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1. Abstract

Although diarrhoea caused by *Cryptosporidium* is prevalent in livestock species throughout the world relatively little is known about the species and subtypes of *Cryptosporidium* found in cattle on Scottish farms. In particular, little is known about the shedding profiles (age when calves become infected and duration of shedding) of the different species found in cattle and how calves become infected. There are several theories about how neonatal calves first become infected with the parasite but the role which adult cattle play in the transmission of the parasite has not been fully addressed. It was previously thought that adult cattle did not become infected with the same species of *Cryptosporidium* which causes disease in the young calves. Some studies have shown that this may not be true and with the advance of new techniques to discriminate species this is an area which should be revisited.

In addition, it is known that it is possible for humans to become infected with *Cryptosporidium* and show clinical disease early in life and then again later in adulthood. In livestock however, diarrhoea caused by the parasite is generally only seen in neonatal livestock while older animals tend to be asymptomatic. It is not known if this resistance to clinical disease at an older age is due to changes in the host with an increase in age or if prior infection “immunises” the animal and provides protection against re-infection. It is also not known if infection with one isolate of *C. parvum* will provide protection against infection with another or if the protection formed is species/isolate specific.

The main aims of this thesis were to: determine the species and subtypes of *Cryptosporidium* found in calves on a study farm over a one year period from birth; assess the role which adult cattle play in the transmission of the parasite to newborn calves; develop new typing tools to enable the rapid and easy differentiation of *Cryptosporidium* species found in cattle and to examine the host-pathogen interactions in animals given serial experimental challenges with distinct *Cryptosporidium parvum* isolates to determine if the resistance seen in older animals on farms is due to an increase in age and or as a result of prior infection.

A variety of different approaches were taken to achieve these aims. Longitudinal experiments carried out on a study farm revealed that in calves <9 weeks of age the most common species of *Cryptosporidium* is *C. parvum* and that all calves in the group became infected with *Cryptosporidium* within the first two weeks of life. Sample collection from the same animals later in life (at 6 months of age) showed that contrary to most previous studies the most common species detected at in this age group was also *C. parvum* although, interestingly, the subtype which the calves were shedding was not the same subtype that they were shedding as previously.

The longitudinal study which investigated the role of adult cattle in the transmission of *Cryptosporidium* also yielded some interesting results. It was found that most of the adult cattle on this farm were shedding *Cryptosporidium* albeit intermittently. Speciation of the positive samples revealed that, on this farm, the most predominant species of *Cryptosporidium* in adult cattle was also *C. parvum.* This is very unusual as most previous studies have not found this level of infection in older cattle and *C. parvum* is not usually found in this age group. A number of different subtypes were found in adult cattle and some animals shed more than one subtype over the course of the study. This contradicts prior findings which demonstrated that only one subtype is found on a single farm.

The experimental infection trial involving infection of young (<1 week old) and older (6 week old) lambs with distinct *C. parvum* isolates demonstrated that an increase in age at primary infection reduces the effect of clinical disease. Animals which were infected at <1 week of age were re-challenged at 6 weeks of age with either a homologous or heterologous infection. Results revealed that previous exposure does not protect against re-infection with the same or a different isolate of *C. parvum.* This study also demonstrated that an increase in infective dose leads to a shorter pre-patent period and that there are variations in the clinical manifestations of different isolates of the same *Cryptosporidium* species.

2. Industry messages

- Cattle can be infected with and shed multiple species and subtypes of *Cryptosporidium* at any one time (study 1 & 2) but only one species, *C. parvum*, causes disease.

- Adult cattle shed more *C. parvum* than previously thought (study 2). The majority of adult cattle in this study shed *C. parvum* at some point, they have potential to be an important source of infection for other susceptible hosts such as calves.

- Adult cattle, though shedding *C. parvum,* may not be an important source of infection for calves (study 2) as they seem to shed different subtypes of *C. parvum.*

- Clinical disease does not correlate with oocyst shedding (study 1, 2 & 3). Animals can be shedding oocysts without diarrhoea and can potentially contaminate the environment with infective oocysts.

- Cattle do not develop complete sterile immunity to infection with *Cryptosporidium* (study 1 & 2) and can become infected with *Cryptosporidium* as a young calf and again as an adult. Older animals tend to be asymptomatic.

- Lambs do not develop immunity to infection with *Cryptosporidium* in the first six weeks of life although there is a reduction in disease severity at 6 weeks of age (study 3).

- Infection with one *C. parvum* subtype does not provide protection against challenge with the same or a different subtype (study 3).

- Different *C. parvum* subtypes can manifest differently in the host in terms of oocyst output and clinical disease (study 3).

- Evidence that there is extensive variation in the disease outcome in individual animals in terms of oocyst output and disease manifestation (study 3) indicating that host factors are very important in the outcome of disease.

3. Introduction

3.1. Study One (*Cryptosporidium* species and subtypes found in calves from birth to 1 year)

A longitudinal study was conducted to assess the shedding profile of *Cryptosporidium* species in a group of calves from birth for twelve months. Faecal samples were collected from 30 calves three times per week for the first six weeks of their lives and then again at 3, 6, 9 and 12 months. Samples were tested for *Cryptosporidium* species and genotypes by nested species specific multiplex PCR amplification and sequencing.

3.2. Study Two (Role of adult cattle in the transmission of *C. parvum*)

It has been suggested that adult cattle may be a source of infection with *Cryptosporidium* for neonatal calves. A few studies have been carried out to test this theory but many of them assumed that the presence of *Cryptosporidium* in the adult faecal samples (most usually by microscopy or sometimes ELISA – to detect *Cryptosporidium* antigens in faecal supernatant using an anti-*Cryptosporidium* antibody) meant that they were the source from which calves became infected. However, the methods used were unable to distinguish between different *Cryptosporidium* species present and only confirm the presence of oocysts.

A longitudinal study was conducted to assess the shedding of *Cryptosporidium* oocysts in adult cattle, to identify the species shed and to determine if these adult cattle were the source of infection for their calves. Faecal samples were collected from 30 in-calf adult dairy cattle (heifers and cows) three times per week for up to ten weeks pre-calving. After calving faecal samples were then collected from the calves for at least six weeks three times per week and again at 3 and 6 months. Samples were tested for *Cryptosporidium* species and genotypes by nested species specific multiplex PCR (nssm-PCR) amplification and sequencing.

3.3. Study Three (Can infection with one isolate of *C. parvum* protect against infection with another)

Currently there is little knowledge about the development of immunity to *Cryptosporidium*, in humans it is possible to become infected with *Cryptosporidium* early in life and then again later in adulthood. In livestock however, diarrhoea caused by the parasite is generally only seen in neonatal livestock and not older animals. It is not known if infection with one isolate of *C. parvum* will provide protection against infection with another isolate. It is possible that if one isolate does provide protection against another then there is potential for the development of control measures using a less virulent (if one is identified) isolate to protect against infection from severe disease caused by a more virulent one.

There are very few studies which have looked at the effect of sequential infection of *Cryptosporidium parvum* on neonatal livestock. Two studies, one in sheep [1] and one in cattle [2] have looked at the effect of age on infection with *Cryptosporidium* species. In the sheep study , there was an age-related susceptibility to infection with younger animals shedding larger numbers of oocysts for a more prolonged period compared with those infected a few weeks later. Both of these studies showed that animals exposed to the parasite at a younger age do not have such severe disease when exposed a second time showing that the animals were able to develop protective immunity to a homologous challenge. Most of the animal studies investigating immune responses to infection were carried out before the discovery of many of the species, which are now known to infect farm livestock. At this time there was not the knowledge of different genotypes of *C. parvum* that we have now.

