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Differences in virulence and oocyst shedding profiles in lambs experimentally infected with different isolates of *Cryptosporidium parvum*

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ABSTRACT

A wide spectrum of disease severity associated with cryptosporidiosis has been described, ranging from asymptomatic to fatal in both human and animal hosts. The reasons for the variations in severity are likely to be multifactorial, involving environmental, host and parasite factors. This paper describes two experimental infection trials in lambs, a symptomatic host for the parasite, to investigate variation in the clinical manifestations following infection with two distinct isolates of *Cryptosporidium parvum*. In the first experiment, groups of naïve lambs were challenged with one of two isolates (*CP1* or *CP2*) at < 1 week of age, to test the effect of the isolates on disease outcome. In a second experiment one group of lambs challenged at < 1 week of age (*CP1*) was then re-challenged with the same isolate at 6 weeks of age (*CP1*), while a second group was challenged for the first time at 6 weeks of age (*CP1*). This experiment examined age-related disease symptoms, oocyst shedding and the effect of prior exposure to the parasite on a subsequent homologous challenge. The two isolates were associated with significant differences in the demeanour of the animals and in the numbers of oocysts shed in the faeces. There were also differences in the duration and severity of diarrhoea, though these were not significant. The age of the lamb, at the time of a primary challenge (<1 week or 6 weeks), also resulted in differences in clinical outcomes, with younger lambs showing more severe clinical disease than the older lambs (feeding profiles and presentation of diarrhoea), while older lambs showed virtually no signs of infection but still produced large numbers of oocysts.

1. Introduction

Cryptosporidium parvum is a well-known cause of enteritis in many species including humans (Robinson et al., 2022). This species is also one of the most important causes of neonatal enteritis in farm livestock (Thomson et al., 2017; Shaw et al., 2021). Young farm animals can become infected with the parasite very early in life and suffer from profuse watery diarrhoea, which can result in severe disease and sometimes in the death of the animal. Present control measures are limited, mainly relying on good farm hygiene to minimise the risk of infection and rehydration therapy to treat the clinical signs (Thomson et al., 2017).

Many distinct isolates of *C. parvum* exist, but very little information is available on how these isolates differ in their pathogenicity and virulence. It has been recognised that animals and humans infected with *Cryptosporidium* vary in the severity of disease; some are asymptomatic while in others the disease may be fatal. Some of this variation is likely to be due to environmental and host factors (Chalmers and Davies, 2010). Two trials have provided some evidence of variation in virulence of *C. parvum* isolates in healthy human volunteers (Okhuysen et al., 1999) and calves (Pozio et al., 1992). Another study carried out using mice by Sayed et al. (2016) showed a difference in the virulence of two different *C. parvum* isolates; one was the IOWA strain and the other an isolate obtained from a local water supply. This study showed that the locally sourced *C. parvum* isolate was more virulent than the IOWA strain and resulted in more severe clinical disease, a shorter incubation period and greater numbers of oocysts being shed (Sayed et al., 2016). A recent study

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by Audebert et al. (2020) identified variants in a number of membrane and secretory proteins of *C. parvum* that may account for differences in isolate pathogenicity and virulence.

Previous studies have also demonstrated that isolates of the human-adapted species *Cryptosporidium hominis* can manifest differently in infected people, both healthy and immune-compromised (Okhuysen and Chappell, 2002; Cama et al., 2007, 2008). These studies showed that *C. hominis* subtype family Id was associated with an increased risk of diarrhoea (Cama et al., 2007), while subtype family Ia was not associated with diarrhoea, and that *C. hominis* subtype family Ib was associated with nausea and vomiting (Cama et al., 2008).

To test the effect of two different *C. parvum* isolates on clinical disease as well as oocyst shedding, two experimental infections in lambs (a symptomatic host of *C. parvum*) were carried out. Experiment 1 demonstrates that a multi-factorial approach is needed to accurately describe differences in neonatal lambs experimentally challenged with *C. parvum*. The individual use of common indicators of disease, e.g. the presence of diarrhoea, time to onset of diarrhoea and a reluctance to feed, may not be informative on their own; however, when all combined they demonstrate clear (if not always statistically significant) differences between pathogenicity of individual strains. In Experiment 1, naïve lambs < 1 week-old were challenged with two different isolates (*CP1* or *CP2*). In the second experiment, one group of lambs were re-challenged with the same parasite isolate (*CP1*) at 6 weeks of age, to test the effect of previous exposure to a homologous challenge while another group of lambs received a primary challenge at 6 weeks-old with the same isolate (*CP1*), to examine the age-related effects of infection.

