication occurred in these birds. The pheasants in 2017 seemed to reflect the multilocus genotype present in the cattle in 2016, and so the pheasants could be reflecting the environmental parasite load from cattle from the previous year.

Other wildlife species such as deer are known to carry zoonotic species of *Cryptosporidium* (Wells et al., 2015). Therefore, further work should include research into other wildlife species which may pose a risk of *Cryptosporidium* transmission to calves. From this, barriers could be introduced to reduce this transmission route on farm.

## 4.6 Conclusion

In conclusion, *C. parvum* is present in both wild rabbits and pheasants in Scotland, albeit likely at low levels. However, due to the low infectious dose of *Cryptosporidium* (Zambrisky et al., 2013), farms with a high wildlife abundance should take care as these species do have the potential to act as a transmission vector of *C. parvum* to livestock. Wildlife is likely to facilitate the spread of oocysts between farms even if it is purely through mechanical transmission and so precautions should be taken to reduce the incidence of this transfer route to young calves by using barns and pens which provide a barrier to wildlife.

# Chapter 5 Disinfectants against *Cryptosporidium*

## 5.1 Introduction

One of the main difficulties in the control of cryptosporidiosis, is removing the environmental load of the parasite on the farm. *Cryptosporidium* generates very hardy, difficult to destroy oocysts which the host releases into the environment where oocysts can survive for several months. *Cryptosporidium* oocysts have the ability to survive best in temperatures ranging from 22°C to 38 °C (Shahiduzzaman et al., 2010). Various environmental pressures affecting the viability of *Cryptosporidium* oocysts have been analysed, and the results showed that some oocysts still remained viable after being exposed to temperatures as low as -22 °C for 750 hours, being submerged in seawater, 2 hours of air drying and many of the processes associated with water treatment (Robertson et al., 1992). These water treatment processes include alum floccing, addition of lime and ferric sulphate floccing. Although the majority of the oocysts died after exposure to these environmental stresses, some still remained viable.

Since an outbreak of cryptosporidiosis was associated with contaminated salad leaves, a study was done to assess the viability of *Cryptosporidium* oocysts in similar conditions to evaluate the risk of future outbreaks. This study found that *Cryptosporidium* oocysts were still viable having been stored on lettuce leaves on the bench top, in a cupboard and in a fridge at 4 °C for 14 days (Utaaker, Skjerve & Roberston., 2017).

It is clear from these studies that *Cryptosporidium* oocysts are able to survive for long periods of time in the environment and they have well developed mechanisms to allow them to do this. It has been found that oocysts can also survive on field crops at 20 – 30 °C (World Health Organisation, 2006). Therefore, livestock are very likely to ingest these oocysts from the environment.

There are few effective disinfectants against *Cryptosporidium* due to the hardy and robust nature of the parasite. Many commonly used iodine and glutaraldehyde-based disinfectants have limited or no ability to kill *Cryptosporidium* oocysts (Weir et al., 2002; Wilson & Margolin, 1999). Of the disinfectants which do work, many are dangerous to use such as chloro-cresol and hydrogen peroxide and therefore require protective clothing and cannot be used while animals are present. This means the farmer would need to empty out the shed or pens before using the disinfectant. Many of the disinfectant companies recommend that the pen is fully cleaned out before the disinfectant is used for maximum efficacy. All of this makes disinfectant use very labour intensive.

A disinfectant known as Steriplex SD+ (sBioMed) is used in hospitals in the United States of America as a disinfectant. It was proposed that this product may have a good efficacy against *Cryptosporidium* oocysts (Williams, 2016. Personal communication). Not only would this potentially bring a new product onto the market, but Steriplex SD+ prides itself as being completely safe to handle with the ability for it to be used while animals are still in the pens. Their method of application (electrostatic spray) claims to ensure the whole area would be covered and the pens would not need to be cleaned out (Williams, R. 2017. Personal communication). As one of the main ingredients is silver nanoparticles, the company thought there was also likely to be a residual effect of the disinfectant which would continue to kill *Cryptosporidium* oocysts for some time after application. Silver nanoparticles have been found to be effective in previous work done between Novo Science Ltd, The Moredun Research Institute and Heriot-Watt University with the ability to reduce oocyst viability from 83 % to 33 % (Cameron et al., 2016). Steriplex SD+ has hydrogen peroxide as its main ingredient and this has already been proven to have excellent efficacy at killing *Cryptosporidium* (Barbee et al., 1999) and so Steriplex SD+ could be a candidate for *Cryptosporidium* disinfection on farm.

Disinfectants KENO™COX (CID Lines) and Neopredisan 135-1 © (Menno Chemie, Norderstedt, Germany) have proven efficacy against *Cryptosporidium* with published data available in research journals. Work done at the Institut National de la Recherche Agronomique in France showed that incubating *Cryptosporidium* oocysts with 2 % KENO™COX for 2 hours has been shown to result in a 89 % reduction in the number of oocysts present, of those which remained only 48.6 out of 500,000 remained viable which was analysed by inoculating mice with the oocysts. This is therefore a 99.99 % reduction in viability (Naciri et al., 2011). Mice infected with *Cryptosporidium* oocysts which were exposed to 0.25 % Neopredisan 135-1 © for 2 hours were found to have a reduced number of infected animals when compared to the control animals (Joachim et al., 2003). Application of Neopredisan 135-1 © at 4 % concentration for 2 hours to *Cryptosporidium* oocysts resulted in a 99.5 % inactivation according to work done at The University of Leipzig, Germany (Shahiduzzaman et al., 2010).

A couple of products on the market Progiene Coxicur ® (Progiene Dairy Hygiene, Rumenco Ltd) and Cyclex ® (Kilco International Ltd) state that their products have good efficacy against *Cryptosporidium* oocysts. However there is no data to prove this in the available scientific literature. Both products are DEFRA approved; Progiene Coxicur ® for Diseases of Poultry Order, Tuberculosis Orders and General Orders, and Cyclex ® is approved for Diseases of Poultry Orders and General Orders.

Hydrogen peroxide and hydrogen peroxide-based disinfectants are highly effective at killing *Cryptosporidium* oocysts and these are very commonly used in laboratory disinfectant protocols (Delling et al., 2016; Quilez et al., 2005). Hydrogen peroxide at low concentrations (>3%) is considered as a safer alternative to chlorine-based disinfectants as when it degrades, it forms oxygen and water. At high concentrations (>30%) however it can be dangerous as it is an aggressive oxidiser, and can be explosive when it comes into contact with organic material (Occupational Safety & Health

Administration, 2000). The problem with this method of disinfection is that low concentrations of the product have a relatively short shelf life and must be kept in the dark.

There is currently no available study comparing the efficacy of commercially available disinfectants on the viability of *Cryptosporidium* oocysts. This information would be useful to compare one product against another in order to make an informed decision as to which is the best product. In some cases, there is no published evidence that commercially available disinfectants can affect the viability of *Cryptosporidium* oocysts at all. In this study, several different disinfectant products were compared for their efficacy in reducing the viability of *Cryptosporidium* oocysts.

The disinfectant Steriplex SD+ was analysed for its efficacy against *Cryptosporidium* oocysts along with comparing other commercially available disinfectants on their ability to reduce *Cryptosporidium* viability according to their manufacturers guidelines; Progiene Coxicur ®, Cyclex ®, Neopredisan 135-1 ©, KENO™COX, Steriplex SD+, Hydrogen Peroxide and FAM-30 ((Evans Vanodine) a commercially available, commonly used iodine-based disinfectant which was included for comparative purposes). Information regarding all of these disinfectants can be seen in Table 26.

**Table 26 Disinfectants available for the inactivation of *Cryptosporidium* oocysts. All information provided by product providers. WL – working litre (1 litre of working solution)**

| Disinfectant | Active Ingredient | Price | Amount | £ per L | Working concentration | £ per WL | Contact time | Shelf life | Safety | Instruction |
|---|---|---|---|---|---|---|---|---|---|---|
| Steriplex SD + | Hydrogen peroxide, silver | £200 | 5L | £40 | None | £40 | 5 mins | 7 days | No PPE required, safe for environment | Mix Part A and Part B before use. Apply using a conventional sprayer 0.2L per $m^2$. Or 0.1L using their own electrostatic sprayer. |
| Neopredisan 135-1 © | Chlorocresol | £372.60 | 10L | £37.26 | 3% | £1.12 | 1 hour | 2 years | Safe to use in the presence of animals and humans. Environmentally friendly, biologically degradable. | Apply to clean surface, 0.4L per $m^2$ |
| Cyclex ® | Chlorocresol | £117 | 5L | £23.40 | 3% | £0.70 | 4 hours | 2 years | Burns, eye damage, skin reactions, toxic to aquatic life | Apply to clean surface, 0.3L per $m^2$ |
| KENO™COX | N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine, alcohol | £207.59 | 10L | £20.76 | 2% | £0.42 | 2 hours | 2 years | Corrosive, dangerous to environment | Apply to clean shed 0.4L per $m^2$ |
| Hydrogen Peroxide | Hydrogen peroxide | £24.35 for 35% | 2.5L | £9.74 | 3% | £0.84 | 4 mins | 1 month | Irritating to the respiratory system and skin. Irritating to eyes. Irritation of the mouth. Environmentally friendly. | Dilute 3% and use as a spray. |
| FAM - 30 | Acid based Iodine | £24.51 | 5L | £4.90 | 2% | £0.15 | 30 min | 3 years | Skin Corr. 1B - H314 Eye Dam. 1 - H318<br>Not dangerous to environment | Dilute 1:50 (based on use for coronavirus) |
| Progiene Coxicur ® | Chlorocresol | £90 | 5L | £18 | 3% | £0.54 | 30 min | 12 months | Burns, eye damage, skin reactions, toxic to aquatic life | Apply to clean surface, 0.3L per $m^2$ |

## 5.2 Aims

- Determine the efficacy of Steriplex SD+ on *C. parvum* oocysts under a range of conditions.

- Assess the suitability of Steriplex SD+ for use against *C. parvum* in a farm setting.

- Analyse commercially available disinfectants to determine which is the best at inactivating oocysts.

- Determine whether prepared disinfectants lose their efficacy over time.

- Make recommendations on the best disinfectant to use based on disinfectant efficacy, safety and costs.

## 5.3 Materials and Methods

### 5.3.1 *Cryptosporidium* Excystation

*Cryptosporidium* oocysts used during this study were obtained by concentrating oocysts from experimentally infected animals. These were provided in two separate batches and so one batch was used for the first study of examining the effect of commercially available disinfectants on oocyst viability using manufacturer's guidelines, and another batch used for examining whether or not disinfectants lose their efficacy over time against *Cryptosporidium* oocysts seven days after they have been prepared, ready for use. Samples containing 1 x $10^6$ *Cryptosporidium* oocysts in 0.25 ml tubes were centrifuged at 12,500 x g for 30 seconds and the supernatant discarded. If disinfectant was present, 50 µl phosphate buffered saline (PBS) was added and mixed before another spin at 12,500 x g for 30 seconds. This was repeated until 3 PBS washes had taken place and supernatant discarded. Afterwards 40 µl of 1 x Hanks Buffered Salt Solution (HBSS) was added and the pellet resuspended. Fifty microliters of 1 x Trypsin (prepared in HBSS at pH 3 (add 7.5 µl 2% HCL)) was added and mixed. The tubes were then placed into a water bath at 37°C for 1 hour. Once the hour had elapsed the eppendorfs were centrifuged at 12,500 x g for 30 seconds and the supernatant discarded.

The addition of 90 µl of 1 x HBSS, 10 µl 2.2% Sodium Bicarbonate and 10 µl 1% Sodium Deoxycholate at this stage changes the pH to mimic intestinal conditions. The contents in the tubes were mixed and placed in a water bath at 37°C for 40 minutes. After this time the tubes were centrifuged at 12,500 x g for 30 seconds and the supernatant discarded. Oocysts, shells and sporozoites were then suspended in 50 µl of 3% glutaraldehyde in PBS to fix and spot onto a microscope slide. A coverslip was placed over the spotted liquid and the edges of the cover slip were sealed using mineral oil. The slides were then left to settle for a few minutes.

Slides were examined under a microscope using x 40 phase contrast magnification. The oocysts, shells and sporozoites were then counted to a combined total count of 250. The sporozoite per shell ratio was calculated as follows:-

(Sporozoite Count)/(Shell Count) = Sporozoites per Shell

The excystation percentage was calculated as follows:-

(Shell Count)/(Oocyst Count+Shell Count) ×100 = Percentage Excystation

### 5.3.2 Steriplex SD+ Trial

The effect of Steriplex SD+ (Active ingredients – Silver 00:015%, Hydrogen Peroxide 22%, Peroxyacetic Acid 15%) on *Cryptosporidium* oocyst excystation was performed in order to determine:

1. **The contact time required to achieve inactivation**

   The time that the disinfectant is in contact with *Cryptosporidium* oocysts.

2. **How the disinfectant degrades over time**

   Whether the working concentration of the disinfectant loses its efficacy over time.

3. **The ability of the disinfectant to inactivate oocysts when used as a spray**

   Does the use of a spray rather than suspending oocysts in the disinfectant reduce disinfectant efficacy?

4. **The ability of the disinfectant to inactivate oocysts in a 'dirty' environment**

   Effect of the disinfectant on *Cryptosporidium* oocysts still in faeces

5. **The residual effect of the disinfectant**

   Disinfectant sprayed on surface and left for 12 hours before *Cryptosporidium* oocysts added to determine if left over disinfectant affects oocyst excystation rate

These experiments were considered a pilot study for the bigger experiment looking at many commercially used disinfectants to see which tests would be suitable to perform. When Steriplex SD+, a two-part product, is activated properly by using the directions of use on the label, the solution meets the criteria for a toxicity Category IV product. The category IV rating is the lowest rating possible for toxicity levels requiring no precautionary or first aid statements.

Steriplex SD+ was made up according to the manufacturer's instructions which was 1:59 ratio for part A to part B of the solution. This was added to approximately 1 x $10^6$ *Cryptosporidium* oocysts inside a 0.25 ml microcentrifuge tube at 1:2 ratio and mixed using a vortex.