An experiment challenge was carried out to test the development of resistance to homologous and heterologous *Cryptosporidium* infections in lambs using two distinct *C. parvum* isolates. Thirty-six neonatal lambs were split into six groups: two to test age-related susceptibility, two homologous challenge groups and two heterologous challenge groups. The lambs were kept for 8 weeks until oocyst shedding stopped, total faecal output was collected from each lamb along with weekly blood samples. Clinical data (feed intake, demeanour and faecal consistency) was recorded daily. Oocyst counts are used to determine the shedding profile of each group. Antibody levels will be compared with shedding data to confirm infection status.

4. Materials and methods

4.1. Study One and Study Two

4.1.1. Sample Collection

Calves

Faecal samples were collected directly from the rectum of calves from the day of birth until six weeks of age (study one) or eight weeks of age (study two), three times per week (Monday, Wednesday and Friday). The samples were mixed thoroughly and aliquoted into bijoux tubes and stored at -20°C for further processing.

Adults

Faecal samples were collected from adult dairy cattle (heifers and cows) three times per week for up to ten weeks pre-calving. Cows were observed in the cattle shed until they defecated and then the entire motion was collected in a plastic bag. Due to the method of collection it was not always possible to collect a sample from each cow at each sampling point. After collection the samples were transferred to the laboratory where the entire motion was mixed and a sub-sample aliquoted into a 125ml tub and stored at 4°C.

4.1.2. Sample processing and DNA isolation

Faecal samples from calves up to, and including, 9 months were processed using the Macherery-Nagel NucleoSpin Tissue Kit as described by the manufacturer except that 10 × freeze-thaw cycles were added prior to the proteinase-K incubation. Samples from calves aged 12 months and adults were processed by acid flocculation prior to DNA extraction as described for calf samples obtain DNA for PCR amplification.

4.1.3. PCR amplification of the 18S rRNA gene

All DNA samples were amplified using the 18S PCR described by Xiao et al., (2000) and visualised on a 1.5% agarose gel stained with GelRed (Biotium, UK). All reactions were carried out in triplicate and a sample was considered positive if a band corresponding with ~840 bp was seen. Several controls, both negative (no template and extraction controls) and positive (*Cryptosporidium* DNA) were run with each set of reactions. A positive result was only accepted when all negative controls were negative and positive controls were positive.

4.1.4. Species identification by nssm-PCR (nested species specific multiplex-PCR)

For the differentiation of *Cryptosporidium* species an nssm-PCR (Thomson et al., 2015 – In Prep) was carried out using the first round PCR product from the 18S PCR as a template. The PCR products were visualised on a 1.5% agarose stained with GelRed (Biotium, UK). All reactions were carried out in triplicate and a sample was considered positive if a band corresponding with 840 bp and any combination of 625 bp, 415 bp, 305 bp or 241 bp was seen. Positive controls for each *Cryptosporidium* species tested for were included as well as extraction controls and no template controls. A positive result was only accepted when all negative controls were negative and all positive controls were positive.

4.1.5. PCR amplification and sequencing of the GP60 gene for *C. parvum* subtyping

Subtyping of *C. parvum* positive samples was carried out by amplification and sequencing of the GP60 gene [3]. The PCR products were visualised on a 1.5% agarose stained with GelRed (Biotium, UK). Reactions were originally carried out in duplicate and if amplification was not successful the reactions were repeated. A sample was considered positive if a band corresponding with ~375 bp was seen. Positive, negative and extraction controls were included for each set of reactions. A positive result was only accepted when all negative controls were negative and all positive controls were positive.
GP60 positive samples were sequenced in the forward and reverse orientation by GATC Biotech (GATC, Germany). The sequences were analysed using Chromas Lite (version 2.01 Technelysium) and the subtypes named as according to [4].

4.2. Study Three

4.2.1. Production of inoculum for experimental trial

Faecal samples were collected from calves on three different farms known to have a problem with *Cryptosporidium* (history of cryptosporidiosis diagnosed by a veterinarian and regular occurrence of scour in neonatal calves). Samples were collected from 2-6 scouring calves on each farm. Oocysts were extracted from faeces that were found to be positive for *Cryptosporidium* by sucrose flotation. *Cryptosporidium* oocysts were surface sterilised using 2 ml 70% EtOH (ethanol) for 30 mins before being resuspended in phosphate buffered saline (PBS). Oocysts were then counted and the oocyst suspension diluted with PBS to give the desired infective dose.

4.2.2. Study animals

All experimental animals were used in accordance with Home Office regulations and approval of the experimental design sought before the study commenced from the Moredun Research Institute's ethics committee. Thirty-six newborn (<24 h old) Texel and Texel cross male lambs were obtained

from Firth Mains farm, Roslin and were separated into six groups of six depending on experimental challenge.

4.2.3. Clinical Scoring

Lambs were monitored a minimum of three times per day for the first six weeks and then a minimum of twice daily for the remainder of the study. Daily monitoring involved the recording of the volume of milk each lamb drank along with how well the animal drank, the demeanour of the animal and the appearance of the faeces produced.

4.2.4. Faecal Collection and processing

Total faecal output was collected from each lamb using a specially designed harness and bag system which the lamb wore continuously. This enabled the lambs to be penned in groups rather than separately but still allowed collection of samples from each lamb individually. This system also ensured that after inoculation the lambs were not exposed to any faecal matter and were not re-infected by oocysts shed in the faeces. Bags were taken off and replaced with a new one clearly marked with group/animal/sample when required. Samples were stored in the bags at 4°C until they were processed. The sample was weighed and the weight recorded, water was then added to the sample to make the faeces liquid enough to pipette. If the sample was already liquid then no additional water was added. The sample was then re-weighed and the new weight recorded. Using a food processor the sample was homogenised completely and a 2 ml sample aliquoted into a tube for storage for use in DNA extraction. These aliquots were stored at -20°C. A small subsample ~850 µl was taken and used to quantify the oocysts present in the sample. The remainder of the faecal material was discarded.

4.2.5. Quantification of oocysts

Oocysts numbers in faecal samples were counted by malachite green staining and microscopy at 400 × magnification.

5. Results

5.1. Study One

5.1.1. *Cryptosporidium* Positive Samples

The overall percentage of positive samples for the whole study was 59.7% (n=255). In the first six weeks of life each calf tested positive for *Cryptosporidium* on at least six occasions, giving a period prevalence of 100% and the overall percentage of samples positive in calves <6 weeks was 64% (n=232). The earliest observation of oocyst shedding occurred at 2 days, which was observed in three calves. In the majority of calves the first observation of oocyst shedding occurred at day 4 (4 animals) or day 6 (4 animals). Peak shedding of *Cryptosporidium* oocysts occurred in week 3 with 100% of the calves tested shedding oocysts. Oocyst shedding tailed off towards the end of week 6 (Figure 1).

Samples from the calves at 3, 6, 9 and 12 months showed a prevalence of 24%, 63%, 41% and 18.65% respectively (Figure 1). Five of the animals did not test positive for *Cryptosporidium* at any point after the initial 6 week period and no animals tested positive at all age groups though two tested positive at <6 weeks, 3, 6 and 9 months.

The percentage of positive samples collected from each calf ranged from 38.9% to 87.5% (Figure 2).