2. Materials and methods

2.1. Experimental animals

Thirty-four male Texel and Texel cross lambs were collected at birth and housed in 7 groups of 2–6 animals in separate concrete sided isolation rooms (pens), bedded with straw. The lambs were fed on commercial ultra high temperature (UHT)-treated milk three times per day *via* bottle and teat in a specially designed lamb feeder to ensure that each lamb could access only the milk intended for it. This feeding system enabled accurate recording of the milk intake of each individual animal. Animal handlers wore protective clothing (waterproofs, wellington boots and nitrile gloves) and passed through a disinfectant footbath before coming into contact with the lambs; handlers were blinded to animal groupings. Separate protective clothing was used for handling each group (pen) of lambs.

2.2. Cryptosporidium parvum isolates

The *C. parvum* isolates used in the experimental trial were obtained from naturally infected calves from two separate dairy farms with known histories of cryptosporidiosis. Management practices and animal husbandry were very similar on both of these farms, but the multi-locus genotype (MLG) of each isolate was different, as were the clinical manifestations in the animals. Calves on Farm 1 generally suffered from mild or asymptomatic infections whilst still shedding large numbers of oocysts, whereas calves on Farm 2 suffered from more severe clinical disease with profuse watery diarrhoea.

Isolate *C. parvum 1* (*CP1*) was collected from Farm 1 and isolate *C. parvum 2* (*CP2*) was collected from Farm 2. Faecal samples were collected from calves on each farm and tested for the presence of *C. parvum* by 18S nssm-PCR (nested species-specific PCR) (Thomson et al., 2016), as well as determination of the multi-locus genotype (MLG) using six of the markers (MM5, MM18, MM19, TP14, MS1 and *gp60*) as previously described (Hotchkiss et al., 2015). Oocysts were purified from calf faeces by sucrose flotation (Bukhari and Smith, 1995). These fresh purified oocysts of each isolate were passaged in neonatal lambs (5×10^6 oocysts per animal) (total $n = 4$, $n = 2$ for each strain), in order to produce sufficient oocyst numbers for use in the experimental infections. Both isolates caused clinical disease in the donor animals. Oocysts were purified from infected lamb faeces by acid flocculation (Ortega-Mora and Wright, 1994) and stored in phosphate-buffered saline (PBS) at 4 °C. Prior to challenge, viability of oocysts was assessed by carrying out a maximised *in vitro* excystation assay (Cameron et al., 2015). The same *CP1* oocyst stocks were used in infections at < 1 week and 6 weeks of age; the excystation percentage for both isolates was $> 90.0\%$ and the sporozoite to shell ratio was > 2.00. The *Cryptosporidium* oocysts used as challenge inocula were surface-sterilised using 2 ml of 70% ethanol for 30 min before being resuspended in PBS. Oocysts were counted and the oocyst suspension diluted with PBS to give the desired infective dose.

2.3. Experimental design

2.3.1. Experiment 1

Twenty-four lambs were used in experiment one. Lambs were randomly divided into two groups; these were housed in 4 pens each containing 6 lambs (as described above). Lambs in two pens (Pens 1 and 2) were challenged at 3–6 days of age (referred to as < 1 week) with 1×10^6 *C. parvum* oocysts (*CP1*). The lambs in the other two pens (Pens 3 and 4) were challenged at age < 1 week with 1×10^6 *C. parvum* oocysts (*CP2*) and observed for six weeks. Those animals not being used in Experiment 2 (Pens 2, 3 and 4) were euthanized at six weeks post-challenge.

2.3.2. Experiment 2

The six lambs housed in Pen 1, were challenged for a second time at 6 weeks of age with 5×10^6 *C. parvum* oocysts (*CP1*), while another 6 lambs, housed in a separate isolation pen (Pen 5), received a primary challenge with 5×10^6 *C. parvum* oocysts (*CP1*) at age 6 weeks. The group of lambs (Pen 5) were acquired at the same time as all of the other lambs used for Experiment 1 and housed separately. Faecal samples were collected twice weekly (and screened by microscopy) from the Pen 5 lambs from age one day-old through to 6 weeks of age, to ensure that the lambs remained free from *Cryptosporidium* infection. All of the animals used in Experiment 2 were maintained for three weeks following the challenge at age 6 weeks, after which time they were euthanized.