**Contact time**

Steriplex SD+ was made up according to the manufacturer's instructions. Contact times tested for were 30 sec, 1.5 minutes, 2.5 minutes, 3.5 minutes, 4.5 minutes and 5.5 minutes. After the contact time had elapsed an oocyst excystation was performed (6.2.1).

**Degradation**

Steriplex SD+ was made up according to 'Contact time' experiment and tested on oocyst excystation (6.2.1 with a 5-minute contact time) after 0, 3, 7, 10 and 14 days post mixing both parts of the disinfectant together. The disinfectant was stored at 4 °C in the dark. These experiments were done in triplicate.

**Use as a spray**

Steriplex SD+ was made up according to 'Contact time' experiment except the oocysts (increased to 3 x $10^6$) were placed into a weigh boat and then sprayed with the disinfectant using 2 x sprays (approximately 2-3 ml). After

a 5-minute contact time the oocysts and solution were collected and placed into two separate 2 ml eppendorfs and centrifuged at 11,000 xg for 1 minute. The supernatant was discarded and oocysts resuspended in 100 µL TE buffer before being transferred to a single 0.25 ml tube and oocyst excystation tested (6.2.1). This experiment was done in duplicate.

**Use on dirty oocysts**

Diarrheic samples collected from calves were split into six 2g samples, each of which were spiked with 3 x $10^6$ *Cryptosporidium* oocysts. Equal volume of spiked faeces with Steriplex SD+ was put into in sample 1 and 2, spiked faeces was spread on over a weigh boat and then sprayed with Steriplex SD+ for samples 3 and 4, and samples 5 and 6 had no disinfectant added and used as a control. A 5-minute contact time was allowed before all samples underwent a salt flotation to concentrate the oocysts from the faeces (2.3.4). Collected oocysts then underwent excystation (6.2.1). This experiment was performed in duplicate.

**Residual effect**

In order to test the residual effect of Steriplex SD+, Steriplex was sprayed onto a weigh boat using 2 x sprays (approximately 2-3 ml) and left for 12 hours. After this time approximately 1 x $10^6$ *Cryptosporidium* oocysts were added for a 5-minute contact time. The oocysts and solution were collected and placed into two separate 2 ml eppendorfs and centrifuged at 11,000 xg for 1 minute. The supernatant was discarded and oocysts resuspended in 100 µL TE buffer before being transferred to a single 0.25 ml tube and oocyst excystation tested (6.2.1). This experiment was done in duplicate with a control where oocysts were added to a clean weigh boat.

## 5.3.3 Disinfectant Comparisons

### Manufacturers guidelines

**(Disinfect used according to the instructions provided by the manufacturer)**

Disinfectants were sourced through donations from participating companies which can be seen in Table 27. Each disinfectant was made up fresh according to the manufacturer's guidelines on the same day it was to be used for the experiment. Disinfectant concentration was made up according to Table 27.

**Table 27 Disinfectants used in efficacy studies**

| Disinfectant | Active Ingredient | Working concentration | Contact time |
|---|---|---|---|
| Steriplex SD+ | Hydrogen peroxide, silver | None | 5 minutes |
| Neopredisan 135-1 © | Chlorocresol | 3% | 1 hour |
| Cyclex ® | Chlorocresol | 3% | 4 hours |
| KENO™COX | N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine, alcohol | 2% | 2 hours |
| Hydrogen Peroxide | Hydrogen peroxide | 3% | 5 minutes |
| FAM – 30 | Iodine | 2% | 30 minutes |
| Progiene Coxicur ® | Chlorocresol | 3% | 30 minutes |

Approximately 1 x $10^6$ *Cryptosporidium* oocysts were placed into 8 separate 0.25 ml microcentrifuge tubes, one for each of the 7 disinfectants and 1 control tube with no disinfectant added. Each disinfectant was added at manufacturers recommended concentration (Table 27) 1:2 with oocysts. After the recommended contact time (Table 27) had elapsed, excystation were done to determine the oocyst viability according to 6.2.1. This was replicated 5 times.

**Disinfectant degradation**

**(Does prepared disinfectant have reduced efficacy after 7 days)**

Degradation of disinfectants over a 7-day period was examined by making up each disinfectant according to the manufacturer's instructions and left for 7 days before conducting the experiment 'Manufacturers guidelines' (described above) was repeated and was also repeated on 5 separate occasions so that each disinfectant had 5 replicates. All disinfectants were kept at 4 °C in the dark during the 7-day period.

# 5.4 Results

## 5.4.1 Steriplex SD+ Pilot Study

### Contact Time

Steriplex SD+ was examined for its ability to inactivate *Cryptosporidium* oocysts using excystation as a measure of oocyst viability. These results can be seen in Table 28 and visualised in Figure 20. Initial oocyst excystation rate was 62.6% which drops to 14.5% after only 30 seconds contact time with Steriplex SD+. All *Cryptosporidium* oocyst excystation is halted by 4.5 minutes contact time with Steriplex SD+.

**Table 28 Determination of the best contact time to give Steriplex SD+ in order to inactivate *Cryptosporidium* oocysts**

| Contact time with *Cryptosporidium* oocysts | Oocyst count | Shell count | Sporozoite count | Shell to Sporozoite ratio | Excystation rate |
|---|---|---|---|---|---|
| No disinfectant added (Control) | 55 | 92 | 103 | 1.12 | 62.6% |
| 30 seconds | 200 | 34 | 16 | 0.47 | 14.5% |
| 1.5 minutes | 221 | 21 | 8 | 0.38 | 8.7% |
| 2.5 minutes | 243 | 7 | 0 | 0.00 | 2.8% |
| 3.5 minutes | 243 | 7 | 0 | 0.00 | 2.8% |
| 4.5 minutes | 250 | 0 | 0 | 0.00 | 0.0% |
| 5.5 minutes | 250 | 0 | 0 | 0.00 | 0.0% |

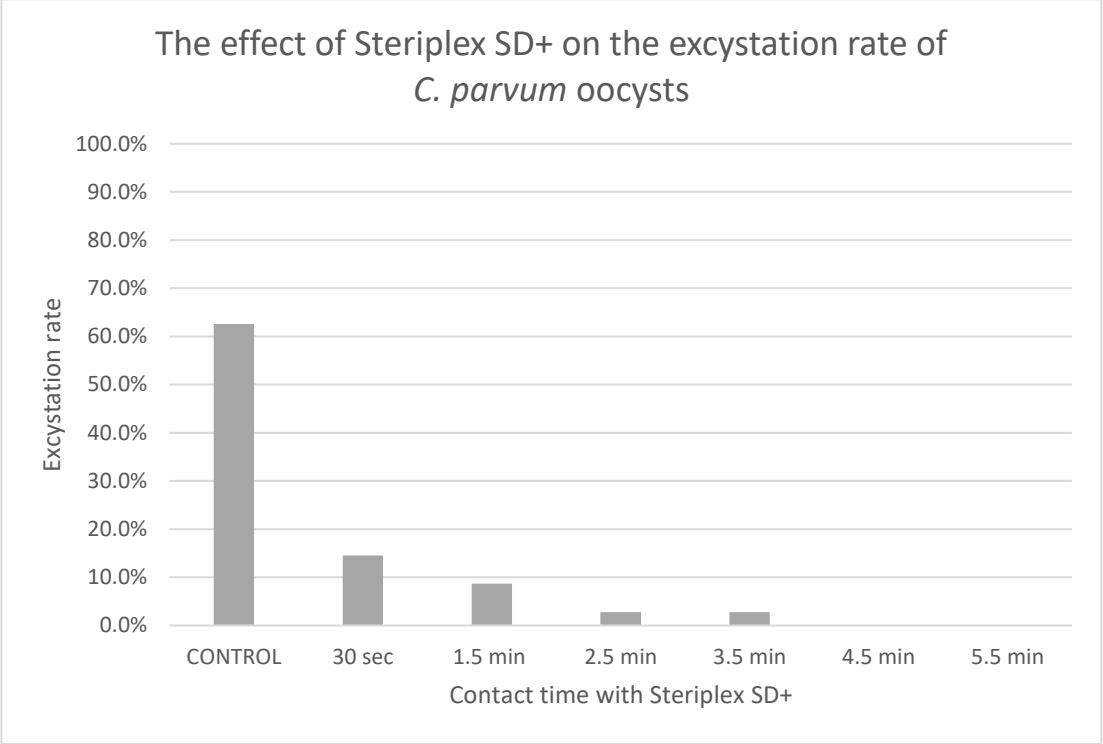


**Figure 20 The percentage reduction in *Cryptosporidium* oocyst viability (based on the reduction in excystation rate compared to the control) following exposure to Steriplex SD+ at various contact times from 30 seconds - 5.5 minutes. Control in this case is the excystation rate of untreated oocysts.**

Examining the effectiveness of Steriplex SD+ is best visualised by determining the effect of contact time on *Cryptosporidium* oocyst inactivation. Therefore, assuming the control excystation rate of 62.6% is 100%, oocyst inactivation was calculated using the following equation:

Inactivation % = 1 – (excystation rate/control excystation rate) x 100

The results for oocyst inactivation can be seen in Table 29 and Figure 21. Complete inactivation is achieved by 4.5 minutes and so this is the best contact time to give Steriplex SD+ to ensure full inactivation of oocysts.

**Table 29 Determination of oocyst inactivation based on excystation rate of *Cryptosporidium* oocysts compared to the control**

| Contact time with *Cryptosporidium* oocysts | Excystation rate | Oocyst Inactivation compared to the control |
|---|---|---|
| 30 seconds | 14.5% | 76.8% |
| 1.5 minutes | 8.7% | 86.1% |
| 2.5 minutes | 2.8% | 95.5% |
| 3.5 minutes | 2.8% | 95.5% |
| 4.5 minutes | 0.0% | 100.0% |
| 5.5 minutes | 0.0% | 100.0% |

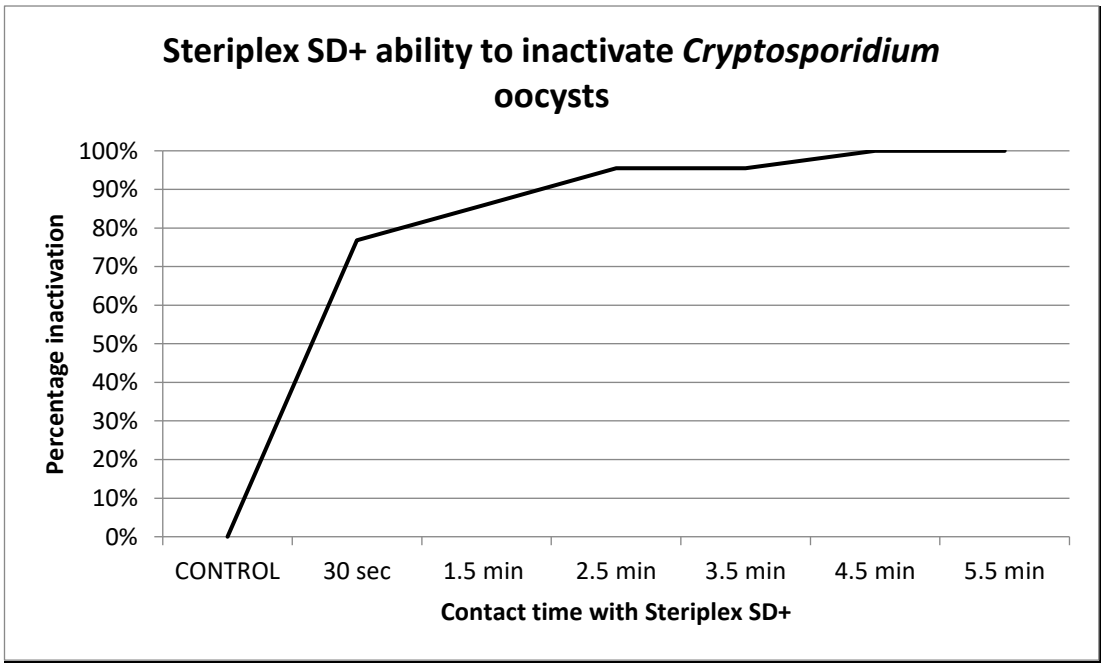


**Figure 21 Steriplex SD+ ability to inactivate *Cryptosporidium* oocysts based on excystation rate compared to the control. In this case the control was where no disinfectant was added.**

**Further testing**

The initial experiment examining contact time showed that 30 seconds contact reduced the oocyst excystation rate by 76.8% compared to the control, 1.5 minutes reduced excystation by 86.1%, 2.5 minutes by 95.5%, 3.5 minutes by 95.5% and by 4.5 minutes 100.0% reduction was achieved.

Further testing of Steriplex SD+ was done using a 5-minute contact time as this was deemed the best from the above study. Steriplex SD+ was then examined for its effectiveness after the disinfectant had been made up after 3, 7, 10 and 14 days. It was examined for its efficacy as a spray and also its effect on oocysts which were ‘dirty’ (in faecal samples), and also whether or not the disinfectant had a residual effect. These results can be seen in full in Table 30 and summarised in Figure 22.