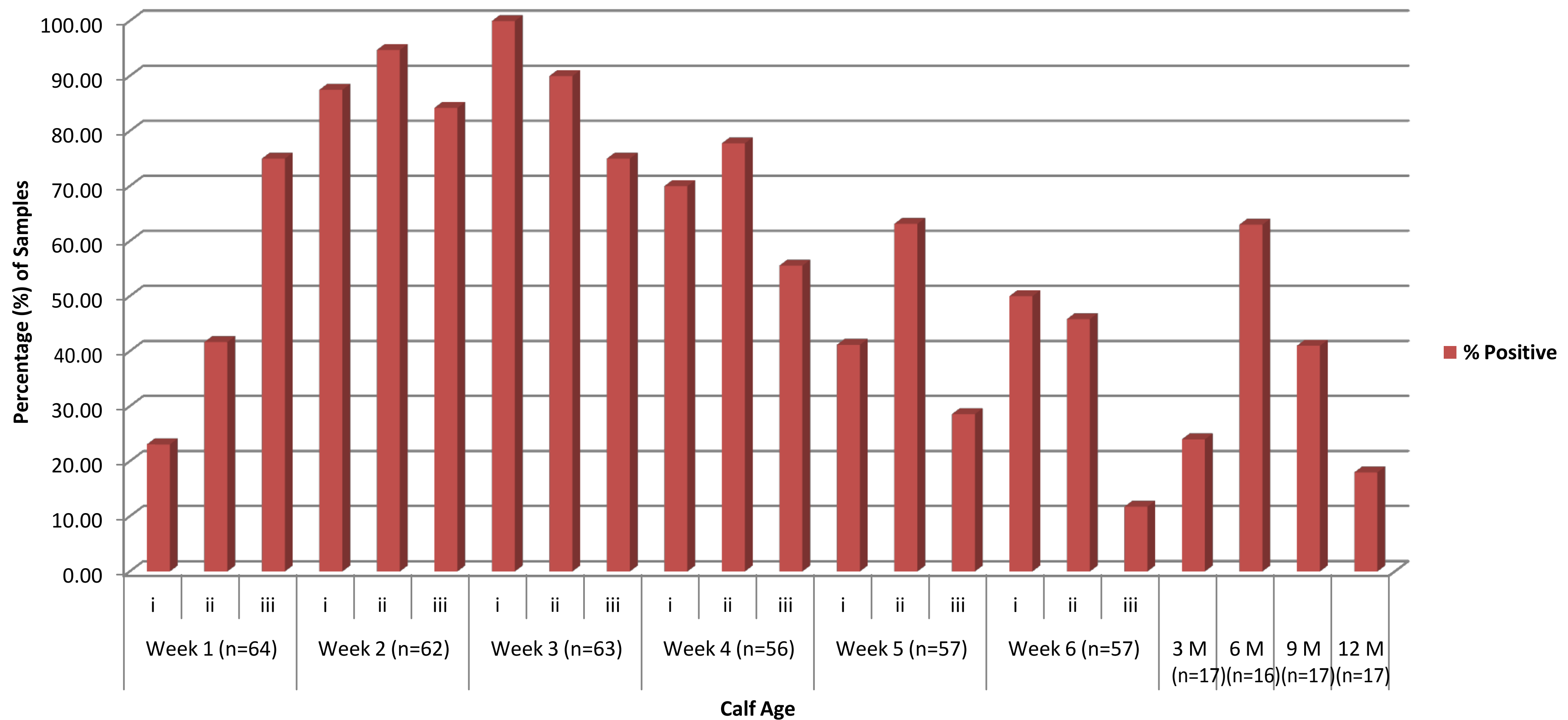

Figure 1: *Cryptosporidium* positive samples from calves from birth to 12 months.

Percentage of positive samples from calves in the first six weeks of life and at 3, 6, 9 and 12 months of age. The percentage of positive samples at each sampling point (i, ii, iii) is indicated by a red bar.

	Calf Age (Weeks)																		Calf Age (Months)				
Calf ID	Week 1			Week 2			Week 3			Week 4			Week 5			Week 6			3 M	6 M	9 M	12 M	% Positive
2384	N	N	P	P	P	P	P	P	P	N	P	0	0	P	N	0	N	N	P	0	P	N	61.1
2385	N	P	P	P	P	N	0	P	P	P	P	0	0	P	P	0	P	N	N	N	N	N	61.1
2386	0	N	P	P	P	N	P	N	P	N	P	P	0	P	P	0	N	0	0	0	0		64.3
2387	0	N	P	P	P	N	0	P	P	P	P	P	0	P	P	0	N	0	0	0	0		76.9
2388	0	P	P	P	P	P	P	N	P	P	P	P	0	P	N	0	N	N	P	P	N	N	68.4
2389	N	N	P	N	N	P	0	P	0	P	P	0	N	N	0	P	0	0	0	0	0		50.0
2390	N	N	P	N	P	0	P	P	N	P	P	0	N	N	0	0	N	N	N	P	N	N	38.9
2393	N	P	N	P	P	0	P	P	P	P	0	0	N	0	N	N	P	N	N	P	P	N	55.6
2394	N	N	P	N	P	0	0	P	P	P	0	0	P	0	P	N	P	N	0	0	0		61.5
2395	N	N	N	P	0	P	P	P	P	P	0	N	N	0	N	P	P	N	0	0	0		53.3
2396	N	N	N	P	P	P	P	P	P	P	0	N	N	0	N	N	P	N	0	0	0		50.0
2397	0	P	P	P	P	P	P	P	P	0	0	N	0	P	0	P	P	P	N	P	P	0	87.5
2398	N	N	P	P	0	P	P	P	P	0	P	N	0	0	N	0	P	N	P	P	P	N	64.7
2399	0	0	N	P	P	P	P	0	N	0	N	P	0	P	N	P	N	N	0	0	0		53.8
2402	P	P	P	P	P	P	P	0	N	N	0	N	N	N	N	N	N	0	N	N	P	N	42.1
2403	P	P	P	P	P	P	P	0	P	N	0	0	N	P	N	0	N	N	P	P	P	N	66.7
2404	0	P	N	P	P	0	0	0	P	0	P	P	P	0	N	N	N	P	N	P	N	N	56.3
2405	0	N	N	P	P	P	P	0	P	P	P	P	P	P	N	P	N	N	N	P	N	N	60.0
2407	0	N	P	P	P	P	0	P	N	0	P	P	P	P	P	P	P	N	N	P	N	N	68.4
2409	0	P	0	0	P	0	P	P	P	P	N	N	N	N	N	N	N	N	0	0	0	0	42.9
2411	0	P	P	P	P	0	P	P	P	N	P	N	P	P	N	N	P	N	N	N	N	N	55.0
2412	P	N	P	P	0	P	P	P	N	P	N	P	P	P	0	P	P	0	N	P	P	N	73.7
2413	0	P	P	P	0	P	P	P	P	P	P	N	N	N	N	N	N	0	N	N	N	N	47.4
2415	N	N	P	P	0	P	P	P	P	N	P	P	N	N	N	0	N	0	N	N	N	P	47.4
2417	0	N	P	P	0	P	P	P	N	P	N	P	P	N	P	P	P	0	N	N	N	P	63.2
% Positive	23.1	41.7	75.0	87.5	94.7	84.2	100.0	90.0	75.0	70.0	77.8	55.6	41.2	63.2	28.6	50.0	45.8	11.8	23.5	62.5	41.2	12.5	

Figure 2: Positive and negative samples collected from each calf.

Samples collected from each calf at each time point. A positive sample is denoted by a red "P" while a negative sample is denoted by a blue "N", "0" indicates that a sample was not collected from that calf at on that day. The percentage of positive samples at each time point are shown at the bottom of each column while percentage of positive samples from each individual is shown in the right-hand column.

5.1.2. Species of Cryptosporidium

Three species were identified (*C. parvum, C. bovis* and *C. ryanae*) in 228, 21 and 12 samples respectively. In four samples the species present could not be determined. Fourteen mixed infections were identified: these were *C. parvum* and *C. bovis* (n=7) and *C. bovis* and *C. ryanae* (n=7). In this study *C. parvum* was the predominant species detected in calves <6 weeks old with seven calves showing evidence of mixed infections (Figure 3). In calves and humans only *C. parvum* causes disease.