The dose of oocysts used for Experiment 2 was higher (5×10^6) than that used in Experiment 1, as the animals are larger and we wanted to increase the chances of producing a clinical infection in the older lambs.

2.4. Clinical assessment

All lambs were monitored a minimum of three times per day for the first three weeks (21 days) post- infection (PI) and then a minimum of twice daily for the remainder of the study. Daily monitoring involved recording the volume of milk each lamb consumed, along with scoring how well the animal fed: 0, drank vigorously; 1, complete feed with interruptions; 2, complete feed with assistance of a handler; 3, partial feed; 4, partial feed with assistance; and 5, complete reluctance to feed). The general demeanour of the animals was also scored as follows: 1, happy to rise (or already standing when handler enters pen); 2, reluctant to rise; 3, needs assistance to rise/unsteady on feet; and 4, cannot remain standing).

2.5. Collection and processing of samples

In both experiments one and two the total faecal output from each lamb was collected using a specially designed harness and bag system, which the lamb wore continuously from Day 1. Total faecal weight per day was recorded for each lamb. The harness and bag system ensured that after infection the lambs were not exposed to any faecal matter from other lambs and were not re-infected by oocysts shed in the faeces. The faecal collection bags were taken off and immediately replaced when required.

Faecal consistency was scored against the following criteria: 1, firm

pellets; 2, soft pellets; 3, soft mass (forms a pat); 4, liquid (forms a puddle); 5, liquid (flows freely). A faecal score of ≥ 4 was considered as diarrhoeic.

Quantitative examination of oocyst numbers involved homogenising the entire faecal sample in a food processor (with the addition of H_2O to make the sample liquid, if required). A sub-sample diluted 1:5 (0.16% malachite green/1% SDS) was prepared for counting using an improved Neubauer haemocytometer. From each lamb, every sample produced by the lamb during the clinical stage of infection (days 0–21 PI) was counted. Additionally, 1–2 samples per week in the non-clinical stages were also processed and examined for each lamb. The researcher carrying out the counts was blinded to the identity of the samples.

2.6. Statistical analysis

All statistical analyses were performed using R version 4.2.1 (R Core Team, 2022).

2.6.1. Clinical signs

Demeanour data were recorded as ordinal categorical data, from 1 to 4, with 1 being good, and reduced to binary data (1, normal; 2–4, abnormal) but data were not analysed statistically because only 10 abnormal observations were recorded over the entire study. Feed intake was scored as ordinal categorical data, from 0 to 5, then reduced to binary, with scores of 0–2 being considered to be complete and scores of 3–5 as incomplete feeding. Frequency tables of counts of days of incomplete feeding for each lamb during the experimental period were analysed using Fisher's exact test. Faecal consistency score was recorded as ordinal categorical data from 1 to 5, with ≥ 4 indicating diarrhoea and analysed using frequency tables with Fisher's exact test. Diarrhoea duration was defined as the count of days on which each lamb had a faecal score ≥ 4, whether these days were continuous or discontinuous. The association of diarrhoea duration with isolate and pen was tested using the Kruskal-Wallis rank sum test. The time between infection and the onset of diarrhoea was right-censored and analysed using the functions "surv" and "survreg" from the *survival* package in R, with a Weibull distribution, with "pen" and "isolate" as potential explanatory variables. The faecal consistency scores over time were analysed using a generalized additive model (GAM) using the "gam" function of the *mgcv* package in R (Wood, 2011), with "smoothed time", "isolate" and "pen" as fixed effects and "lamb" as a random effect. Residuals were plotted and checked for normality using the "qnorm" function in R.

2.6.2. Oocyst shedding

The time from infection until oocyst shedding was not right-censored, because all lambs shed oocysts at least once during the study and the effects of pen and isolate were tested using the Kruskal-Wallis rank sum test. As expected, the oocyst counts were not normally distributed (assessed by Shapiro-Wilk test for normality and visual inspection of histograms), with zero inflation, but had a complex, zero-inflated distribution that was not amenable to transformation. For each lamb, a total oocyst output over the 21 days was estimated and used as the dependent variable in a Kruskal-Wallis rank sum test for association with isolate and pen.