**Table 30 Results of varying conditions of Steriplex SD+ on the excystation rate of *Cryptosporidium* oocysts. Controls highlighted in grey. Post preparation (PP). Hours (HR).**

| Experiment | Oocyst Type | Oocysts | Shells | Sporozoites | Sporozoite to Shell ratio | Excystation Rate |
|---|---|---|---|---|---|---|
| SUSPENDED | CLEAN OOCYSTS SUSPENDED 1:2 | 250 | 0 | 0 | 0.00 | 0.0% |
| | CLEAN OOCYSTS NO STERIPLEX | 55 | 92 | 103 | 1.12 | 62.6% |
| DIRTY OOCYSTS | DIRTY OOCYSTS SUSPENDED 1:2 | 135 | 86 | 29 | 0.34 | 38.9% |
| | | 143 | 79 | 28 | 0.35 | 35.6% |
| | DIRTY OOCYSTS STERIPLEX AS SPRAY | 82 | 95 | 73 | 0.77 | 53.7% |
| | | 93 | 83 | 74 | 0.89 | 47.2% |
| | DIRTY OOCYSTS H20 SPRAY | 72 | 103 | 75 | 0.73 | 58.9% |
| | DIRTY OOCYSTS NO STERIPLEX | 92 | 140 | 18 | 0.13 | 60.3% |
| 10 AND 14 DAYS POST PREPARATION | CLEAN OOCYSTS NO STERIPLEX | 29 | 176 | 45 | 0.26 | 85.9% |
| | 10 DAYS PP STERIPLEX | 138 | 107 | 5 | 0.05 | 43.7% |
| | 10 DAYS PP STERIPLEX | 130 | 117 | 3 | 0.03 | 47.4% |
| | 10 DAYS PP STERIPLEX | 60 | 37 | 3 | 0.08 | 38.1% |
| | 14 DAYS PP STERIPLEX | 132 | 112 | 12 | 0.11 | 45.9% |
| | 14 DAYS PP STERIPLEX | 122 | 113 | 15 | 0.13 | 48.1% |
| | 14 DAYS PP STERIPLEX | 129 | 112 | 9 | 0.08 | 46.5% |

| | | | | | | |
|---|---|---|---|---|---|---|
| SPRAY | CLEAN OOCYSTS STERIPLEX SPRAY | 167 | 76 | 7 | 0.09 | 31.3% |
| | | 178 | 70 | 2 | 0.03 | 28.2% |
| | CONTROL NO STERIPLEX | 35 | 189 | 26 | 0.14 | 84.4% |
| | CONTROL SPRAY WITH H20 | 30 | 171 | 49 | 0.29 | 85.1% |
| 3 AND 7 DAYS POST PREPARATION | CLEAN OOCYSTS NO STERIPLEX | 62 | 154 | 34 | 0.22 | 71.3% |
| | 3 DAYS PP STERIPLEX | 173 | 70 | 7 | 0.10 | 28.8% |
| | 3 DAYS PP STERIPLEX | 163 | 79 | 8 | 0.10 | 32.6% |
| | 3 DAYS PP STERIPLEX | 163 | 85 | 2 | 0.02 | 34.3% |
| | 7 DAYS PP STERIPLEX | 149 | 95 | 6 | 0.06 | 38.9% |
| | 7 DAYS PP STERIPLEX | 138 | 106 | 6 | 0.06 | 43.4% |
| | 7 DAYS PP STERIPLEX | 140 | 103 | 7 | 0.07 | 42.4% |
| RESIDUAL EFFECT | WATER SPRAY 12 HR BEFORE CLEAN OOCYST CONTROL | 98 | 148 | 4 | 0.03 | 60.2% |
| | STERIPLEX SPRAY 12 HR BEFORE SPRAY ON CLEAN OOCYSTS | 75 | 175 | 0 | 0.00 | 70.0% |
| | STERIPLEX SPRAY 12 HR BEFORE SPRAY ON CLEAN OOCYSTS | 51 | 105 | 94 | 0.90 | 67.3% |

**Suspended**

Oocyst inactivation at 5-minute contact time when the oocysts are mixed 1:2 with the disinfectant.

**Disinfectant efficacy over time**

Oocyst inactivation was calculated in the same way as the Steriplex SD+ experiment using the excystation compared to the control which underwent the same protocol but with no disinfectant added (details for each control described in Table 30). When used immediately, with a contact time of 5 minutes, 100 % of oocysts were inactivated. After three days, this fell to 55% inactivation. After seven days 42 % of oocysts were inactivated. This fell to 39% by day 10 and then 26 % by day 14.

**Steriplex SD+ as a spray**

When the product is used as a spray and the disinfectant is fresh with a 5-minute contact time, the inactivation of the *Cryptosporidium* oocysts was 65 %.

**Dirty conditions**

When used on oocysts which were in faeces (dirty) the inactiavtion was 38 % when oocysts in faecal samples were suspended 1:2 in the disinfectant and 16 % when oocysts in faecal samples were sprayed with the disinfectant.

**Residual effect**

The disinfectant had no residual effect (Allowing the disinfectant to dry on surface which is then contaminated with *Cryptosporidium* oocysts) on the excystation rate of the *Cryptosporidium* oocysts when compared to a control which had no disinfectant added.

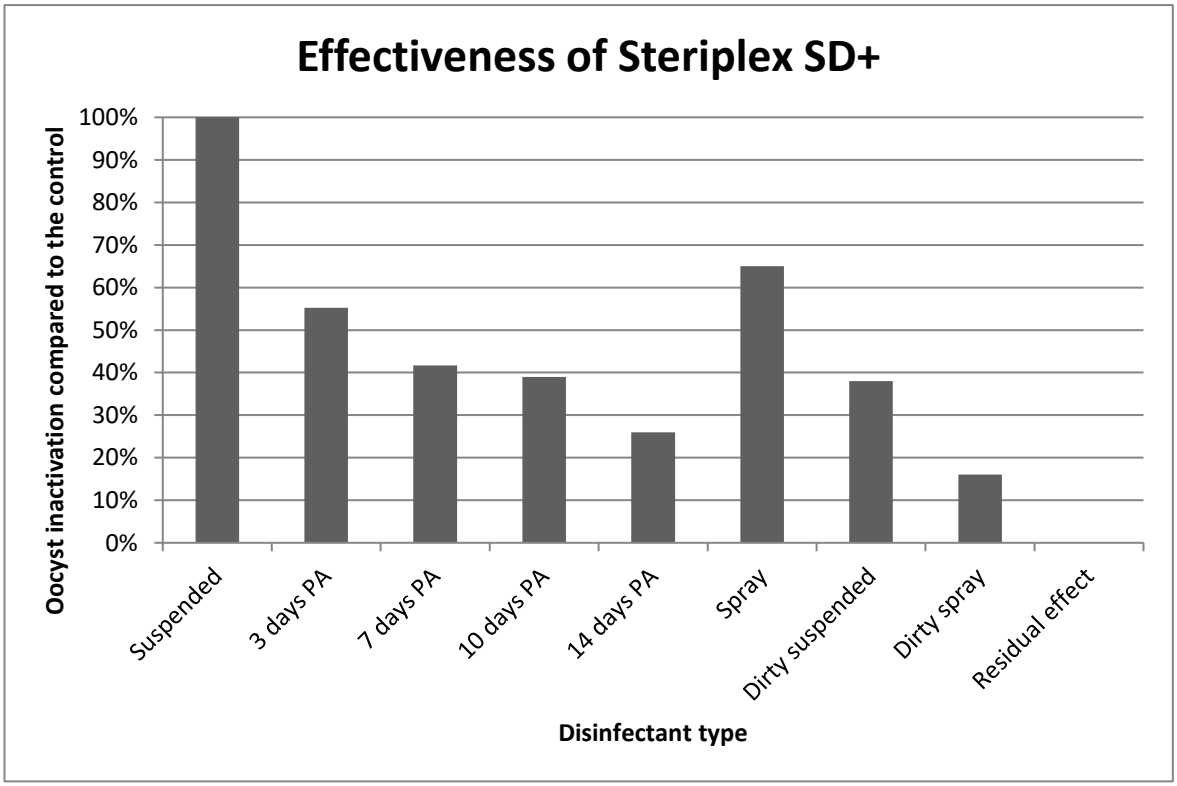


**Figure 22 Effectiveness of Steriplex SD+ on *Cryptosporidium* oocyst viability, based on excystation rate compared to a control (excystation rate of untreated oocysts of a similar state, described in Table 30). This includes 3 – 14 days after making up Steriplex SD+ (PA – post activation), Use of the disinfectant as a spray, use in a dirty environment (on oocysts in faecal samples both in 1:2 suspension with Steriplex SD+ and as a spray) and the residual effect of the disinfectant. Suspended is oocysts which are suspended 1:2 with fresh disinfectant.**

## 5.4.2 Disinfectant Comparisons

Disinfectants were tested for their efficacy against *Cryptosporidium* oocysts based on the manufacturer's guidelines for use. The best performing disinfectant with regard to excystation rate alone was Hydrogen peroxide which after 5 repeats gave a mean excystation rate of 0.40 % with a standard deviation (STDEV) of 0.40 %. Steriplex SD+, a hydrogen peroxide-

based disinfectant, also performed very well with a mean oocyst excystation rate of 4.97 % after exposure, with a standard deviation of 3.55 %. The worst performing based on oocyst excystation rate alone was Progiene Coxicur which had an oocyst excystation rate of 33.68 % (STDEV 10.25 %) following exposure, closely followed by FAM-30 with an excystation rate of 30.62 % (STDEV 12.16 %). A full table of results can be seen in Table 31 for excystation rate and a summarised boxplot in Figure 23.

With regard to sporozoite to shell ratio, all disinfectants except FAM-30 performed very well. Steriplex SD+ had a mean sporozoite to shell ratio of 0 with a standard deviation of 0, meaning no sporozoites were seen in any of the five replicates. Cyclex and KENOCOX both had a mean sporozoite to shell ratio of 0.1, Neopredisan 135-1 and Progiene Coxicur had a mean sporozoite to shell ratio of 0.2 and Hydrogen Peroxide had a mean ratio of 0.3. All of these results can be seen in Table 32 and a summarised boxplot in Figure 24.

**Table 31 The mean value, standard deviation, interquartile ranges, median value and minimum and maximum value for the excystation rate of *Cryptosporidium* oocysts after exposure to each disinfectant.**

| Disinfectant | Mean | STDEV | Q1 | Median | Q3 | Minimum | Maximum |
|---|---|---|---|---|---|---|---|
| Control | 84.47% | 3.69% | 81.56% | 85.00% | 87.12% | 78.91% | 89.25% |
| Cyclex | 12.52% | 9.38% | 3.60% | 11.65% | 21.87% | 3.20% | 24.79% |
| Fam - 30 | 30.62% | 12.16% | 23.69% | 26.80% | 39.48% | 20.99% | 51.91% |
| Hydrogen peroxide | 0.40% | 0.40% | 0.00% | 0.40% | 0.80% | 0.00% | 0.81% |
| KENO™COX | 6.31% | 5.52% | 2.41% | 4.40% | 11.17% | 1.20% | 15.51% |
| Neopredisan 135-1 | 6.08% | 4.85% | 2.81% | 4.05% | 10.37% | 2.40% | 14.35% |
| Progiene Coxicur | 33.68% | 10.25% | 24.63% | 32.23% | 43.45% | 21.90% | 48.37% |
| Steriplex SD+ | 4.97% | 3.55% | 2.20% | 2.86% | 8.80% | 2.00% | 9.60% |

**Table 32 The mean value, standard deviation, interquartile ranges, median value and minimum and maximum value for the shell to sporozoite ratio of *Cryptosporidium* oocysts after exposure to each disinfectant.**

| Disinfectant | Mean | STDEV | Q1 | Median | Q3 | Minimum | Maximum |
|---|---|---|---|---|---|---|---|
| Control | 1.7 | 0.3 | 1.5 | 1.8 | 1.9 | 1.2 | 1.9 |
| Cyclex | 0.1 | 0.1 | 0.0 | 0.1 | 0.1 | 0.0 | 0.1 |
| Fam - 30 | 2.0 | 0.2 | 1.8 | 2.0 | 2.2 | 1.8 | 2.4 |
| Hydrogen peroxide | 0.3 | 0.7 | 0.0 | 0.0 | 0.8 | 0.0 | 1.5 |
| KENO™COX | 0.1 | 0.1 | 0.0 | 0.1 | 0.2 | 0.0 | 0.3 |
| Neopredisan 135-1 | 0.2 | 0.2 | 0.0 | 0.3 | 0.3 | 0.0 | 0.4 |
| Progiene Coxicur | 0.2 | 0.4 | 0.0 | 0.1 | 0.5 | 0.0 | 0.9 |
| Steriplex | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

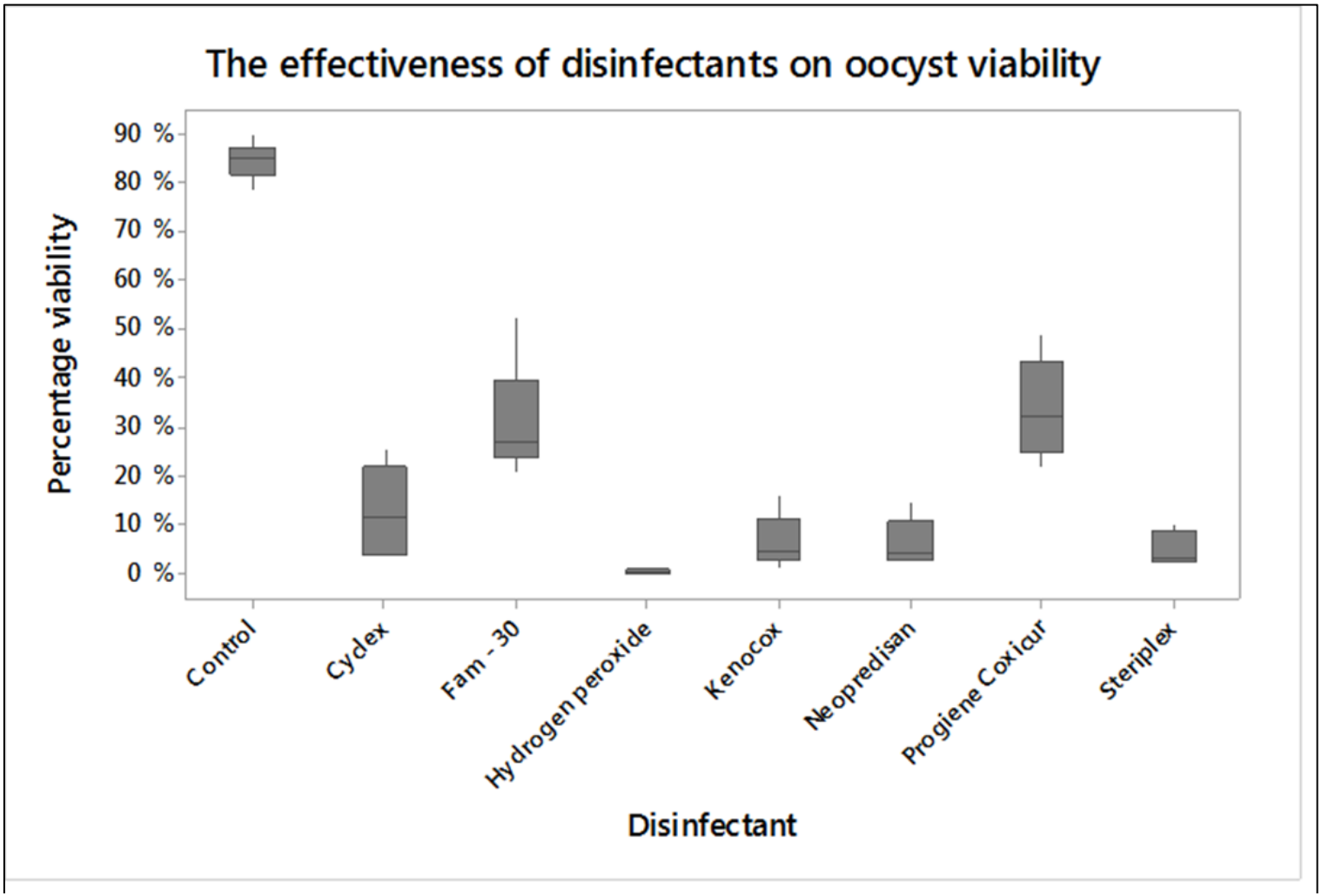


**Figure 23 Excystation rate for *Cryptosporidium* oocysts undergoing excystation following exposure to various disinfectants used according to the manufacturers guidelines. The rectangle represents the second and third quartiles, the horizontal line inside indicates the median value and the lower and upper quartiles are shown as vertical lines either side of the rectangle. The control is made up of 5 repeats done with the 5 repeats of disinfectant efficacy.**