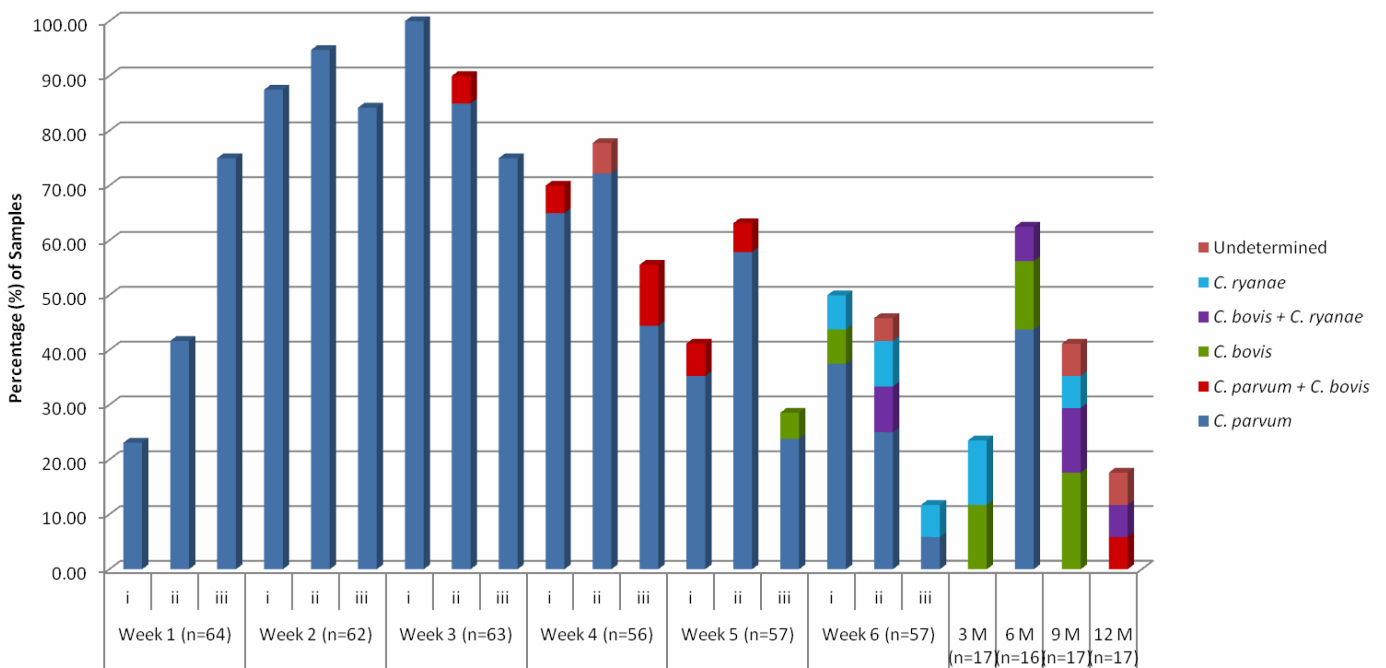

Figure 3: Prevalence of *Cryptosporidium* species in each age group of cattle.

Percentage of each species of *Cryptosporidium* at each time point.

5.1.3. Subtypes of *C. parvum*

Two different subtypes of *C. parvum* were identified. In all of the samples from the calves <6 weeks the subtype identified was IIaA19G2R1 and in the samples from the same calves at 6 months the subtype identified was IIaA15G2R1 (Table 1).

Table 1: *C. parvum* types found in calves at different ages.

Calf ID	< 1 Weeks	2-3 Weeks	6 Weeks	6 Months
2384		IIaA19G2R1		
2385		IIaA19G2R1	IIaA19G2R1	
2388	IIaA19G2R1	IIaA19G2R1	IIaA19G2R1	IIAa15G2R1
2393	IIaA19G2R1	IIaA19G2R1		IIAa15G2R1
2395	IIaA19G2R1	IIaA19G2R1		
2397		IIaA19G2R1		IIaA15G2R1
2398	IIaA19G2R1	IIaA19G2R1		
2404	IIaA19G2R1	IIaA19G2R1	IIaA19G2R1	IIaA15G2R1
2405	IIaA19G2R1	IIaA19G2R1	IIaA19G2R1	IIaA15G2R1
2407	IIaA19G2R1	IIaA19G2R1	IIaA19G2R1	
2417	IIaA19G2R1	IIaA19G2R1	IIaA19G2R1	

5.2. Study Two

5.2.1. *Cryptosporidium* positive samples

Adults

Overall 27.9% (n=57) of samples from adult cows tested positive for *Cryptosporidium*. Four cows did not test positive for *Cryptosporidium* at any point throughout the study, the remaining 25 cows tested positive on at least one occasion and two cows that tested negative for *Cryptosporidium* pre-calving tested positive post-calving (Figure 5).

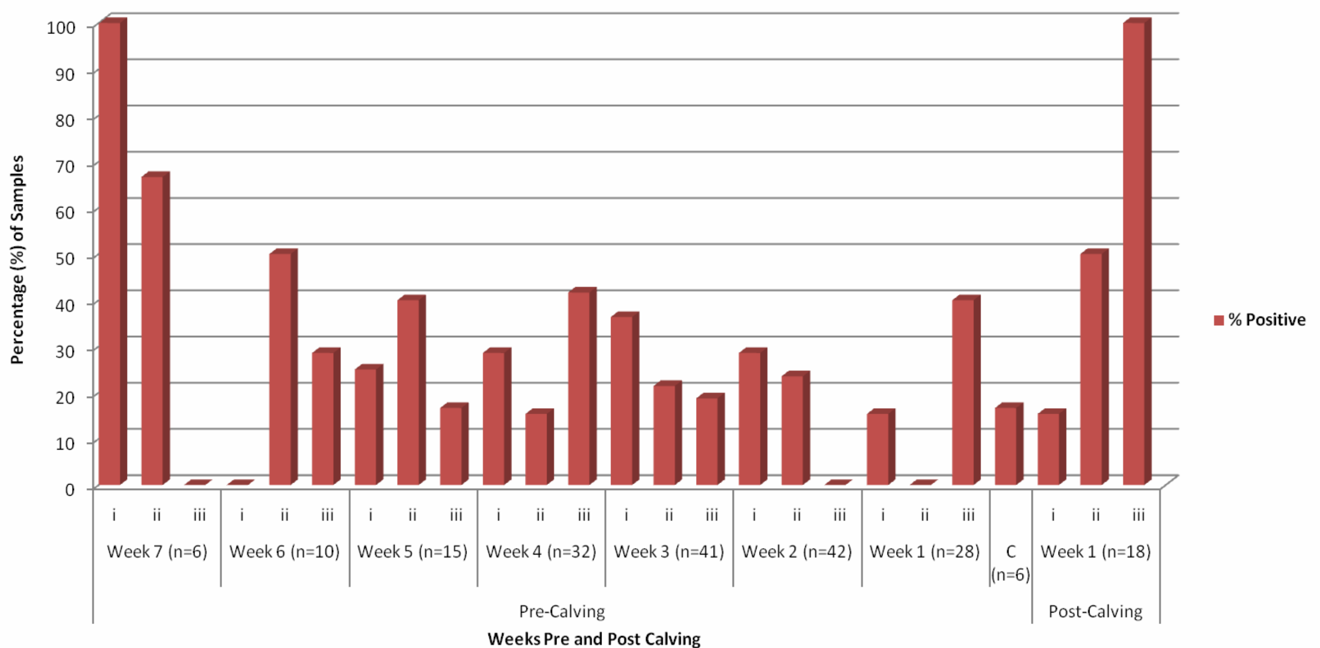

Figure 5: Positive samples detected in adult cows pre- and post-calving.

Percentage of positive samples detected in cows in the weeks pre- and post-calving. The percentage of positive samples at each sampling point (i, ii, iii) is indicated by a red bar. Weeks 8 and 9 pre-calving and week 2 post-calving have been omitted from this graph as only two samples were collected in each of these week. “C” indicates the day of calving, it was not possible to collect a sample from every cow on the day it calved.