3. Results

3.1. Cryptosporidium parvum: Multi-locus genotype analysis to identify suitable isolates

Microsatellite analysis of six loci including *gp60* was carried out to ensure that the selected two isolates (*CP1* and *CP2*) were genetically distinct. Results showed that the *CP1* (IIaA15G2R1) and *CP2* (IIaA19G2R1) were genetically distinct with three markers (MM5, MM19 and *gp60*).

All animals in both experiments became infected with *C. parvum*, with all animals shedding oocysts at least once during the experiment. A selection of samples produced from both *CP1*- and *CP2*-infected animals following the primary challenge at age < 1 week were analysed using *gp60* and microsatellite markers. All of these excreted oocyst samples had identical MLG to the challenge strain that each of the animals had received.

3.2. Experiment 1: A comparison of virulence of two distinct isolates of C. parvum

3.2.1. Clinical signs

The number of days on which feed intake was incomplete did not differ during the study period, either according to pen (Kruskal-Wallis $\chi^2 = 1.10$, $df = 3$, $P = 0.78$) or with isolate (*CP1* = 2.5 days, *CP2* = 1.25 days; Kruskal-Wallis $\chi^2 = 0.9546$, $df = 1$, $P = 0.3286$). Though there were no statistical differences in the feed intake observed in Experiment 1, 8/12 of the *CP1*-challenged lambs (Pens 1 and 2) recorded incomplete feeds on at least one occasion between days 3–10 post-challenge, with 3 out of 12 animals demonstrating a reluctance to feed on six or more consecutive occasions. During the same period (3–10 days post-challenge) 5 out of 12 of the *CP2*-challenged lambs recorded incomplete feeds with two animals being reluctant to feed on four or more consecutive occasions. After Day 10 post-challenge there was only one single record of a lamb feeding reluctantly.

The total number of occasions when each lamb was identified as reluctant to rise differed significantly between isolates (Fisher's exact test, $P = 6.4 \times 10^{-05}$), with lambs infected with *CP2* being more affected. All lambs in the *CP2*-treatment group were scored as abnormal on Day 5 after infection, compared with no lambs considered abnormal in the *CP1*-challenge group on Day 5.

Most lambs (20/24, 83.3%) infected at age < 1 week had diarrhoea post-challenge (faecal score ≥ 4) on at least one occasion during the experiment. The mean daily faecal consistency scores for the lambs challenged with *CP1* and *CP2* at < 1 week of age are illustrated in Fig. 1. The *CP2*-challenged lambs demonstrated a higher mean faecal consistency than *CP1* between 7 and 19 days after challenge, though these differences were not statistically significant.

The time from challenge to the onset of diarrhoea did not differ significantly between isolates or pens when these were considered in the same survival regression model (Fig. 2; isolate, $P = 0.50$; pen, $P = 0.27$).

There was no significant difference in the duration of diarrhoea post-infection in lambs challenged with either isolate ($P = 0.36$, means *CP1* = 3.2 days and *CP2* = 4.7 days), but tended to differ with pen ($P = 0.055$, being 3.0, 3.3, 2.7 and 7.2 days for pens 1, 2, 3 and 4, respectively).

3.2.2. Faecal oocyst shedding

There was no difference between isolates for the interval from challenge until the day when faecal oocysts were first detected (*CP1* = 6.17 ± 3.48 days; *CP2* = 7.58 ± 3.48 days; $P = 0.16$). There was also no significant difference among pens ($P = 0.12$).

The total oocyst output per lamb over 21 days differed significantly between the two isolates (mean *CP1* = $356 \times 10^6 \pm 85.8 \times 10^6$ oocysts; *CP2* = $78.1 \times 10^6 \pm 85.9 \times 10^6$ oocysts, Kruskal-Wallis test, $P = 0.00034$). The difference in the mean daily oocysts counts from the lambs challenged with *CP1* and *CP2* are illustrated in Fig. 3 and the time series plots of mean daily oocyst counts are shown in Fig. 4A. As suggested from the time series plot of mean oocyst counts (Fig. 4A), the evolution of oocyst counts over time is complex and non-linear. The generalized additive model (GAM) of oocyst shedding, using "smoothed time", "isolate" and "pen" as fixed effects and "lamb" as a random variable explained only 14.1% of the deviance, with "smoothed time" being a highly significant variable ($P < 2.2 \times 10^{16}$), "isolate" also significant ($P = 0.0047$), and "pen" not significant ($P = 0.41$) (Fig. 4B). No transformations improved the approximation to normality of the raw data, or of the residuals, so caution is required in the interpretation of the GAM.