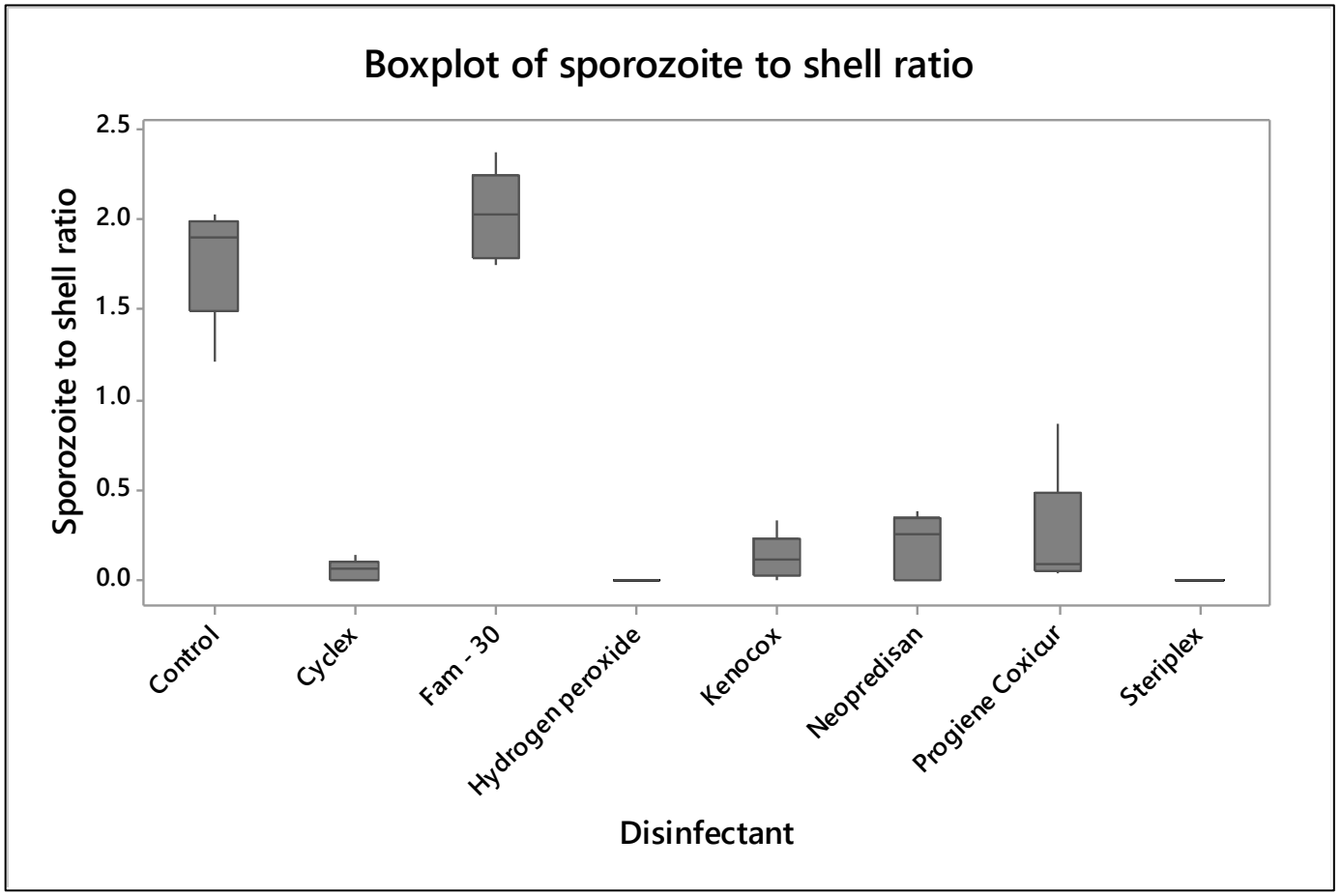


**Figure 24 Shell to sporozoite ratio for *Cryptosporidium* oocysts undergoing excystation following exposure to various disinfectants used according to the manufacturers guidelines. The rectangle represents the second and third quartiles, the horizontal line inside indicates the median value and the lower and upper quartiles are shown as vertical lines either side of the rectangle. The control is made up of 5 repeats done with the 5 repeats of disinfectant efficacy.**

### 5.4.3 Disinfectant Degradation

The effect of time following preparation of disinfectants on the efficacy of the various commercial disinfectants was analysed to see how the excystation rate and sporozoite to shell ratio is altered 7 days after the disinfectant had been prepared. Overall, every disinfectant had a worse performance when the 7-day old disinfectant was used with a higher excystation rate being observed. The excystation rate increased by 64.98 % to 71.06% for Neopredisan 135 – 1, which was the worst performing disinfectant for this test. Steriplex SD+ increased by 49.82 % to 54.79%, Cyclex by 42.01 % to 54.53 %, Progiene Coxicur by 24.97 % to 58.65%, KENO™COX by 20.25 % to 26.56 % and Hydrogen peroxide by 10.73 % to 11.13 %. Only FAM-30 had a reduction in excystation rate which was a reduction of 13.88 % to 16.75 % 7 days after the disinfectant was made up. Overall the disinfectant with the smallest excystation rate using disinfectant made up 7 days prior to analysis was Hydrogen peroxide. All of this data is summarised in Table 33.

**Table 33 The mean excystation rate of *Cryptosporidium* exposed to disinfectants made up on the day (fresh) or 7 days prior (7 days) based on 5 repeats.**

| Disinfectant | Mean (fresh) | Mean (7 days) | Reduction in efficacy |
|---|---|---|---|
| Cyclex | 12.52% | 54.53% | 42.01% |
| Fam - 30 | 30.62% | 16.75% | -13.88% |
| Hydrogen Peroxide | 0.40% | 11.13% | 10.73% |
| KENO™COX | 6.31% | 26.56% | 20.25% |
| Neopredisan 135 -1 | 6.08% | 71.06% | 64.98% |
| Progiene Coxicur | 33.68% | 58.65% | 24.97% |
| Steriplex SD+ | 4.97% | 54.79% | 49.82% |

### 5.4.4 Statistics

Determination of the best disinfectant to use was done by using a Tukey multiple comparisons test, which allowed for the analysis of each disinfectant against the others in multiple statistical tests. The best performing disinfectant for excystation rate compared to the control is hydrogen peroxide which is statistically significant at p= <0.001. Each disinfectant for both fresh and 7-day old disinfectants were grouped according to letter, where a different letter indicates that the disinfectant is significantly different. For excystation, all disinfectants were significantly different from the control when used fresh which can be seen in Table 34. After 7 days however, Neopredisan 135-1 and Progiene Coxicur were not significantly different from the control. For shell to sporozoite ratio (Table 35), all disinfectants apart from FAM-30 were significantly different from the control when used fresh, and this was also the case when the disinfectant was 7 days old. Despite hydrogen peroxide performing well with regards to the excystation percentage, Neopredisan 135 -1, KENO™COX, Cyclex, Steriplex SD+ all performed significantly better with regard to sporozoite to shell ratio. Therefore, even though very few oocysts excyst following treatment with hydrogen peroxide, as the disinfectant gets older the ones which do excyst have are healthier and release more sporozoites.

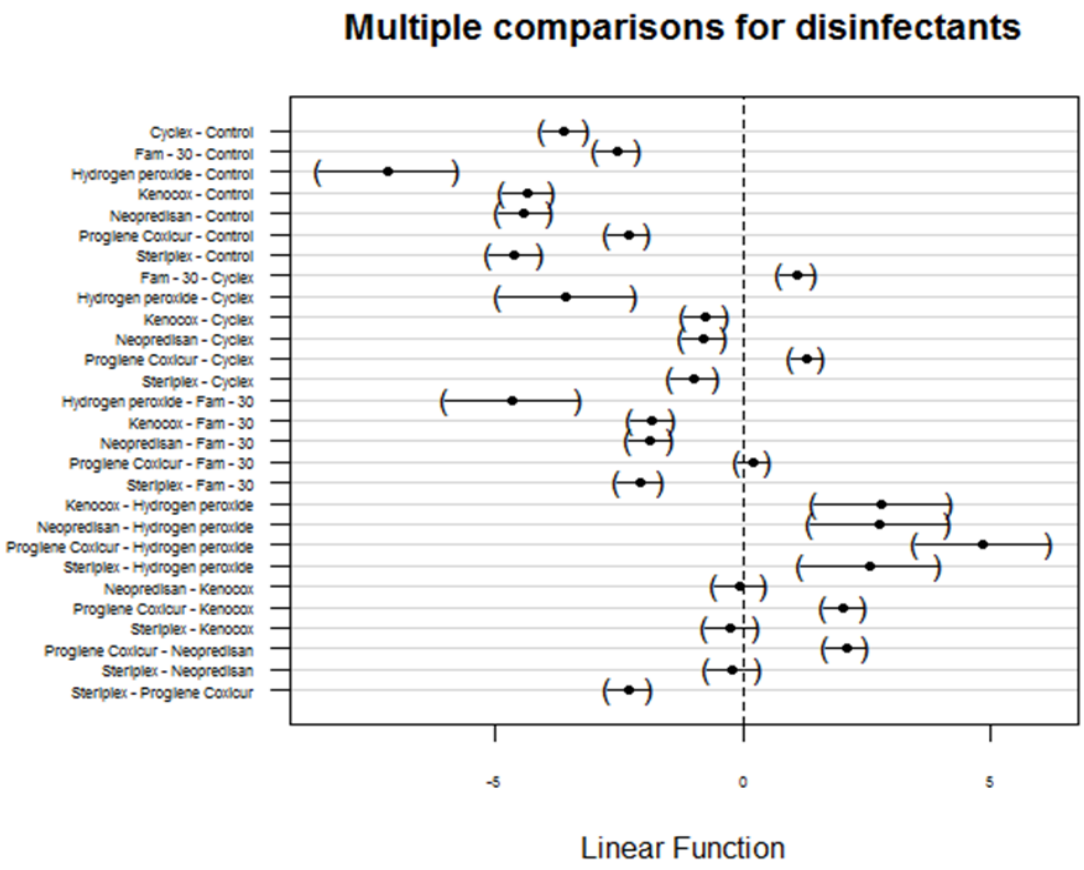


**Figure 25 Multiple comparisons test for the disinfectants used according to the manufacturers guidelines and made up fresh. The black dot indicates the difference in the mean in the two groups, and the horizontal lines and brackets indicate the 95 % confidence interval in this difference. If the horizontal lines do not pass through zero therefore, it is a significant difference. A negative difference in the means indicates that the first listed disinfectant is better than the one it is being compared to and a positive difference indicates it is worse.**

**Table 34 A Tukey comparison test to show the grouping of each disinfectant on their effect on *Cryptosporidium* oocyst excystation. A letter is assigned to each disinfectant; a shared letter indicates that the disinfectants are not statistically different from each other.**

| Disinfectant | Excystation rate (fresh) mean | Grouping | Excystation rate (7 day) mean | Grouping |
|---|---|---|---|---|
| Control | 80.59% | A | 80.59% | A |
| Progiene Coxicur | 33.68% | B C | 58. 65 % | A B |
| FAM - 30 | 30.62% | B C | 16.75 % | C D |
| Cyclex | 12.52% | C D E | 54.50 % | B |
| KENO™COX | 6.31% | D E | 26.56 % | C |
| Neopredisan 135-1 | 6.08% | D E | 71.06 % | A B |
| Steriplex SD+ | 4.97% | D E | 54.79 % | B |
| Hydrogen peroxide | 0.40% | E | 11.13 % | C D |

**Table 35 A Tukey comparison test to show the grouping of each disinfectant on their effect on *Cryptosporidium* oocyst sporozoite to shell ratio following excystation. A letter is assigned to each disinfectant; a shared letter indicates that the disinfectants are not statistically different from each other.**

| Disinfectant | Sporozoite to shell ratio (fresh) mean | Grouping | Sporozoite to shell ratio (7 day) mean | Grouping |
|---|---|---|---|---|
| FAM - 30 | 2.0 | A | 1.9 | A |
| Control | 1.7 | A | 2.0 | A |
| Progiene Coxicur | 0.2 | B | 0.2 | B C |
| Neopredisan 135 -1 | 0.2 | B | 0.1 | C |
| KENO™COX | 0.1 | B | 0.0 | C |
| Cyclex | 0.1 | B | 0.1 | C |
| Steriplex SD+ | 0.0 | B | 0.0 | C |
| Hydrogen peroxide | 0.3 | B | 0.8 | B |

## 5.5 Discussion

Hydrogen peroxide and hydrogen peroxide based (Steriplex SD+) disinfectants were found to be the most effective at reducing excystation rate and sporozoite to shell ratio in these excystation studies when used fresh. Hydrogen peroxide has performed well in many previous experimental studies on *Cryptosporidium*. Exposure of *Cryptosporidium* oocysts to 10 % Hydrogen peroxide for 30 minutes resulted in over 97 % inactivation (Delling et al., 2016) based on infectivity of *Cryptosporidium* of HCT-8 cells. Gas plasma sterilization using hydrogen peroxide led to inactivation of *C. parvum* oocysts (Vassal et al., 1998). A 1000 fold reduction in *Cryptosporidium* oocyst viability was reported when oocysts were exposed to 6 % hydrogen peroxide for 4 minutes (Weir et al., 2002), Hydrogen peroxide at 6 % for 20 minutes inactivated *C. parvum* (Barbee et al., 1999), and it has also been reported that *C. parvum* was unable in infect HCT-8 cells after oocysts were treated with hydrogen peroxide at 0.3 mg/ml (Kniel et al., 2004).