Calves

The overall percentage of positive samples was 51.0% (n=194). In the first nine weeks of life each calf tested positive for *Cryptosporidium* on at least 2 occasions. The earliest evidence of oocyst shedding occurred in one calf one day after birth, the majority of calves began shedding oocysts at day 4 (7 calves) or day 5 (6 calves) after birth. Peak shedding of *Cryptosporidium* oocysts occurred in week two with 95% of calves tested shedding oocysts (Figure 6).

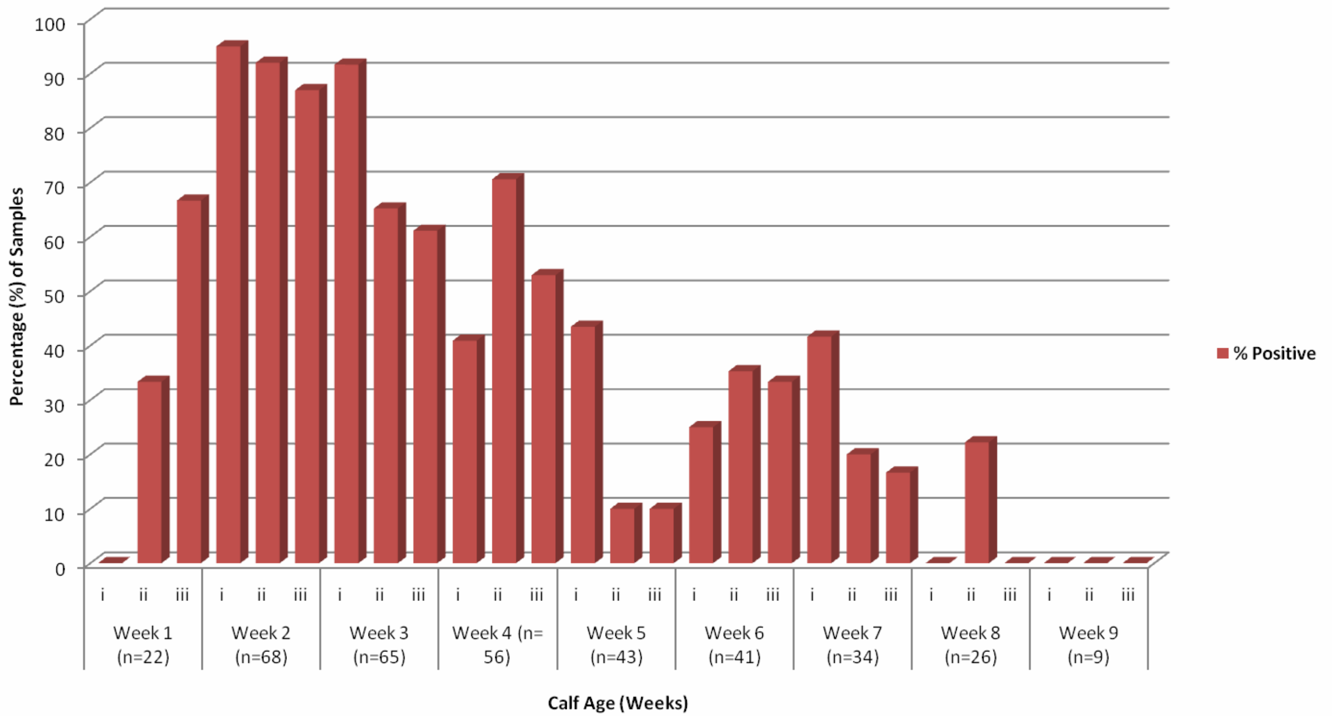

Figure 6: Shedding profile of *Cryptosporidium* oocysts in calves <9 weeks.

Percentage of positive samples detected in calves in the first nine weeks of life. The percentage of positive samples at each sampling point (i, ii, iii) is indicated by a red bar. Peak shedding of oocysts occurred in week 2 of life and tailed off towards the end of week 8/9.

5.2.2. Cryptosporidium species

Adults

Ninety-one point two percent (n=52) of the samples were identified as *C. parvum*, 3.5% (n=2) as a mixed infection of *C. parvum* and *C. ryanae,* 1.75% (n=1) as a mixed infection of *C. ryanae* and *C. bovis*, 1.75% (n=1) as a mixed infection of *C. parvum* and *C. andersoni* and 1.75% (n=1) as a mixed infection of *C. parvum, C. ryanae* and *C. bovis* (Figure 7).

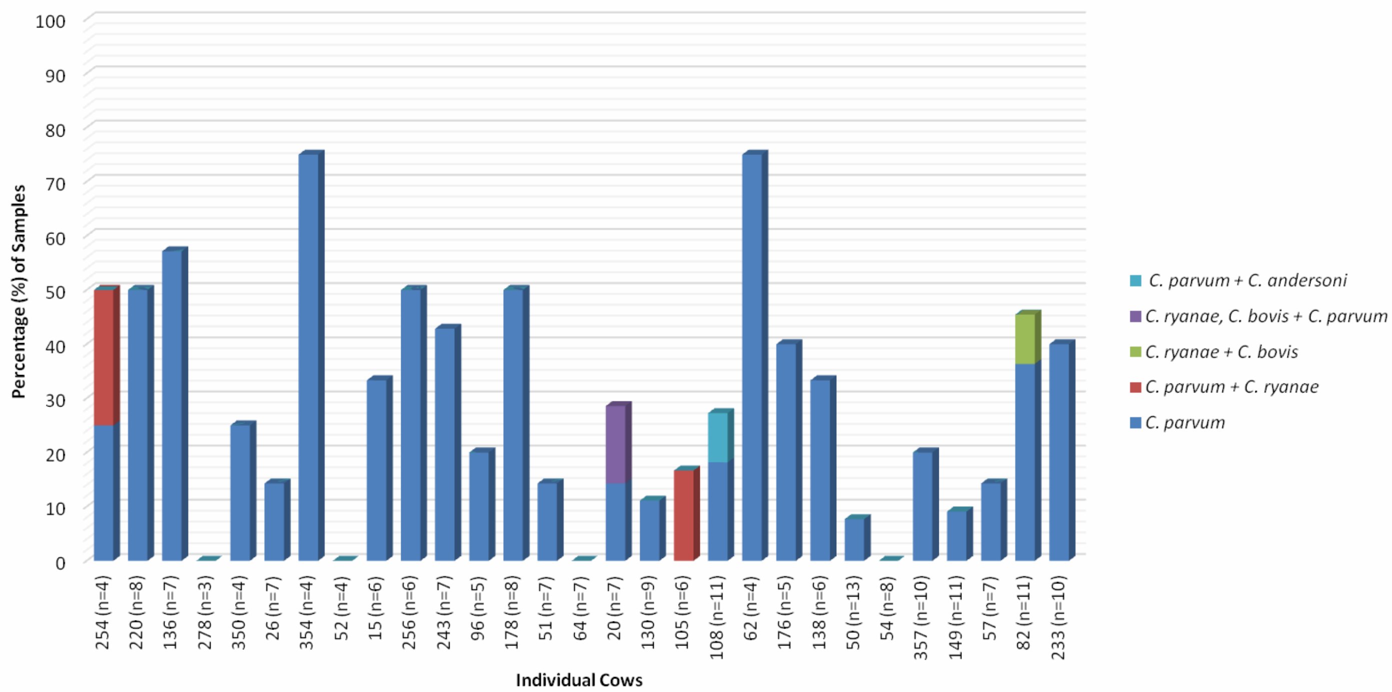

Figure 7: Species detected in individual cows.

Percentage of different species detected in positive samples from each individual cow. *C. parvum* was the most frequently detected species in these samples. Four of the cows produced no *Cryptosporidium* positive samples.

Calves

All of the samples from calves <9 weeks old were *C. parvum*. Of the five positive samples from the calves at 6 months old, 20% (n=1) were *C. parvum* positive and 80% (n=4) were mixed infections of *C. parvum* and *C. bovis*.