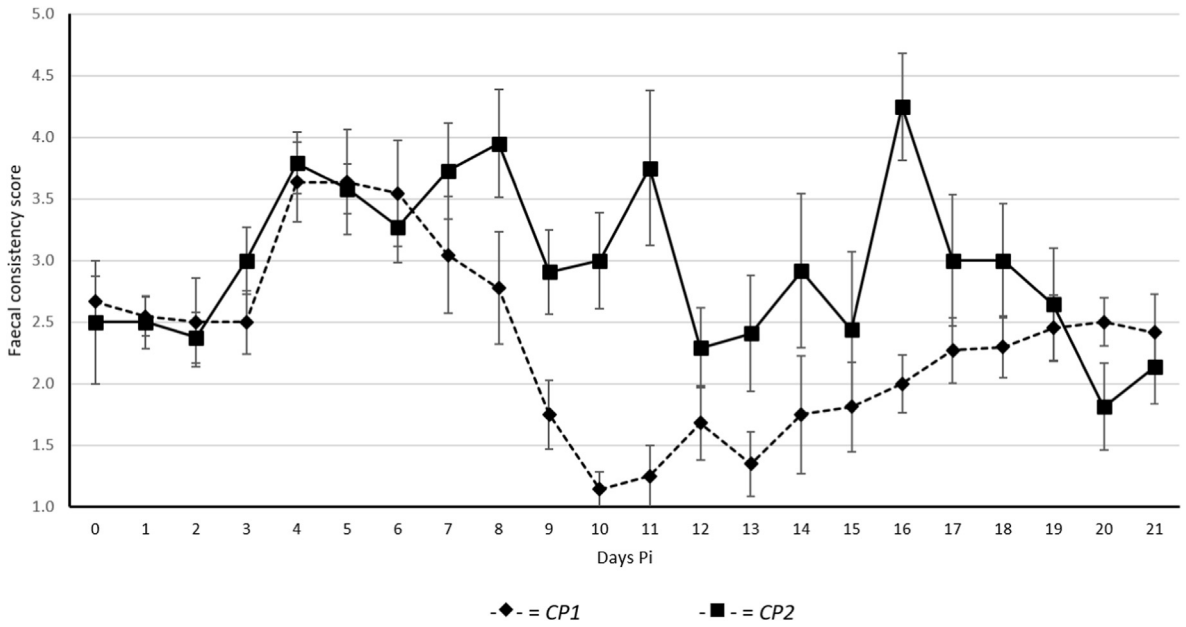

Fig. 1. Mean daily faecal consistency scores of lambs following a primary challenge at age < 1 week with two distinct isolates of *Cryptosporidium parvum*, either *CP1* or *CP2*. Error bars: ± standard error of the mean (SEM).

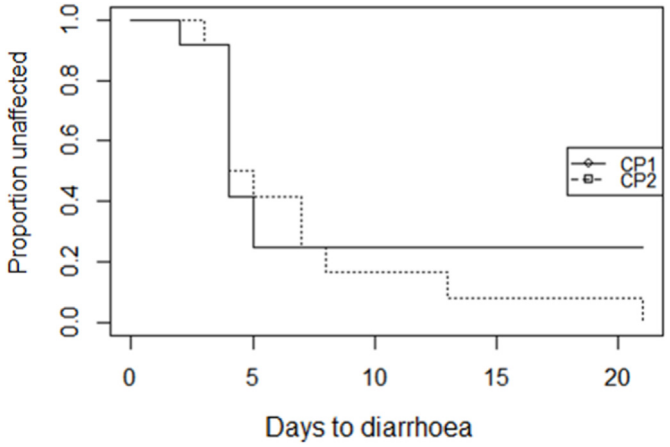

Fig. 2. Time until the onset of diarrhoea of lambs following a primary challenge at age < 1 week with two distinct isolates of *Cryptosporidium parvum*, either *CP1* or *CP2*.