In Germany, guidelines state that an effective disinfectant must reduce infectivity by 99.5 % after 2 hours of exposure to be considered appropriate for use against protozoa (Delling et al., 2017). Currently no information on disinfectant guidelines is available for the United Kingdom. Assuming similar regulations, this would mean that only hydrogen peroxide, when used fresh, would meet these German guidelines for appropriate to use against protozoa, based on the results of this study.

Typically, many *Cryptosporidium* viability studies involve using oocysts to infect either animals or cells. There is no standardized method for assessing C*ryptosporidium* viability; however *Eimeria tennela* has a standardized protocol to assess viability which involves a chicken infection model which can be used to test disinfectants (Daugschies, Bose, Marx, Teich & Friedhoff., 2002). This does however involve extensive animal experimentation and so many research groups opt for *in vitro* studies for

oocyst viability tests to bypass the ethical implications of *in vivo* testing (Delling, Lendner, Müller & Daugschies., 2017). *In vitro* work has successfully been done for *C. parvum* at the University of Leipzig in Germany which combined cell culture with real time PCR (qPCR). This group successfully managed to get *C. parvum* to infect human ileocecal adenocarcinoma cells (HCT-8 cells) and so could then use this method for drug and disinfectant testing (Shahiduzzaman et al., 2009). This therefore would be an effective measure of viability of *Cryptosporidium* oocysts.

Excystation is a traditionally used technique to determine oocyst viability and is proven to be an effective measure of oocyst and sporozoite health (Pecková et al., 2016). It allows for the imitation of host signals in order to induce the release of sporozoites from oocysts and therefore determine oocyst health and viability. This is a preferred method to animal infectivity assays as it is considered more precise, cheaper to conduct, can be done over a shorter time frame and is not susceptible to genetic variation (Pecková et al., 2016). However, recently research has shown that it is not as precise as using cell culture. If an oocyst fails to excyst under excystation protocols, it does not necessarily mean that the oocyst is no longer infectious (Neumann, Gyurek, Finch & Belosevic., 2000). This is according to research which showed disinfectant treated oocysts which did not excyst under excystation protocols (seen as intact oocysts under the microscope) were still able to infect HCT-8 cells (Kniel et al., 2004). It was noted that the 'treated' sporozoites used to infect the HCT-8 cells lacked motion and so proved that the presence of sporozoites alone does not necessarily indicate viability. Excystation was used as a measure of viability in this study due to time, labour and costs. Excystation analysis under the microscope is a quick way of determining oocyst and sporozoite health. This allowed for more repeats of the experiment to be carried out to gain statistically significant results. If the work in this chapter could be taken further however, it would be really useful to assess viability by infecting cell lines such as HCT-8 to better determine the viability after exposure to

disinfectants. Especially as many of these disinfectants do not release information as to their mode of action and so excystation, while able to determine oocyst survivability, is likely not enough to a determine infectivity when used alone.

Excystation rates are known to vary with the age and strain of *Cryptosporidium* oocysts (Hijjawi, 2003) and so it is therefore important to note that the oocysts used for the manufacturer's guidelines results were a different batch from the manufacturers guidelines after 7 days results. This is why no direct statistical analysis could be done between fresh and 7-day old disinfectants although each experimental group was compared to its own control. However, based on the results obtained (Table 33) it is highly unlikely that variation in the oocysts would account for the differences observed. This second batch was collected and prepared from faeces fresh before the experiment and so would be not as old as the previous batch which was used a month following preparation. The excystation rate of FAM-30 decreased after exposure to 7-day old disinfectant. The difference in these results between fresh and 7-day old FAM-30 were not statistically significant.

There is a potential issue when it comes to washing off the disinfectant properly from the oocysts to test for excystation. In this study, oocysts were washed three times with phosphate buffered saline (PBS) and the supernatant discarded. Despite this, the sporozoite to shell ratio remained very low for all disinfectants, despite the excystation rate increasing after 7 days of making up the disinfectant. Sporozoites are very fragile and so any remaining disinfectant could have destroyed them. It was found during this study that vigorous mixing was also very damaging to sporozoites and so it is likely the sporozoite count is not representative of what would appear in a natural setting. Steriplex SD+ for example has silver particles in its ingredients list and is likely that these particles were spun down with the oocysts and underwent the excystation protocol in the tube. As it has

been proven that silver nanoparticles destroy *Cryptosporidium* oocysts, it is possible that the results would differ in a farm setting or using cell culture methods as the concentration of these nanoparticles would have been more higher in a tube.

Results from the pilot study on Steriplex SD+ indicates that oocysts in a 'dirty' environment (in faeces) are harder to inactivate (Figure 22). It is essential therefore that thorough cleaning takes places on farm for the disinfectant to work at its highest efficacy. As Steriplex SD+ has hydrogen peroxide as one of its main components, and it is also true that organic material oxidizes hydrogen peroxide into water and oxygen (Safety & Administration, 2000), then it could be said that this result may be specific to hydrogen peroxide based products. However, the user guidelines for all disinfectants include washing the area to be disinfected first and so it is essential that this step is carried out properly. Further work should include 'dirty' environment testing for the other commercially used disinfectants to see which is most likely to perform the best (inactivate the most *Cryptosporidium* oocysts) in a farm environment.

## 5.6 Conclusion

Based on the pilot study work with Steriplex SD+, hydrogen peroxide-based disinfectants are best used when made up fresh and also in a clean environment. When used fresh, hydrogen peroxide and hydrogen peroxide-based products (Steriplex SD+) are the best at inactivating *Cryptosporidium* oocysts based on excystation rate and sporozoite to shell ratio. However, after the product has been made up for 7 days; hydrogen peroxide has a higher sporozoite to shell ratio than other disinfectants Neopredisan 135 -1, KENO™COX, Cyclex, and Steriplex SD+. The efficacy of 7-day old Neopredisan 135-1, Cyclex and Steriplex SD+ reduced by 64.98 %, 42.01 % and 49.82 % respectively and so their efficacy reduces considerably over 7 days. The disinfectant KENO™COX performed the best with regard to

degradation over time, maintaining the highest efficacy 7 days post preparation.

# Chapter 6 General Discussion

Cryptosporidiosis, caused by the protozoan parasite *Cryptosporidium*, is a very important diarrhoeal disease as it is a major cause of animal mortality and economic loss on the farm (Ralston, Thompson, Pethick, McAllister & Olson., 2010; Sweeny et al., 2011; Goater et al., 2014) and is widespread throughout the world. Prevalence in UK cattle herds varies from 28 – 80% (Brook et al., 2008; Wells et al., 2015) and it is thought that all calves in infected herds will shed *Cryptosporidium* oocysts at some point during the first few months of life (Santin et al., 2008). Not only is *Cryptosporidium* a problem for livestock on farms, but it is a risk to public health, being responsible for many human diarrhoeal outbreaks (Chalmers, 2012). It has been estimated that there are around 8.9 cases of cryptosporidiosis per 100,000 people in the UK, based on Health Protection Agency (HPA) reports, most of these being young children (Nichols et al., 2006). There are currently 37 reported species of *Cryptosporidium* with many more genotypes, each of which has its own host range with different clinical manifestations (Ryan et al., 2014). It is likely that significant under-reporting takes place with regard to *Cryptosporidium* due to the fact that disease tends to be limited to 1-2 weeks in otherwise healthy individuals (Thomson et al., 2017). There are other causes of diarrhoea in calves on the farm other than *Cryptosporidium,* such as milk scours (Okada et al., 2009) which occur in neonatal calves, and other pathogens such as rotavirus, coronavirus and *E.coli* (Mawly et al., 2015). Clinical disease severity is determined by both species and genotype of *Cryptosporidium* (Bouzid et al., 2013). Therefore, unless veterinary diagnosis takes place, *Cryptosporidium* could be misidentified and therefore the problem is likely to be bigger than the statistics available.

The aims of the PhD included determining the risk that adult cattle and wildlife, specifically rabbits and pheasants, pose on the transmission of *Cryptosporidium* oocysts to naïve calves. A further aim was to determine whether there is a long-term effect on growth rate following

cryptosporidiosis in beef calves when calves are infected with *Cryptosporidium* in the first 16 days of life. The control of the parasite on the farm is very difficult, and so an analysis on the available disinfectants was performed in order to determine which would be the best one to use on the farm environment.

Typically, neonatal livestock show clinical signs of cryptosporidiosis when they are infected with the species *C. parvum*, although other species *C. bovis, C. ryanae* and *C. andersoni* are also found in cattle (Thomson et al., 2016). Work completed in Chapter 2 shows that neonatal and pre-weaned calves predominantly shed *C. parvum* with the occasional mixed infection with *C. bovis* and *C. ryanae*. The species *C. bovis* and *C. ryanae* did not occur as a single infection until the calves were at least one month of age. The adult cattle are shedding both *C. parvum* and *C. andersoni*. This provides further evidence to the conclusions drawn following a longitudinal study of the species of *Cryptosporidium* found in calves; that the predominant species present tends to follow an age-related distribution (Thomson, 2015). Peak shedding of *C. parvum* occurred between weeks 2 and 3 of age which supports previous findings that young calves tend to show clinical signs of disease in the second week of life (Faubert & Litvinsky, 2000; Sanford & Josephson, 1982). A second peak of infection occurred when the calves were 5 weeks of age and this was with another genotype of *C. parvum*. This indicates that infection with one genotype in calves does not provide protection against another, which has been shown before when lambs still suffered clinical cryptosporidiosis following a heterologous challenge of *C. parvum* genotypes (Thomson, 2015).

The role that adult cattle could play in the transmission of *C. parvum* to calves has been addressed in the work in Chapter 2. Using sensitive concentration techniques and highly discriminatory genotyping tools, it appears that adult cattle do shed *C. parvum* which supports work done by Faubert & Litvinsky (2000) and also work looking at *Cryptosporidium*

species in livestock in a Scottish water catchment (Wells et al., 2015) both of which found *C. parvum* in adult cattle. Although this work contradicts two studies undertaken by Atwill & Pereira (2003) which found no *C. parvum* in adult cattle (Atwill & Pereira, 2003; Atwill et al., 1998). It is likely that the new sensitive techniques, which help to address the common problems posed by adult cattle faeces (large starting volume and high fibre content) (Wells et al., 2016), are responsible for this difference and adult cattle do shed more *C. parvum* than previously thought. In this work 33 % of adult dairy cattle and 56 % of adult beef cattle were shedding *C. parvum*. Results from Chapter 2 showed that only 2/38 (5.26%) adult dairy cattle were shown to be shedding the same genotype (MLG 1) as calves. This was following multi-locus genotyping on the dairy farm which showed the majority of the adults (12) were shedding a genotype which was different to MLG 1 at more than two loci. So therefore, it is unlikely adult dairy cattle play a major part in transmission of *C. parvum* to their calves. Further work needs to be undertaken to determine the role that adult beef cattle play in the transmission of *Cryptosporidium* to their calves. The results for the beef cattle and calf transmission in Chapter 2 were inconclusive. However, as the rearing system means that adults and calves are kept together, it is more likely that transmission will occur between them. Performing this work in a similar way to the dairy farm (sampling the adult cattle before the calves are born) would allow for a more confident conclusion as to the role of adult beef cattle in the transmission of *C. parvum*. However, this is much more difficult to achieve on a beef farm and would require either bringing all cattle in early before calving or sampling cattle while they were in the field. This would make it difficult to identify which sample came from each animal.

The parasite is known to persist within the calf population on farm and commonly recurs each calving season. As *Cryptosporidium* is a very hardy environmentally ubiquitous parasite (Goater et al., 2014), this persistence could potentially be the *Cryptosporidium* oocysts persisting in the calving

area for subsequent years. Many commonly used disinfectants are ineffective at killing *Cryptosporidium* oocysts (Weir et al., 2002) which also increases the chances that the parasite will persist in the environment. It is likely that the environmental load of oocysts increases as the calving season progresses, as more calves are born, become infected and start to shed oocysts. The first calves would likely receive a low infectious dose and therefore suffer reduced clinical disease compared to those born later in the season which are met with a much higher infectious dose following amplification in the first-born calves.

Multi-locus genotyping using microsatellite analysis allowed for a more discriminatory look at the genotypes present in both the adult cattle, calves and wildlife. Previous work has focussed on GP60 genotyping alone (Brook et al., 2009; Thomson, 2015) although work done in Chapter 2 shows this is not enough to determine genotype. Sixteen adult dairy cattle shared the same GP60 genotype as the calves, which may indicate they could play a role in transmission. Further typing, however, revealed that the *C. parvum* present was actually different and the final conclusion was that only two of the adult dairy cattle shared that genotype with their calves. More regions of the genome do exist for molecular typing and further work should include examining these in more detail. Previously published work listed MM5, MM18, MM19 and TP14 as being most discriminatory and useful for cattle infections (Hotchkiss et al., 2015), however more could be analysed to make epidemiological analysis more accurate. A communal research database listing *Cryptosporidium* species, genotypes, locations and hosts would be a useful tool for further epidemiological work. This would allow for transmission studies to be more powerful by gaining more of an insight into the species and genotypes found in particular locations. It would also be useful for public heath, to determine if zoonotic species are present in certain high-risk water catchments.