5.2.3. Subtypes of *C. parvum*

Adults

Four different types were identified; these were IIaA15G2 (n=2), IIaA15G2R1 (n=11), IIaA18G2R1 (n=3) and IIaA19G2R1 (n=1). The most common subtype in the adult cattle was IIaA15G2R1 which was identified in 11 samples from nine cows The other subtypes were identified in fewer samples, IIaA15G2 in two samples from two cows, IIaA18G2R1 in three samples from three cows and finally IIaA19G2R1 in a single sample from one cow which was also the most common subtype in calves <9 weeks of age.

Calves

Two different types were identified IIaA19G2R1 (n=75) in the samples from animals <9 weeks of age and IIaA15G2R1 (n=5) in samples from the same animals at 6 months of age (Table 2).

Table 2: *C. parvum* types found in calves at different ages.

Calf ID	< 1 Weeks	2-4 Weeks	5-8 Weeks	6 Months
2604	IIaA19G2R1	IIaA19G2R1		
2609		IIaA19G2R1	IIaA19G2R1	IIAa15G2R1
2612	IIaA19G2R1			IIAa15G2R1
2613	IIaA19G2R1	IIaA19G2R1	IIaA19G2R1	
2617	IIaA19G2R1	IIaA19G2R1	IIaA19G2R1	
2619		IIaA19G2R1		IIaA15G2R1
2620		IIaA19G2R1		IIaA15G2R1
2626		IIaA19G2R1		IIaA15G2R1
2633	IIaA19G2R1	IIaA19G2R1		

5.3. Study Three

5.3.1. Age-related susceptibility to disease

To determine whether animals become less susceptible to disease as their age increases two groups (Group I (BB wk6) and Group II (LH wk6)) were infected with *C. parvum* at 6 weeks of age and the disease outcome and oocyst shedding was compared with lambs which were infected at <1 week of age (Group III (BB wk1), Group IV (BB wk1), Group V (LH wk1) and Group VI (LH wk1)). Lambs infected at 6 weeks of age were given with a higher dose (5×10^6) than animals infected at <1 week of age (1×10^6).

Clinical data showed that animals which were infected for the first time at 6 weeks of age showed reduced clinical disease (number of diarrhoeic samples and duration) compared with animals infected for the first time at <1 week of age (Figure 8).

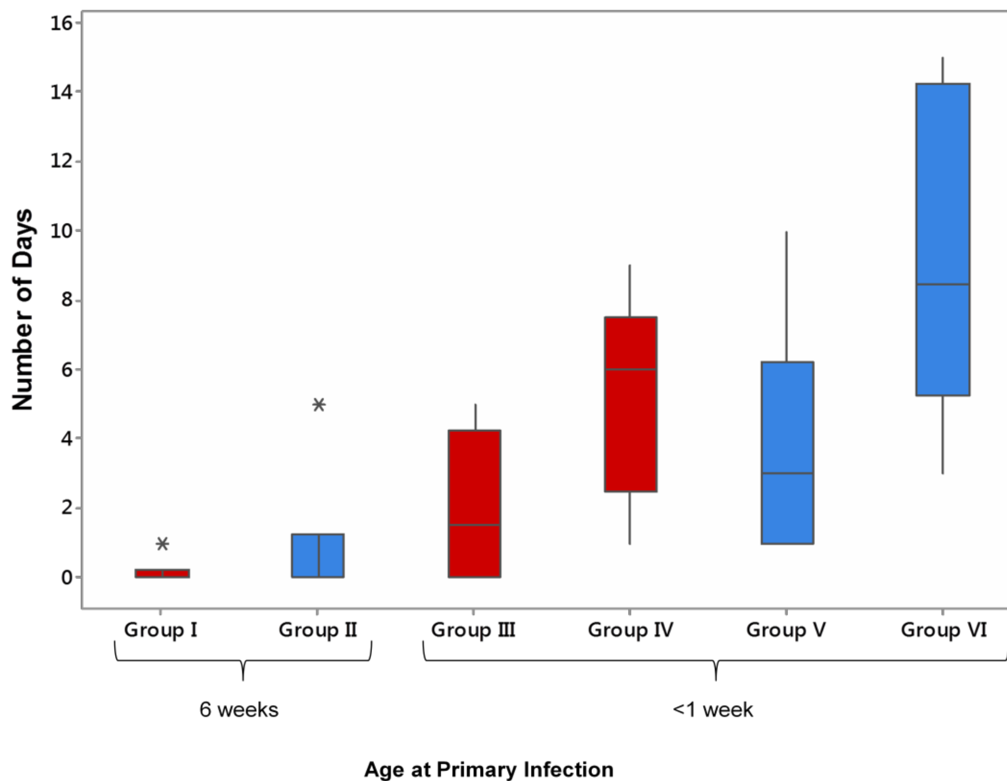

Figure 8: Number of days lambs produced a diarrhoeic sample following primary-infection at <1 week (Group III, IV, V & VI) and 6 weeks (Group I & II) of age.

Lambs infected at 6 weeks of age produced fewer diarrhoeic samples than lambs infected at 6 weeks of age. Red bars show primary infection with BB isolate and blue bars show primary infection with LH isolate. (* indicates outliers, the horizontal line the median and the vertical line indicates that the median is zero).

Animals challenged at 6 weeks (Group I & II) had a shorter duration of diarrhoea than animals challenged at <1 week of age (Group III, IV, V & VI) and produced fewer diarrhoeic samples ($P<0.001$). Demonstrating that lambs become less susceptible to disease caused by *C. parvum* as they increase in age despite an increase in infective dose.

5.3.2. Difference between isolates

To compare the pathogenicity of the two isolates one age-related susceptibility, one homologous and one heterologous challenge per isolate was carried out. Results show that the isolate used in the primary infection at <1 week of age does not significantly affect the probability of diarrhoea during the first three weeks post-infection although there is a trend ($P<0.1$) towards LH-challenged animals having a greater probability of diarrhoea. There was no significant effect of isolate on diarrhoea following challenge at 6 weeks of age.

Isolate had a significant effect on the interval to onset of diarrhoea ($P < 0.05$) such that isolate LH resulted in earlier onset diarrhoea following primary infection. There was no effect of isolate on days to diarrhoea after the second challenge ($P = 0.434$).

Challenge with LH isolate at <1 week of age showed a trend ($P=0.08$) towards an increase in the number of days of diarrhoea (5.1 days vs. 2.9 days) following primary infection compared to a primary infection at <1 week of age with isolate BB (Figure 9).