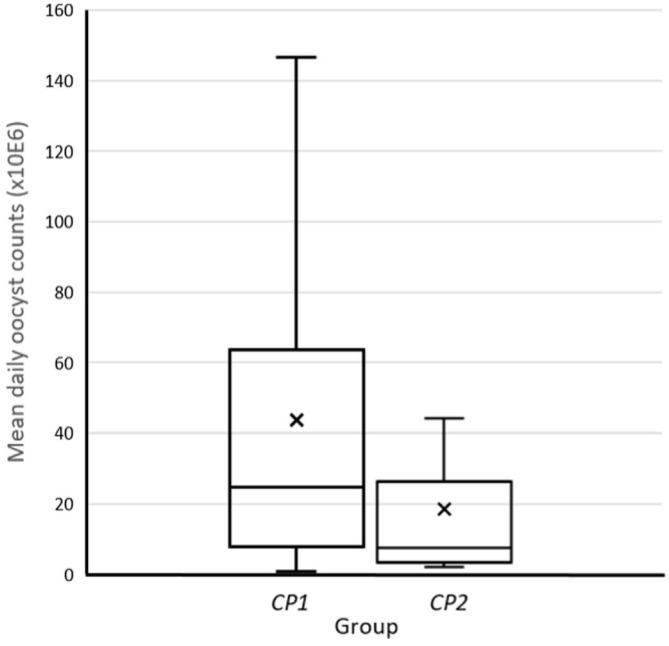

Fig. 3. Box-and-whisker plots of mean daily oocyst counts from lambs challenged age < 1 week with two distinct isolates of *Cryptosporidium parvum*, either *CP1* or *CP2*.

3.3. Experiment 2: Homologous C. parvum challenge and age-related effect on C. parvum infection

3.3.1. Clinical disease

One lamb, from the group of lambs infected for the first time at week 6 of age with *CP1* (Pen 5), was excluded from the experiment as it presented with diarrhoea prior to infection. Faecal samples from this animal were screened routinely (twice weekly) between age of 0–6 weeks and no *Cryptosporidium* oocysts were ever counted. The scores for faecal consistency and oocyst counts from this lamb were excluded from all calculations from this group.

All lambs challenged at 6 weeks old *CP1*-1-6w (challenge isolate *CP1* – primary challenge – 6 weeks of age) and *CP1*-2-6w (challenge isolate *CP1* – secondary challenge – 6 weeks of age) consumed all of the milk offered to them. However, four animals infected at age < 1 week (*CP1*-1-1w) (challenge isolate CP1 – primary challenge – < 1 week of age) fed incompletely, which was a significantly different proportion (Fisher's exact test, $P = 0049$). None of the *CP1*-1-1w, *CP1*-2-6w or *CP1*-1-6w animals showed any changes in demeanour, with all animals being happy to rise to their feet and remain standing during feeding and faecal sample collection. Three out of six lambs infected at age < 1 week (*CP1*-1-1w) developed diarrhoea (faecal score ≥ 4), compared with no lambs infected first at age 6 weeks (*CP1*-1-6w) (Fisher's exact test, $P = 0.074$). Mean faecal consistency scores over time are illustrated in Fig. 5. Survival analysis using a Weibull distribution (Fig. 6) confirmed that the probability of diarrhoea was significantly higher ($P = 0.015$) when the challenge occurred at age < 1 week than when the exposure occurred at 6 weeks.