Clinical cryptosporidiosis is thought to have a long-term effect on calf growth as it has already been proven to be detrimental to the growth of children (Ajjampur et al., 2010; Checkley et al., 1998), and the weight gain and carcass condition of lambs (Jacobson et al., 2016; Sweeny et al., 2011). Work done in Chapter 3 has proved that calves with severe clinical disease have a significantly reduced weight gain when compared to calves with no clinical disease at 6 months of age. Those animals suffering from a mid-range disease still suffered a reduction in weight gain and so any form of clinical cryptosporidiosis could have longer-term effects. This is supported by similar work which was done in children, which found that children which suffered a single episode of cryptosporidiosis had similar weight-for-age and height-for-age scores as children which suffered multiple infections, which was significantly lower than children with no infections (Ajjampur et al., 2010). The impact of these findings could be much larger than just having smaller cattle. A reduction in growth rate is likely to result in a poor body condition score, which is associated with poor reproductive efficiency (Kadivar, Ahmadi & Vatankhah., 2014) with cattle taking longer to ovulate after having a calf. A low body condition score can also predispose cattle to lameness (Randall et al., 2015) and other infectious diseases (Roche, Kay, Friggens, Loor & Berry., 2013). Reduced growth rate would also impact dairy cattle as milk production is known to decrease with reduced body condition and body weight (Roche et al., 2013).

Further work should include following beef calves for a longer period of time until they reach slaughter age. This will allow for an analysis of cryptosporidiosis on the carcass quality and score. Those animals which are kept could have their reproductive performance and milk yield analysed in order to determine if the changes that cryptosporidiosis causes at a young age in calves could have a larger economic impact on the farm. Calves showed a range of clinical manifestations of the disease despite being kept under the same management in the same shed and so are likely to suffer

similar exposures to *Cryptosporidium* oocysts. In fact, in Chapter 3 it was shown that almost all calves tested positive for *C. parvum* following PCR. Therefore, genetic studies to determine why some calves are more affected by *C. parvum* than others would be a very useful area of research and may point towards selective breeding opportunities.

It was found that a high level of *C. parvum* oocysts were being shed by both wild rabbits and pheasants. A surprising find for the rabbits, as previously the most common species shed has been reported to be *C. cuniculus* (Robinson, & Chalmers. 2010). The high prevalence of *C. parvum* in the rabbit is likely due to the rabbits living in close proximity to farmland. These rabbits were sampled initially for the examination of paratuberculosis transmission to cattle (Fox et al., 2018), and so were selected based on their proximity to cattle. The pheasants too were also located very close to the calving shed on the farm they were sampled from. Despite this, the genotypes present in the pheasants were mixed and in the first sampling year of 2016, none of the pheasants were shedding the same genotype of *C. parvum* as the calves. It would be interesting to expand this work to determine if the pheasants were infected with *C. parvum* or if they are acting as a transport host. The rabbits did present with typical clinical signs of cryptosporidiosis (weight loss, lethargy, soft faeces) (Fox., 2017, Personal communication) however without ruling out other infectious diseases it is impossible to attribute that to *C. parvum*. Overall due to the difficulty in concentrating enough oocysts for genotyping and the wide range of genotypes that were present in both rabbits and pheasants, the risk that they pose to the transmission of *C. parvum* to calves may be minimal. However, taking samples from the rabbits at a single time point makes it less likely that you would sample a rabbit during an acute infection and therefore further longitudinal studies would be required to confirm this. Also, due to the low infectious dose required to infect a calf (Zambrisky et al., 2013), transmission of oocysts from rabbits and pheasants is still a possibility.

*Cryptosporidium* is very difficult to manage on farms due to the parasite's ability to survive many of the commonly used disinfectants and environmental conditions. This is further discussed in Chapter 5. However in summary, the lack of control means that once a farm has a problem with cryptosporidiosis, it is currently almost impossible completely inactivate the parasite in the environment. Chapter 5 has shown that some disinfectants do exist which are capable of inactivating the oocysts, however, none of them is 100 % effective. It is therefore essential for farmers to follow the guidelines provided by disinfectant manufacturers, abiding by usage, concentration, contact time and storage recommendations. The pilot study in chapter 5 on Steriplex SD+ shows that the disinfectant is much less effective when used in dirty environments (oocysts in faeces). Therefore, it is essential to make sure pens are cleaned out before disinfectants are used for the product to be the most effective.

Overall, many factors should be considered when determining the best disinfectant to use on the farm. Ease of use and short contact times are most desirable on a working farm in order to reduce the time spent with empty pens and sheds. The cost and shelf life of the disinfectant is also very important as some farms work with only limited budgets. Risk to user and to the environment should also be considered as it could pollute the surrounding environment. Despite hydrogen peroxide performing the best according to the results in Chapter 5, the prepared product has a relatively short shelf life, losing efficacy after only 7 days following preparation. Not only this but there are restrictions on what percentage concentration can be purchased, which is 12% (less than the 30% stock used for this study) which is likely to reduce the shelf life even further. Therefore, a better alternative would be KENO™COX which has a much longer shelf life of the prepared product. Unfortunately, this product has one of the longest required contact times (2 hours) although it is cheaper, working out at £0.42 pence per litre of working solution, compared to £0.84 pence for hydrogen peroxide. Neopredisan 135-1 is commonly used against

*Cryptosporidium* oocysts and did perform well in Chapter 5. However, this disinfectant works out to be the most expensive at £1.12 per litre of working solution. Both are used at 0.4 litres per $m^2$. Another consideration is safety, as Neopredisan 135-1 is considered safe to use in the presence of animals and humans, environmentally friendly and biologically degradable. KENO™COX, on the other hand, is corrosive, requires protective clothing for the user and is considered dangerous to the environment.

The newer products Cyclex, Progiene Coxicur and Steriplex SD+ are currently not the best options for the inactivation of *Cryptosporidium* oocysts. Progiene Coxicur performed the worst out of all the tested disinfectants in Chapter 5. Cyclex, although effective, requires a 4 hour contact time, costs £0.70 pence per litre of working solution and can cause burns, eye damage, skin reactions and is toxic to aquatic life. Steriplex SD+ did perform very well, although only when used fresh. It is not currently on the market for sale to farmers in the UK but in the future may be a good option, although currently, the disinfectant costs the equivalent of £40 per litre of working solution.

What the farming community really requires is an effective drug or vaccine to combat cryptosporidiosis. Research in this area has been lacking for some time owing to the difficulties with maintaining the parasite in laboratory conditions without the use of animals. This means that less parasite is available for the tests required for drug and vaccine development. Despite this, progress has been made and new drugs could be on the horizon. A newly developed bumped kinase inhibitor which targets the calcium-dependant protein kinases in *Cryptosporidium* has effectively cured cryptosporidiosis in 5 out of 6 mice with no side effects (Castellanos-Gonzalez et al., 2016). These bumped kinase inhibitors were also used in a calf model where treatment resulted in a reduction in diarrhoea severity, *Cryptosporidium* oocyst shedding, and overall health of the calves (Schaefer et al., 2016).

**PhD Outcomes**

Overall this PhD has shown that adult cattle, rabbits and pheasants are unlikely to play a major role in the transmission of *Cryptosporidium* oocysts to calves. In order to determine this, in-depth genotyping was required and this work has shown that sequencing at the GP60 locus alone is not discriminatory enough to draw conclusions on transmission. This PhD has shown that cryptosporidiosis has a long-term effect on the growth rate of infected calves, even after they recover from clinical disease with the parasite. This has much wider implications including costs to the farmer and animal's feed conversion efficiency and potentially reproductive efficiency later in life. An analysis of the available disinfectants on the market to farmers has given a useful set of guidelines for disinfectant use including using them when fresh and after a shed has been cleaned. This along with a comparison of cost, contact times required and safety considerations are all very useful when providing information to farmers of the positives and negatives of each option, allowing them to make an informed choice.

**Advice for controlling *Cryptosporidium***

Following from the results of this PhD, it is important to initially control *Cryptosporidium* shedding in the environment by quarantining infected animals and ensuring oocysts are kept as contained as possible. Effective disinfectants such as hydrogen peroxide-based disinfectants and KENOCOX must be used when fresh and used once the area has already been cleaned. Installing barriers against wildlife access, especially in areas where other farms have problems with cryptosporidiosis may help stop *Cryptosporidium* arriving on the farm in the first place. This should help to reduce the number of calves affected with the parasite and also reduce the severity. Both of which should reduce the costs associated with cryptosporidiosis.

**Future Work**

There are still many knowledge gaps which should be addressed with regard to the control of *Cryptosporidium*. Initially microscopy should be carried out on any PCR negative samples in order to rule out false negative results through PCR inhibitors. The work within this thesis has used microsatellite analysis rather than using GP60 alone. However, using whole genome sequencing would allow for further discriminatory ability and allow the identification of suitable microsatellite loci. This work has already started for cattle in Scotland.

A continuation of this research to include a more conclusive study on adult beef cattle and their role in the transmission of *C. parvum* to calves by testing mothers for *Cryptosporidium* oocysts before the calves are born, with the same sample size, and see how the species and genotypes change as calving progresses. A study comparing different calving systems such as indoor and outdoor calving would be interesting to see if oocysts do build up in sheds in consecutive years. It would also be interesting to explore variations in *Cryptosporidium* genotypes present on farm both seasonally and geographically.

Work examining the effect that cryptosporidiosis has on longer term production of cattle such as reproductive performance and time to first ovulation would also be very useful in determining the full economic impact of cryptosporidiosis. This could be extended to include dairy calves as poor weight gain in dairy calves is still likely to cause indirect economic losses from higher susceptibility to disease and reduced milk production.

Further work into other wildlife species such as deer, which are highly prevalent in Scotland, would allow for the determination of the risk they pose to transmitting *C. parvum* to calves. There have also been reports that geese could be a carrier of *C. parvum* oocysts (Paton,. 2018. Personal communication) and so these would also be interesting to look into. Their

migration patterns could result in the wide spread of these oocysts in goose faeces.

Continuing the disinfectant work using disinfectants to treat *Cryptosporidium* oocysts which are then used to infect cells in culture would conclusively show the efficacy of disinfectants.

# Chapter 7 References

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# Appendices

# Solutions

**2% Sulphuric Acid**

| | |
|---|---|
| Sulphuric Acid (98%) 98.08g/mol COSHH RED | 20mls |
| Tap water | 980mls |

Store at room temperature
COSHH: RED

**Saturated Salt Solution**

| | |
|---|---|
| Sodium Chloride (NaCl) | 357g |

Add the salt to a 1 litre bottle.

| | |
|---|---|
| Distilled water make up to | 1 litre |

Store at room temperature
COSHH: GREEN
Stir on magnetic stirrer until dissolved.

**50 X TAE Buffer (2M Trisacetate, 1mM EDTA)**

| | |
|---|---|
| Sigma 7-9 mwt 121.14 (Tris) | 242g |
| 0.5M EDTA (pH 8.0) | 100ml |
| Glacial Acetic Acid (mwt 60.05) | 57.1ml |
| Distilled water | 500mls |

Mix and make up to 1 litre with distilled water.
Store at Room temperature
COSHH: GREEN
Final concentrations: 2.0M tris acetate; 0.05M EDTA; pH 8.2-8.4 (at 25°C)

**EDTA Solution 0.5M**

| | |
|---|---|
| EDTA (mwt 372.2) | 93.05g |

Addition of NaOH until ph8 is reached

| | |
|---|---|
| Distilled water to make up to | 500mls |

COSHH: GREEN

**10X PCR Buffer**

| **Reagent** | **Stock conc.** | **Volume (µl)** | **Final.conc (10X)** | **Conc. In reaction** |
|---|---|---|---|---|
| Tris-HCl (pH 8.8) | 2M | 2250 | 450mM | 45mM |
| Ammonium Sulphate | 1M | 1100 | 110mM | 11mM |
| $MgCl_2$ | 1M | 450 | 45mM | 4.5mM |
| EDTA (pH 8.0) | 10mM | 44 | 44µM | 4.4µM |
| BSA | 10mg/ml | 1130 | 1.13mg/ml | 0.113mg/ml |
| dATP | 100mM | 1000 | 10mM | 1.0mM |
| dCTP | 100mM | 1000 | 10mM | 1.0mM |
| dGTP | 100mM | 1000 | 10mM | 1.0mM |
| dTTP | 100mM | 1000 | 10mM | 1.0mM |

**Appendix 1 Sample number and date of collection for faecal samples collected from adult cattle on a dairy farm. Calf name (if known) is listed along with *Cryptosporidium* species specific PCR results given.**