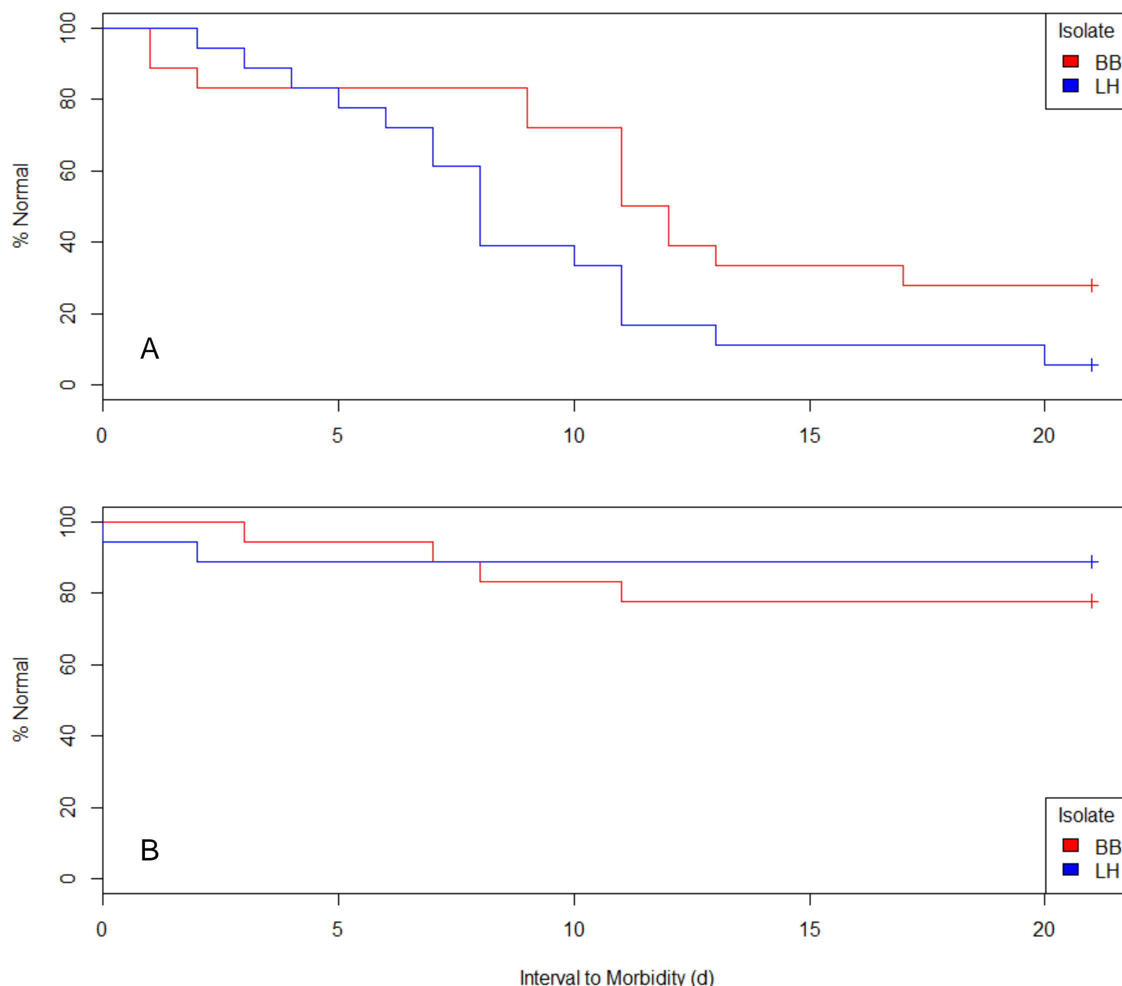

Figure 9: Time to onset of diarrhoea following primary challenge at <1 week (A) and 6 weeks (B) of age.

Lambs challenged with isolate LH at <1 week of age began scouring sooner than animals infected with isolate BB. There was no difference in time to onset of diarrhoea following challenge at 6 weeks of age

5.3.3. Previous exposure (homologous and heterologous challenge)

To determine if previous exposure to *C. parvum* provides protection against a secondary challenge later in life two sequential challenges were carried out to test each isolate in a homologous and heterologous challenge.

All animals receiving a primary infection or secondary challenge at 6 week of age showed a reduction in clinical disease compared with animals receiving a primary infection at <1 week of age. Previous exposure does not provide significant protection (P>0.99) against re-infection and the occurrence of clinical disease. The effect which age has on the occurrence of diarrhoea masks any effect that previous exposure may have. There was no significant difference in the occurrence (P>0.99) or days of diarrhoea (p=0.378) between groups with previous exposure (Group III, IV, V & V) and groups which had no previous exposure (Group I & II) (Figure 10).

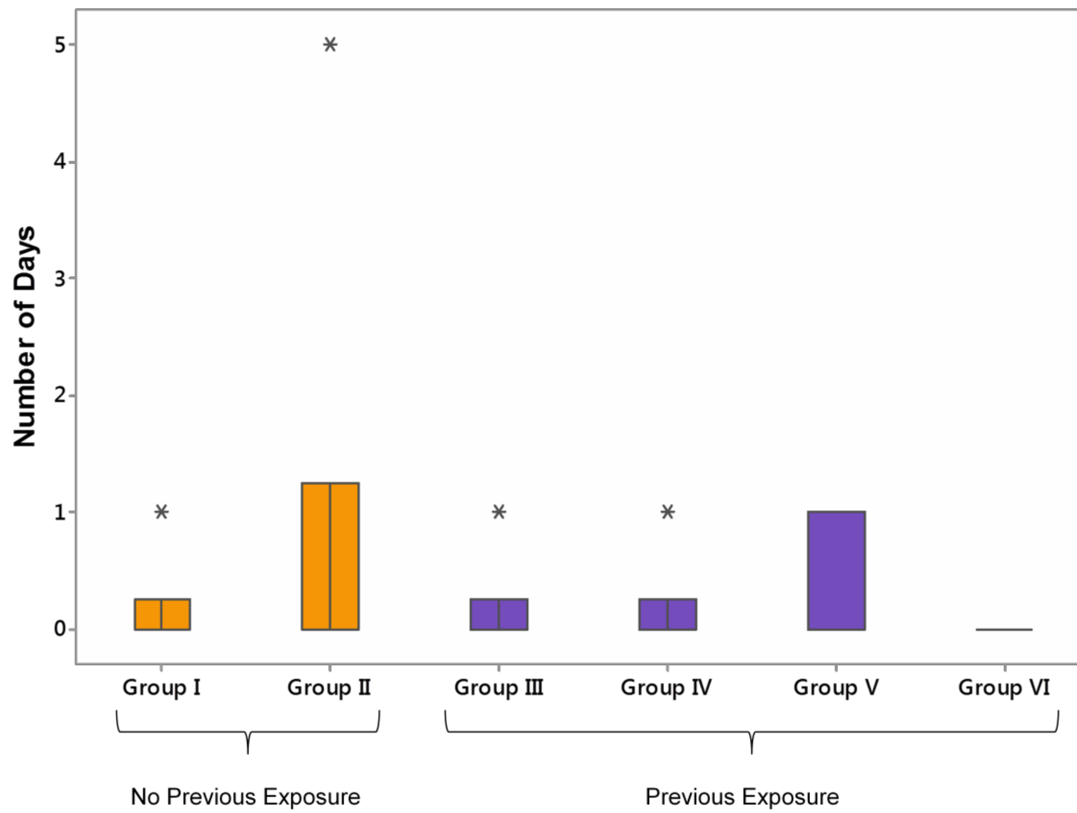

Figure 1: Occurrences of diarrhoea following challenge at 6 weeks

There was no difference in the occurrence or days of diarrhoea following challenge at 6 weeks in lambs receiving (purple boxes) or not receiving (orange boxes) a primary infection. (* indicates outliers, the horizontal line the median and the vertical line indicates that the median is zero).

6. Discussion

The protozoan parasite *Cryptosporidium* is a common cause of diarrhoea in both human and animal hosts. In livestock hosts the disease caused by *Cryptosporidium* has been associated with reduced weight gain in infected animals [5, 6] as well as mortality [7-9]. There are many different species and genotypes of the parasite (~27 species and >60 genotypes currently recognised) which infect different host species [10, 11]. Some of the species and genotypes are believed to be host specific while others can infect several host species [11, 12]. In farm livestock (sheep and cattle) one of the most common causes of neonatal enteritis is *C. parvum* [13, 14], this species of *Cryptosporidium* is also zoonotic and is responsible for ~50% of human cases of cryptosporidiosis in the UK [15]. Unlike several other pathogens associated with neonatal enteritis in sheep and cattle there is no available vaccine to prevent disease caused by *C. parvum* and treatment options are extremely limited and generally rely on rehydration therapy.