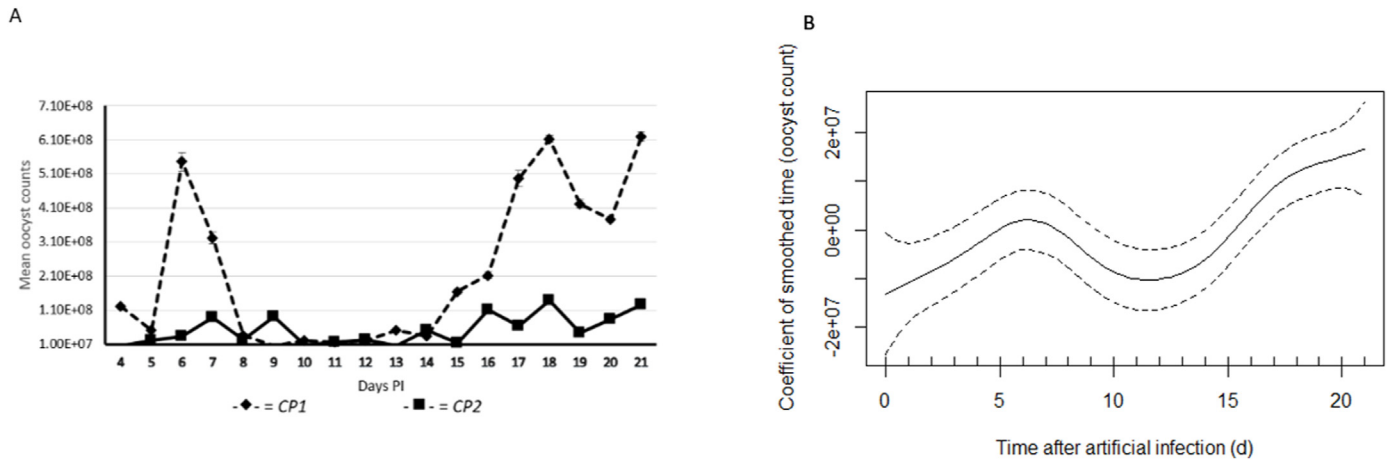

Fig. 4. **A** Mean daily oocyst outputs of lambs following a primary challenge at age < 1 week with two distinct isolates of *Cryptosporidium parvum*, either *CP1* or *CP2*. Error bars: ± standard error of the mean (SEM). **B** The generalized additive model (GAM) of oocyst shedding, using smoothed time, isolate and pen as fixed effects and lamb as a random variable.

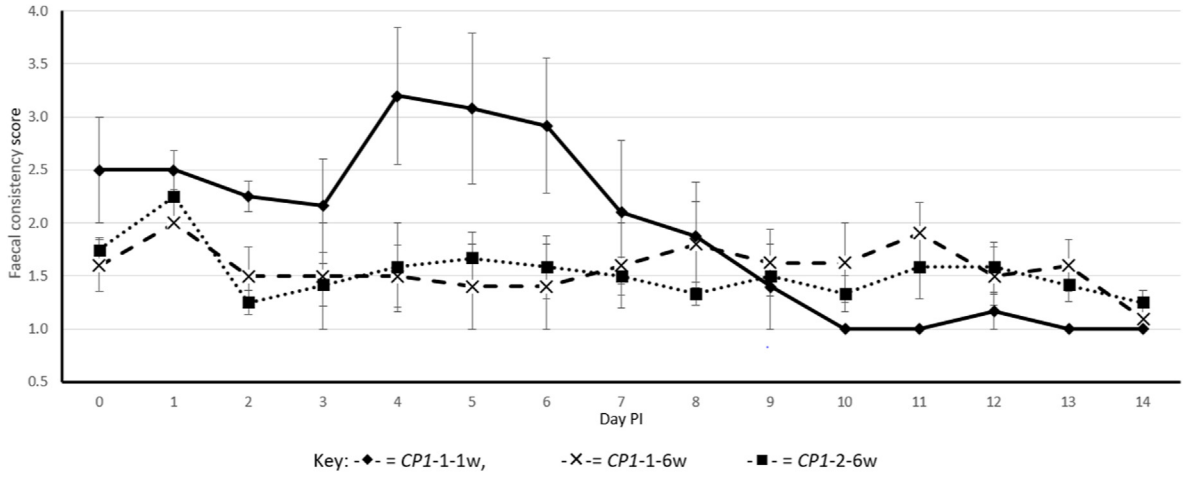

Fig. 5. Mean daily faecal consistency scores of lambs following a primary challenge at age < 1 week (*CP1*-1-1w) or at age 6 weeks (*CP1*-1-6w) or following a secondary challenge at age 6 weeks (*CP1*-2-6w). Error bars: ± standard error of the mean (SEM).

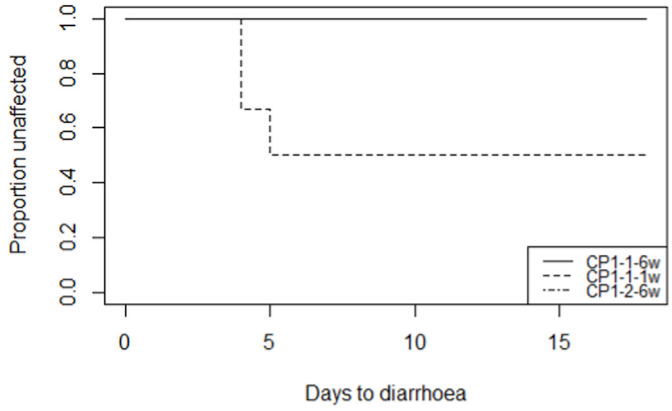

Fig. 6. Survival curve for interval from infection until the onset of diarrhoea for lambs infected with the *CP1* isolate, for the first time at < 1 week of age (*CP1*-1-1w), for the first time at 6 weeks (*CP1*-1-6w) or for the second time at 6 weeks (*CP1*-2-6w). The difference in the probability of diarrhoea was significantly higher for first exposure at < 1 week ($P = 0.015$).