| Lab ID | Date | Animal ID | Info other | Calf Name | *C. parvum* | *C. bovis* | *C. andersoni* | *C. ryanae* |
|---|---|---|---|---|---|---|---|---|
| 3.114 | 20/11/15 | 43 | Adult | Caesarian - Died | NEG | NEG | NEG | NEG |
| 3.54 | 02/11/15 | 500 | Adult | 5993 Aftershock Georgette | POS | NEG | POS | NEG |
| 3.65 | 6/11/15 | 500 | Adult | 5993 Aftershock Georgette | POS | NEG | NEG | NEG |
| 3.201 | 30/11/15 | 1460 | Adult | 6022 Mercure Barbie | NEG | NEG | NEG | NEG |
| 3.47 | 02/11/15 | 1634 | Adult | Bull - Gone | NEG | NEG | NEG | NEG |
| 3.55 | 02/11/15 | 1704 | Adult | Bull - Gone | POS | NEG | POS | NEG |
| 3.319 | 14/12/15 | 1796 | Adult | Twins/Gone | NEG | NEG | NEG | NEG |
| 3.342 | 22/12/15 | 1796 | Adult | Twins/Gone | POS | NEG | NEG | NEG |
| 3.119 | 20/11/15 | 1960 | Adult | Bull - Gone | NEG | NEG | NEG | NEG |
| 3.200 | 27/11/15 | 1960 | Adult | Bull - Gone | POS | NEG | POS | NEG |
| 3.68 | 6/11/15 | 2112 | Adult | Bull Calf - Gone | POS | NEG | NEG | NEG |
| 3.18 | 26/10/15 | 2112 | Adult | Calved - Bull | POS | NEG | NEG | NEG |
| 3.290 | 11/12/15 | 2477 | Adult | 6032 Direct Violet 4 | POS | NEG | POS | NEG |
| 3.317 | 14/12/15 | 2477 | Adult | 6032 Direct Violet 4 | NEG | NEG | POS | NEG |
| 3.334 | 18/12/15 | 2477 | Adult | 6032 Direct Violet 4 | NEG | NEG | NEG | NEG |
| 3.349 | 22/12/15 | 2477 | Adult | 6032 Direct Violet 4 | NEG | NEG | NEG | NEG |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 3.103 | 13/11/15 | 2692 | Adult | 6017 Avalanche Maureen | NEG | NEG | POS | NEG |
| 3.123 | 20/11/15 | 2692 | Adult | 6017 Avalanche Maureen | NEG | NEG | POS | NEG |
| 3.166 | 25/11/15 | 2692 | Adult | 6017 Avalanche Maureen | NEG | NEG | NEG | NEG |
| 3.116 | 20/11/15 | 2846 | Adult | 6013 Snowy Judy | POS | NEG | NEG | NEG |
| 3.15 | 26/10/15 | 3126 | Adult | 5816 Moredun + C.parvum | POS | NEG | NEG | NEG |
| 3.42 | 02/11/15 | 3292 | Adult | 5996 Direct Whitney 4 | NEG | NEG | NEG | NEG |
| 3.112 | 20/11/15 | 3299 | Adult | 5834 Moredun | NEG | NEG | NEG | NEG |
| 3.53 | 02/11/15 | 3339 | Adult | Bull - Gone | NEG | NEG | POS | NEG |
| 3.164 | 25/11/15 | 3345 | Adult | 6014 Direct Cherry | NEG | NEG | POS | NEG |
| 3.58 | 02/11/15 | 3510 | Adult | 6006 Direct Trixie | POS | NEG | NEG | NEG |
| 3.82 | 9/11/15 | 3510 | Adult | 6006 Direct Trixie | NEG | NEG | NEG | NEG |
| 3.110 | 13/11/15 | 3537 | Adult | 6023 Winton Mayflower | NEG | NEG | NEG | NEG |
| 3.187 | 27/11/15 | 3537 | Adult | 6023 Winton Mayflower | NEG | NEG | NEG | NEG |
| 3.204 | 30/11/15 | 3537 | Adult | 6023 Winton Mayflower | NEG | NEG | NEG | NEG |
| 3.289 | 11/12/15 | 3734 | Adult | 6029 Dude Lupin | POS | NEG | NEG | NEG |
| 3.337 | 18/12/15 | 3734 | Adult | 6029 Dude Lupin | POS | NEG | NEG | NEG |
| 3.111 | 13/11/15 | 3871 | Adult | Beef 5832 | POS | NEG | NEG | NEG |
| 3.118 | 20/11/15 | 3871 | Adult | Beef 5833 | NEG | NEG | NEG | NEG |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 3.108 | 13/11/15 | 3889 | Adult | Beef 5839 | NEG | NEG | NEG | NEG |
| 3.199 | 27/11/15 | 3889 | Adult | Beef 5839 | POS | NEG | POS | NEG |
| 3.205 | 30/11/15 | 3889 | Adult | Beef 5839 | NEG | NEG | NEG | NEG |
| 3.51 | 02/11/15 | 3917 | Adult | Bull - Gone | POS | NEG | POS | NEG |
| 3.316 | 14/12/15 | 3939 | Adult | 6030 Direct Placida | NEG | NEG | NEG | NEG |
| 3.336 | 18/12/15 | 3939 | Adult | 6030 Direct Placida | POS | NEG | POS | NEG |
| 3.346 | 22/12/15 | 3939 | Adult | 6030 Direct Placida | NEG | NEG | NEG | NEG |
| 3.16 | 26/10/15 | 4077 | Adult | 5818 Moredun + C.parvum | NEG | NEG | NEG | NEG |
| 3.86 | 9/11/15 | 4081 | Adult | 6019 Winbrook Doll | NEG | NEG | NEG | NEG |
| 3.106 | 13/11/15 | 4081 | Adult | 6019 Winbrook Doll | NEG | NEG | POS | NEG |
| 3.161 | 25/11/15 | 4081 | Adult | 6019 Winbrook Doll | NEG | NEG | NEG | NEG |
| 3.213 | 25/11/15 | 4081 | Adult | 6019 Winbrook Doll | NEG | NEG | NEG | NEG |
| 3.283 | 11/12/15 | 4139 | Adult | | NEG | NEG | POS | NEG |
| 3.13 | 26/10/15 | 4196 | Adult | Beef 5838 | POS | NEG | NEG | NEG |
| 3.52 | 02/11/15 | 4196 | Adult | Beef 5838 | POS | NEG | NEG | NEG |
| 3.67 | 6/11/15 | 4196 | Adult | Beef 5838 | POS | NEG | NEG | NEG |
| 3.107 | 13/11/15 | 4196 | Adult | Beef 5838 | POS | NEG | NEG | NEG |
| 3.167 | 25/11/15 | 4196 | Adult | Beef 5838 | POS | NEG | NEG | NEG |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 3.189 | 27/11/15 | 4196 | Adult | Beef 5838 | NEG | NEG | NEG | NEG |
| 3.207 | 30/11/15 | 4196 | Adult | Beef 5838 | POS | NEG | NEG | NEG |
| 3.159 | 25/11/15 | 4216 | Adult | 6016 Winbrook Kitty | POS | NEG | POS | NEG |
| 3.117 | 20/11/15 | 4226 | Adult | 5835 Moredun | POS | NEG | POS | NEG |
| 3.162 | 25/11/15 | 4241 | Adult | 6026 Dude Emma 2 | POS | NEG | POS | NEG |
| 3.246 | 02/12/15 | 4241 | Adult | 6026 Dude Emma 2 | NEG | NEG | NEG | NEG |
| 3.44 | 02/11/15 | 4247 | Adult | Beef 5831 | POS | NEG | NEG | NEG |
| 3.347 | 22/12/15 | 4249 | Adult | 6037 Direct Agnes 3 | NEG | NEG | NEG | NEG |
| 3.193 | 27/11/15 | 4455 | Adult | Bull - Gone | POS | NEG | NEG | NEG |
| 3.208 | 30/11/15 | 4455 | Adult | Bull - Gone | POS | NEG | POS | NEG |
| 3.292 | 11/12/15 | 4455 | Adult | Bull - Gone | POS | NEG | NEG | NEG |
| 3.169 | 25/11/15 | 4455 | Adult | Bull -Gone | NEG | NEG | NEG | NEG |
| 3.286 | 11/12/15 | 4488 | Adult | Bull- Gone | NEG | NEG | NEG | NEG |
| 3.120 | 20/11/15 | 4491 | Adult | 6018 Spades Fiona | POS | NEG | POS | NEG |
| 3.160 | 25/11/15 | 4500 | Adult | 6033 Fantasmic Frota | NEG | NEG | NEG | NEG |
| 3.191 | 27/11/15 | 4500 | Adult | 6033 Fantasmic Frota | POS | NEG | NEG | NEG |
| 3.209 | 30/11/15 | 4500 | Adult | 6033 Fantasmic Frota | NEG | NEG | NEG | NEG |
| 3.247 | 02/12/15 | 4500 | Adult | 6033 Fantasmic Frota | POS | NEG | NEG | NEG |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 3.287 | 11/12/15 | 4501 | Adult | Bull - Gone | NEG | NEG | POS | NEG |
| 3.48 | 02/11/15 | 4517 | Adult | 5994 Direct Bonnie 4 | NEG | NEG | NEG | NEG |
| 3.66 | 6/11/15 | 4517 | Adult | 5994 Direct Bonnie 4 | POS | NEG | NEG | NEG |
| 3.46 | 02/11/15 | 4529 | Adult | 6001 Tiergan Brana | POS | NEG | NEG | NEG |
| 3.83 | 9/11/15 | 4529 | Adult | 6002 Tiergan Brana | POS | NEG | POS | NEG |
| 3.105 | 13/11/15 | 4545 | Adult | 5836 Moredun | POS | NEG | POS | NEG |
| 3.115 | 20/11/15 | 4653 | Adult | Bull - Gone | NEG | NEG | NEG | NEG |
| 3.12 | 26/10/15 | 4662 | Adult | 5992 Savior Vision | POS | NEG | NEG | NEG |
| 3.50 | 02/11/15 | 4662 | Adult | 5992 Savior Vision | POS | NEG | POS | NEG |
| 3.340 | 18/12/15 | 4670 | Adult | Bull - Gone | NEG | NEG | NEG | NEG |
| 3.49 | 02/11/15 | 4673 | Adult | 6005 Urbain Silvergirl | NEG | NEG | NEG | NEG |
| 3.84 | 9/11/15 | 4673 | Adult | 6005 Urbain Silvergirl | NEG | NEG | POS | NEG |
| 3.348 | 22/12/15 | 4678 | Adult | 6040 Direct Belle | POS | NEG | NEG | NEG |
| 3.57 | 02/11/15 | 4687 | Adult | 5995 Doorman Whitney | POS | NEG | POS | NEG |
| 3.45 | 02/11/15 | 4690 | Adult | Bull - Gone | POS | NEG | NEG | NEG |
| 3.350 | 22/12/15 | 4707 | Adult | 6038 Petal | POS | NEG | NEG | NEG |
| 3.318 | 14/12/15 | 4709 | Adult | Twins/Gone | NEG | NEG | NEG | NEG |
| 3.341 | 18/12/15 | 4709 | Adult | Twins/Gone | NEG | NEG | NEG | NEG |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 3.43 | 02/11/15 | 4721 | Adult | 5997 Sundance Amanda | POS | NEG | NEG | NEG |
| 3.345 | 22/12/15 | 4725 | Adult | 6034 and 6035 | POS | NEG | NEG | NEG |
| 3.339 | 18/12/15 | 4725 | Adult | 6034/ 6035 Fantasmic Adeline | NEG | NEG | NEG | NEG |
| 3.320 | 14/12/15 | 4739 | Adult | 6031 Direct Doreen 2 | NEG | NEG | NEG | NEG |
| 3.335 | 18/12/15 | 4739 | Adult | 6031 Direct Doreen 2 | NEG | NEG | NEG | NEG |
| 3.344 | 22/12/15 | 4739 | Adult | 6031 Direct Doreen 2 | NEG | NEG | NEG | NEG |
| 3.343 | 22/12/15 | 4746 | Adult | Bull - Gone | POS | NEG | NEG | NEG |
| 3.85 | 9/11/15 | 4752 | Adult | 6000 Direct Chanel | POS | NEG | NEG | NEG |
| 3.188 | 27/11/15 | 4775 | Adult | 6036 Bossman Clarissa | POS | NEG | NEG | NEG |
| 3.197 | 27/11/15 | 4775 | Adult | 6036 Bossman Clarissa | POS | NEG | POS | NEG |
| 3.285 | 11/12/15 | 4775 | Adult | 6036 Bossman Clarissa | POS | NEG | NEG | NEG |
| 3.321 | 14/12/15 | 4775 | Adult | 6036 Bossman Clarissa | NEG | NEG | NEG | NEG |
| 3.104 | 13/11/15 | 4777 | Adult | Bull - Gone | NEG | NEG | NEG | NEG |
| 3.125 | 20/11/15 | 4777 | Adult | Bull - Gone | NEG | NEG | NEG | NEG |
| 3.168 | 25/11/15 | 4777 | Adult | Bull - Gone | POS | NEG | POS | NEG |
| 3.195 | 27/11/15 | 4777 | Adult | Bull - Gone | POS | NEG | NEG | NEG |
| 3.163 | 25/11/15 | 4783 | Adult | Bull - Gone | POS | NEG | POS | NEG |
| 3.190 | 27/11/15 | 4783 | Adult | Bull - Gone | POS | NEG | NEG | NEG |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 3.284 | 11/12/15 | 4792 | Adult | Bull - Gone | NEG | NEG | NEG | NEG |
| 3.198 | 27/11/15 | 4801 | Adult | Bull - Gone | POS | NEG | NEG | NEG |
| 3.338 | 18/12/15 | 4803 | Adult | 6041 Endure Redrose | POS | NEG | NEG | NEG |
| 3.121 | 20/11/15 | 4986 | Adult | Bull - Gone | NEG | NEG | NEG | NEG |
| 3.211 | 25/11/15 | 5057 | Adult | Bull - Gone | NEG | NEG | NEG | POS |
| 3.212 | 30/11/15 | 5057 | Adult | Bull - Gone | POS | NEG | NEG | NEG |
| 3.109 | 13/11/15 | 5088 | Adult | 6002 Triumph Bonnie | POS | NEG | NEG | NEG |
| 3.194 | 27/11/15 | 5117 | Adult | 6020 Lindsay Rose | POS | NEG | NEG | NEG |
| 3.202 | 30/11/15 | 5117 | Adult | 6020 Lindsay Rose | NEG | NEG | POS | NEG |
| 3.56 | 02/11/15 | 5131 | Adult | Bull - Gone | POS | NEG | POS | NEG |
| 3.81 | 9/11/15 | 5156 | Adult | 5999 Lindsay Beauty | POS | NEG | NEG | NEG |
| 3.171 | 25/11/15 | 5160 | Adult | 6028 Golden Amanda | POS | NEG | POS | NEG |
| 3.196 | 27/11/15 | 5160 | Adult | 6028 Golden Amanda | POS | NEG | POS | NEG |
| 3.288 | 11/12/15 | 5160 | Adult | 6028 Golden Amanda | NEG | NEG | NEG | NEG |
| 3.210 | 30/11/15 | 5166 | Adult | Bull - Gone | POS | NEG | POS | NEG |
| 3.192 | 27/11/15 | 5172 | Adult | Bull - Gone | POS | NEG | POS | NEG |
| 3.203 | 30/11/15 | 5172 | Adult | Bull - Gone | POS | NEG | NEG | NEG |
| 3.215 | 25/11/15 | 5177 | Adult | 6024 Aftershock Tina | NEG | NEG | POS | NEG |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 3.250 | 02/12/15 | 5177 | Adult | 6024 Aftershock Tina | POS | NEG | NEG | NEG |
| 3.291 | 11/12/15 | 5177 | Adult | 6024 Aftershock Tina | POS | NEG | NEG | NEG |
| 3.17 | 26/10/15 | 3917 Detroit Violet | Adult | 5825 Collared Boy | NEG | NEG | NEG | NEG |
| 3.14 | 26/10/15 | 4668 Aladins Lulu | Adult | Twins/Gone | POS | NEG | NEG | NEG |
| 3.21 | 26/10/15 | 4690 Aladins Violet 2 | Adult | Bull - Gone | POS | NEG | NEG | NEG |
| 3.69 | 6/11/15 | 5088 Castor Bonnie | Adult | 6002 Triumph Bonnie | POS | NEG | POS | NEG |
| 3.70 | 6/11/15 | 5156 Uno Beauty | Adult | 5999 Lindsay Beauty | POS | NEG | NEG | NEG |
| 3.22 | 26/10/15 | 5174 Goldwyn Ambrosia | Adult | 5998 Savior Ambrosia | NEG | NEG | NEG | NEG |
| 3.20 | 26/10/15 | Calving Pen | Adult | N/A | NEG | NEG | NEG | NEG |
| 3.23 | 26/10/15 | Calving Pen | Adult | N/A | POS | NEG | NEG | NEG |
| 3.19 | 26/10/15 | Castor Joyce | Adult | Bull/Gone | POS | NEG | NEG | NEG |
| 3.206 | 30/11/15 | Snowgoose | Adult | Bull - Gone | NEG | NEG | NEG | NEG |