The results of both longitudinal studies showed that all calves <9 weeks old on the study farm became infected with C. parvum within 2 weeks of life. Infection with C. parvum caused severe diarrhoea, inappetence and lethargy in several of the affected animals, others suffered from mild disease only and some animals showed only asymptomatic shedding of oocysts. Shedding of C. parvum oocysts occurred for at least 7 weeks PI with peaks in oocysts shedding at 2-3 weeks of age and again at 7 weeks of age. Clinical signs tended to occur in animals <4 weeks of age and by 6/7 weeks of age many of the infected animals showed no signs of clinical infection though they were still shedding oocysts. In both studies any calves which were still present on the farm at 6 months of age were sampled and their faeces tested again. The results showed that the majority of calves in Study 1 (62%) and 32% of calves in Study 2 were positive for *Cryptosporidium* and that the most common species detected was *C. parvum.*

This is unusual as it is generally expected that after initial exposure to the parasite, at a young age, cattle will develop immunity to infection. None of the 6 month old calves were showing any clinical disease at the time of sampling. Subtyping results revealed that the genotype of *C. parvum* identified in the young calves in both studies was IIaA19G2R1 which is an uncommon subtype identified in cattle only a small number of times previously [16-19] while the subtype identified in faeces from 6 month old calves was the "common" subtype IIaA15G2R1 [20, 21]. Perhaps it is possible for cattle to develop immunity to one subtype of *C. parvum* following infection but this immunity may not necessarily protect against a further infections with a different subtype. This possibility was tested in the experimental trial described in Study 3.

Following the results of Study 1 where it was shown that calves can become infected with *Cryptosporidium* almost immediately at birth the second longitudinal study (Study 2) focused on the possible transmission of *C. parvum* from dam to calf. Previous studies investigating *Cryptosporidium* infection in adult cattle reported that adult cattle were not believed to be a

potential source from which young calves became infected [22-24] although there are two studies which disputed these results [25, 26]. These studies found that adult cattle either shed a species other than *C. parvum* or that they shed such low numbers of oocysts that the authors believed that they were not a significant risk. It is likely that the apparent low levels of *Cryptosporidium* found in adult cattle in previous studies could be due to the limitations of the detection methods applied or the number of samples taken.

In this study (Study 2) a group of in-calf cattle were sampled 3 × per week from 10 weeks pre-calving until calving and then the calves born to these cows were sampled 3 × per week for 6-10 weeks. We found that over 80% of the adult cattle involved in this study were shedding *Cryptosporidium* oocysts in their faeces, the majority of positive samples (98.2% (n=56)) were identified as *C. parvum* using nssm-PCR. This finding is unusual as most studies investigating *Cryptosporidium* in adult cattle find only low numbers of positive samples, or animals and the species most commonly detected is *C. andersoni* not *C. parvum*.

Subtyping results of *C. parvum* positive samples from adult cattle showed evidence of four different *C. parvum gp60* subtypes within this farm. One of the subtypes was IIaA19G2R1 which was the only subtype identified in young (<9 week old) calves and another (IIaA15G2R1) which was the only subtype found in older (6 months) calves, these and a further two subtypes (IIaA15G2 and IIaA18G2R1) were also detected in adult cattle on this farm. This age-related distribution of *C. parvum* subtypes in calves on this farm is very interesting; similar results were found in two separate studies in subsequent years on this farm indicating that this age-related distribution of *C. parvum* subtypes occurs frequently on this farm. From other work carried out on this particular farm it is known that the IIaA19G2R1 *C. parvum* subtype has been present and stable since at least 2008 [27]. None of the other subtypes have been previously detected although other studies have only examined young calves.

There are several reasons for this apparent distribution of isolates including the possibility that the *C. parvum* isolate detected in 6 month old animals is another species of *Cryptosporidium*. Another reason for the difference in isolates found in calves of different age groups on this farm could be related to changes in the host themselves. It has been reported in previous experimental studies that some host species (pigs and chickens) become more susceptible to certain *Cryptosporidium* species as they increase in age. The authors of these papers [28-30] have suggested that this may be due to physiological changes in the gut or the gut flora of the host. It could be that the different isolate found in older cattle is somehow better adapted to replicate in the gut conditions of a fully mature bovine than in the undeveloped gut of a neonatal animal. Other studies examining the subtypes of *C. parvum* in different age groups of cattle on the same farms also found that adult cattle may be carrying different genotypes compared to their calves [31] indicating that adult cattle may not be an important source of infection for young calves.

In study 3 a large scale experimental trial was carried out to assess the development of host resistance to distinct *C. parvum* isolates as both a homologous and heterologous challenge. Both isolates which were known to cause clinical disease in naturally infected calves from two dairy farms in Scotland. The results of this study showed that distinct isolates of *C. parvum* can have different clinical manifestations in infected individuals even when those individuals have received an identical infective dose. The study also demonstrated that host factors play a role in the severity of disease and oocyst shedding as even within groups when animals received the same infective dose of the same isolate there was variation in the outcome of disease with some individuals experiencing more severe or prolonged diarrhoea or increased shedding of oocysts compared to others in the same group. This variation in response has been noted in several other experimental trials involving *Cryptosporidium* and other pathogens [1, 2, 32-37]. Although it is clear that there is a difference in the virulence/pathogenicity of isolates there are also host factors which should be investigated further.

The results of Study 3 showed that while lambs do not develop complete resistance to disease by 6 weeks of age they do become less susceptible to clinical infection. If lambs are naïve to *Cryptosporidium* and are infected for the first time at 6 weeks of age then they show significantly fewer clinical signs than when lambs are infected at <1 week of age. Another study carried out in 1994, which was similar, demonstrated that lambs become resistant to disease at 8 weeks of age. The lambs in that trial showed no signs of clinical disease though some did shed oocysts when infected at 8 weeks of age. In the trial described in Study 3 it was shown that while older lambs exhibited fewer clinical signs of disease than younger lambs they still shed huge numbers of potentially infective oocysts in their faeces. This may be due to the dose of oocysts the animals were given; animals infected at <1 week of age were inoculated with 1×10^6 oocysts while animals infected at 6 weeks of age were inoculated with 5×10^6 oocysts. This increase in infective dose may be the reason why the animals showed asymptomatic shedding of oocysts. Ortega-Mora & Wright (1994) orally infected the lambs in their trial with the same infective dose (1×10^6) at each age group. This may be why, in their study, the older lambs did not show any oocyst shedding. It is possible that if an 8 week old lamb had been challenged with a higher dose as in this trial, that asymptomatic shedding of oocysts would have occurred.

This means that in a field setting it would be possible for an older animal to become infected with a large dose of *C. parvum* from a young animal and for the older animal to shed huge numbers of oocysts into the environment while showing no clinical signs of infection itself. The amplification rate of the parasite in a young naïve animal is phenomenal; an animal infected with 1×10^6 oocysts is able to shed up to 2.73×10^9 potentially infectious oocysts in a two week period. In theory, this is enough to infect a further 2730 susceptible hosts with the same number of oocysts as the initial challenge. It is the amplification rate which leads to the rapid spread of disease within a herd or

flock. From this we can see why, in a field setting, it is possible that older lambs, or even adults, may become subclinically infected with *Cryptosporidium* and maintain the parasite between lambing or calving periods.

Overall, work carried out for this PhD has enhanced our understanding of the transmission dynamics of the parasite within cattle on the study farm and has indicated that adult cattle may act as a source of infection for young calves although further work is required to confirm this. Improved techniques developed throughout this thesis also advance our ability to quickly and accurately determine the species of *Cryptosporidium* being shed by individual animals as well as increasing the sensitivity of detection methods from adult cattle faeces. These improved techniques have also been used to examine the faeces of other host species such as deer, sheep and rabbits with great success [31]. The experimental study looking at host resistance has contributed greatly to the field and has answered several questions about the development of immunity as well as the possibility of variation in pathogenicity between isolates of the same *Cryptosporidium* species. This work can be used as a basis to improve our understanding of disease pathogenesis, transmission, development of host resistance and variation in pathogenicity of different *C. parvum* subtypes.

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