**Appendix 2 Multilocus genotypes with loci assignment and mixed infections in beef calves and their mothers.**

| CALF | GP60 | MM5 | MM18 | MM19 | TP14 | MLG | DAM GP60 | DAM MM5 | DAM MM18 | DAM MM19 | DAM TP14 | DAM MLG |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 16-1 | Not collected | | | | | | Not collected | | | | | |
| 16-2 | Not collected | | | | | | Not collected | | | | | |
| 16-3 | Not collected | | | | | | Not collected | | | | | |
| 16-4 | Not collected | | | | | | Not collected | | | | | |
| 16-5 | Not collected | | | | | | Not collected | | | | | |
| 16-6 | Not collected | | | | | | Not collected | | | | | |
| 16-7 | Not collected | | | | | | 18S Neg | | | | | |
| 16-8 | Not collected | | | | | | 18S Neg | | | | | |
| 16-9 | Not collected | | | | | | 18S Neg | | | | | |
| 16-10 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 | Not collected | | | | | |
| 16-11 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 | Not collected | | | | | |
| 16-12 | IIaA17G1R1 | 2 | 1 | 3 + 9 | 1 | 10 | IIaA17G1R1 | 2 | 4 | 3 | 1 | 14 |
| 16-13 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 | GP60 Neg | | | | 1 | |
| 16-14 | IIaA17G1R1 | 2 | 1 | 3 + 9 | 1 | 10 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 16-15 | IIaA17G1R1 | 2 | 1 | 10 + 3 | 1 | 11 | IIaA15R1 | 2 | 1 + 2 | 8 | 1 | 13 |
| 16-16 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 16-17 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 | IIaA16G3R1 | 2 + 3 | 1 | 3 | 1 | 15 |
| 16-18 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 | IIaA17G1R1 | 2 | 1 + 2 | 3 | 1 | 10 |
| 16-19 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 | GP60 Neg | | | | | |
| 16-20 | IIaA17G1R1 | 2 | 1 | 3 + 9 | 1 | 10 | 18S Neg | | | | | |
| 16-21 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 | IIaA17G1R1 | Neg | Neg | Neg | Neg | |
| 16-22 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 | IIaA15R1 | 2 | 1 | 8 | 1 | 13 |
| 16-23 | IIaA17G1R1 | Neg | Neg | Neg | Neg | | 18S Neg | | | | | |
| 16-24 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 16-25 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 | 18S Neg | | | | | |
| 16-26 | Not collected | | | | | | 18S Neg | | | | | |
| 16-27 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 | 18S Neg | | | | | |
| 16-28 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 16-29 | IIaA17G1R1 | 2 | 1 | 3 + 9 | 1 | 10 | IIaA19G2R1 | 2 | 1 | 8 | 1 | 12 |
| 16-30 | IIaA17G1R1 | 2 | 1 | 3 + 9 | 1 | 10 | IIaA19G2R1 | 2 | 1 | 8 | 1 | 12 |
| 16-31 | IIaA17G1R1 | 2 | 1 | 3 + 9 | 1 | 10 | IIaA17G1R1 | 2 + 3 | 1 | 3 | 1 | 10 |
| 16-32 | IIaA17G1R1 | 2 | 1 | 3 + 9 | 1 | 10 | IIaA17G1R1 | 2 | 1 | 3 | 1 | 10 |
| 16-33 | Not collected | | | | | | GP60 Neg | | | | 1 | |
| 16-34 | IIaA17G1R1 | 2 | 1 | 3 + 9 | 1 | 10 | 18S Neg | | | | | |
| T5 | Not collected | | | | | | IIaA17G1R1 (M) | 2 | 1 | 3 | 1 | 10 |
| X3 | Not collected | | | | | | IIaA15R1 | 2 | 1 | 8 | 1 | 13 |

**Appendix 3 Fragment sizes and GP60 assignment for GP60 positive rabbit samples following *C. parvum* identification in rabbit faeces.**

| Rabbit ID | Farm number | MM5 | MM18 | MM19 | TP14 | GP60 |
|---|---|---|---|---|---|---|
| 1 | 1 | 235.21 | 288.2 | 286.11 | 295.91 | llaA15R1 |
| 2 | 1 | 235.31 | 288.92 | 286.07 | 296.1 | Failed sequencing |
| 5 | 1 | NEG | NEG | 286.09 | 295.88 | llcA5G3 |
| 12 | 1 | NEG | 288.24 | 286.02 | 295.79 | llcA5G3 |
| 16 | 1 | 234.94 | 288.2 | 286.06 | 304.45 | llcA5G3 |
| 19 | 1 | NEG | 288.47 | 285.19 | 296.19 | Failed sequencing |
| 21 | 1 | NEG | 288.43 | NEG | NEG | Failed sequencing |
| 24 | 1 | NEG | 288.5 | NEG | NEG | Failed sequencing |

| | | | | | | |
|---|---|---|---|---|---|---|
| 25 | 2 | 234.95 | NEG | NEG | NEG | llcA5G3 |
| 26 | 2 | NEG | 288.08 | 291.67 | NEG | llcA5G3 |
| 28 | 2 | 235.07 | 293.69 | 286.03 | 295.79 | llaA15R1 |
| 29 | 3 | 262.12 | NEG | 293.4 | NEG | llaA15G2R1 |
| 30 | 3 | NEG | 288.22 | 287.09 | 295 | llaA15R1 |
| 31 | 3 | 235 | 288.15 | 286.05 | NEG | llcA5G3 |
| 33 | 3 | NEG | 288.22 | 291.9 | NEG | llaA15R1 |
| 39 | 4 | NEG | 288.49 | 286.12 | 296.28 | llaA15R1 |
| 40 | 4 | 260 | 288.51 | 286.11 | NEG | llaA15R1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 49 | 5 | 235.35 | 288.47 | NEG | 296.19 | Failed sequencing |
| 50 | 5 | 234.84 | 288+293 | 286.19 | 295.95 | llaA15R1 |
| 51 | 5 | 234.65 | 288+293 | 285+291 | 295.91 | llaA15R1 |
| 52 | 5 | 235.56 | 288.5 | 286.21 | NEG | llaA15R1 |
| 68 | 6 | NEG | NEG | NEG | 296.24 | llaA15R1 |
| 69 | 6 | 234.58 | NEG | NEG | NEG | llaA15R1 |
| 70 | 6 | 261.48 | 288.16 | 286.03 | 295.84 | llaA15R1 |
| 71 | 6 | NEG | 288.1 | 286.11 | 295.75 | llcA5G3 |
| 79 | 7 | 234+262 | 293.6 | 291.88 | 286.81 | llaA15R1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 121 | 4 | NEG | NEG | NEG | NEG | llaA15G2R1 |
| 123 | 4 | NEG | NEG | 291.86 | NEG | Failed sequencing |
| 124 | 4 | 235.12 | 288.34 | NEG | NEG | llcA5G3 |
| 125 | 4 | 262+234 | 288.2 | 291+286 | 295.81 | llaA15R1 |
| 126 | 4 | 262.26 | 288.49 | 286.15 | 296.26 | llaA15R1 |
| 143 | 11 | 234+262 | NEG | NEG | 295.1 | Failed sequencing |
| 147 | 2 | 234.74 | NEG | 291.65 | 304.99 | llaA15G2R1 |
| 148 | 2 | 235.14 | NEG | NEG | 295.8 | llaA15R1 |
| 149 | 2 | 234.78 | 287.99 | 315.96 | NEG | llaA15R1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 150 | 2 | NEG | NEG | 291.79 | 305.03 | llcA5G3 |
| 152 | 7 | 234+262 | 287.93 | NEG | NEG | llcA5G3 |
| 153 | 7 | NEG | NEG | NEG | NEG | llaA15R1 |
| 177 | 7 | NEG | NEG | NEG | 296.19 | llaA15R1 |
| 194 | 4 | NEG | NEG | NEG | NEG | Failed sequencing |
| 204 | 10 | 235.55 | NEG | 286.62 | 260.36 | Failed sequencing |
| 233 | 15 | NEG | NEG | NEG | NEG | llcA5G3 |
| 236 | 15 | 234.78 | NEG | NEG | NEG | llaA15R1 |
| 250 | 13 | NEG | NEG | NEG | NEG | llaA15R1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 260 | 12 | 234.74 | NEG | NEG | NEG | llcA5G3 |
| 300 | 16 | NEG | NEG | NEG | NEG | llcA5G3 |
| 342 | 1 | NEG | NEG | 286.09 | NEG | llaA15R1 |

**Appendix 4 Raw data for pheasant faecal samples collected from a farm in Perthshire between April-May of 2016 and 2017. Species and genotypes of *Cryptosporidium* listed. Mixed infections are noted with a (mixed) annotation. Blank cells indicate PCR was not done.**

| Pheasant no | Year | 18S | Species | GP60 | Repeat 18S | Species | MM5 | MM18 | MM19 | TP14 | MLG |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2016 | Negative | | | | | | | | | |
| 2 | 2016 | Positive | *C. bovis* (mixed) | IIaA15G2R1 | Positive | *C. parvum* | Neg | Neg | Neg | 1 | |
| 3 | 2016 | Negative | | | | | | | | | |
| 4 | 2016 | Negative | | | | | | | | | |
| 5 | 2016 | Negative | | | | | | | | | |
| 6 | 2016 | Negative | | | | | | | | | |
| 7 | 2016 | Negative | | | | | | | | | |
| 8 | 2016 | Positive | *C. parvum* | IIaA17G2R1 | | | 1 | Neg | Neg | 1 | |
| 9 | 2016 | Negative | | | | | | | | | |
| 10 | 2016 | Negative | | | | | | | | | |
| 11 | 2016 | Negative | | | | | | | | | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 12 | 2016 | Positive | Failed sequencing | IIaA17G2R1 | Negative | | | | | | |
| 13 | 2016 | Negative | | | | | | | | | |
| 14 | 2016 | Negative | | | | | | | | | |
| 15 | 2016 | Positive | *C. parvum* | IIaA17G2R1 | | | 1 | 1 | 3 | 1 | 16 |
| 16 | 2016 | Positive | *C. bovis* (mixed) | IIaA15G2R1 | Positive | *C. parvum* | | | | | |
| 17 | 2016 | Negative | | | | | | | | | |
| 18 | 2016 | Negative | | | | | | | | | |
| 19 | 2016 | Negative | | | | | | | | | |
| 20 | 2016 | Positive | *C. parvum* | IIaA15G2R1 | | | 1 | 1 | 8 | 1 | 17 |
| 21 | 2016 | Positive | *C. parvum* | IIaA17G2R1 | | | 2 + 3 | 1 | 8 | 1 | 18 |
| 22 | 2016 | Positive | *C. parvum* | IIaA17G2R1 | | | 1 | 1 | 3 | 1 | 16 |
| 23 | 2016 | Positive | *C. bovis* (mixed) | Negative | | | | | | | |
| 24 | 2016 | Negative | | | | | | | | | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 25 | 2016 | Positive | *C. parvum* | IIaA15G2R1 | | | 2 | 1 | 3 | 1 | 19 |
| 26 | 2016 | Positive | *C. parvum* | IIaA15G2R1 | | | 2 + 3 | 1 | 8 | 1 | 20 |
| 27 | 2016 | Negative | | | | | | | | | |
| 28 | 2016 | Negative | | | | | | | | | |
| 29 | 2016 | Positive | *C. parvum* | IIaA15R1 | | | | | | | |
| 30 | 2016 | Negative | | | | | | | | | |
| 31 | 2017 | Positive | *C. parvum* | IIaA17G1R1 | | | | | | | |
| 32 | 2017 | Positive | *C. parvum* | IIaA17G1R1 | | | 2 | 1 | 3 | 1 | 10 |
| 33 | 2017 | Positive | *C. parvum* | IIaA15G2R1 (mixed) | | | 2 | 1 | 3 | 1 | 19 |
| 34 | 2017 | Positive | *C. parvum* | IIaA17G1R1 | | | 2 | 1 | 3 | 1 | 10 |
| 35 | 2017 | Positive | *C. parvum* | | | | | | | | |
| 36 | 2017 | Positive | *C. parvum* | Neg | | | 1 + 2 | Neg | Neg | Neg | |
| 37 | 2017 | Negative | | | | | | | | | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 38 | 2017 | Negative | | | | | | | | | |
| 39 | 2017 | Positive | *C. parvum* | IIaA17G1R1 | | | | | | | |
| 40 | 2017 | Negative | | | | | | | | | |
| 41 | 2017 | Positive | *C. parvum* | IIaA17G1R1 | | | 1 | 1 | Neg | Neg | |
| 42 | 2017 | Positive | *C. parvum* | IIaA17G1R1 | | | 2 | 1 | 3 | 1 | 10 |
| 43 | 2017 | Negative | | | | | | | | | |
| 44 | 2017 | Positive | Negative | | | | | | | | |
| 45 | 2017 | Negative | | | | | | | | | |
| 46 | 2017 | Positive | *C. parvum* | IIaA17G1R1 | | | 1 + 2 | 1 | 8 | 1 | 21 |
| 47 | 2017 | Positive | *C. parvum* | IIaA17G2R1 | | | 1 | 1 | 3 | 1 | 16 |
| 48 | 2017 | Negative | | | | | | | | | |
| 49 | 2017 | Positive | *C. parvum* | IIaA17G1R1 | | | 2 +3 | 1 | 3 | 1 | 10 |
| 50 | 2017 | Negative | | | | | | | | | |