3.3.2. Faecal oocyst counts

The total oocyst counts over the first 16 days from *CP1*-1-1w ($66.2 \times 10^6 \pm 48.6 \times 10^6$), *CP1*-1-6w ($1320 \times 10^6 \pm 766 \times 10^6$) and *CP1*-2-6w ($2130 \times 10^6 \pm 2000 \times 10^6$) differed among groups, being significantly lower in the animals challenged at < 1 week than at 6 weeks (Kruskal-Wallis test, $P = 0.00067$, see Fig. 7). The animals challenged at age < 1 week (*CP1*-1-1w) received 1×10^6 oocysts compared to the dose of 5×10^6 that was given to the lambs at age 6 weeks. Although there were no statistical differences in the numbers of oocysts shed by the animals receiving primary (*CP1*-1-6w) or secondary (*CP1*-2-6w) challenges at age 6 weeks, the *CP1*-1-6w lambs started shedding large numbers of oocysts on Day 3 post-challenge. This was earlier than in *CP1*-2-6w lambs where larger numbers were not seen until Day 6 post-challenge; however, the *CP1*-2-6w lambs continued to shed oocysts for three days longer than the *CP1*-1-6w lambs.

4. Discussion

In this study we have demonstrated a difference in clinical outcome following challenge with two different *C. parvum* isolates (*CP1* and *CP2*) in naïve < 1 week-old lambs.

Prior to the present study, the interval from infection to the onset of oocyst shedding had been associated with the size of the infectious dose.

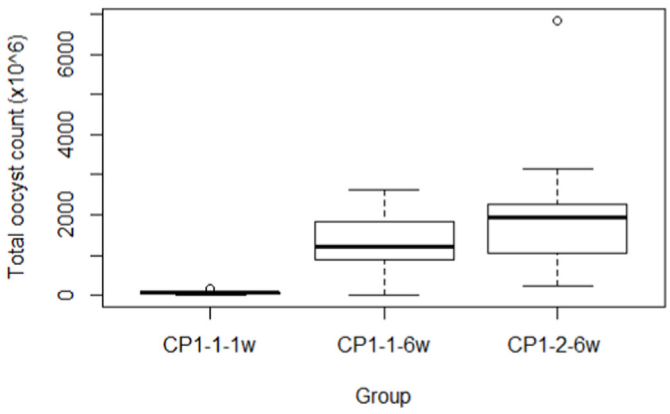

Fig. 7. Box-and-whisker plots illustrating the total oocyst outputs (over the first 16 days post-challenge) for lambs infected with the *CP1* isolate, for the first time at < 1 week of age (*CP1*-1-1w), for the first time at 6 weeks (*CP1*-1-6w) or for the second time at 6 weeks (*CP1*-2-6w). Error bars: ± standard error of the mean (SEM).

Zambriski et al. (2013) showed that calves experimentally infected with higher numbers of oocysts began shedding much quicker than those challenged with a lower dose (Zambriski et al., 2013). In the present study, the lambs infected at age < 1 week all received the same infective dose regardless of the isolate used. The very early detection of oocysts in the faeces of some lambs post-challenge is likely to be undigested and non-excysted oocysts passing through the gut, or in the case of the small number of previously exposed animals (*CP1*-2-6w), oocyst shedding from the primary infection might not have completely ceased.

The isolate (*CP1* or *CP2*) used in the primary challenge at age < 1 week affected the total numbers of oocysts shed by lambs in each group. At age < 1 week, lambs infected with *CP1* shed 4.56 times more oocysts than lambs infected with *CP2* (between 3 and 21 days PI). The naïve lambs challenged with *CP1* at age 6 weeks (*CP1*-1-6w) shed 20.59 times more oocysts following infection compared to *CP1*-1-1w, while the lambs re-infected with *CP1*-2-6w shed 33.73 times more oocysts than were seen in *CP1*-1-1w. However, it should be remembered that the animals challenged with *CP1* at age 6 weeks (*CP1*-2-6w and *CP1*-1-6w) received a higher dose of 5×10^6 oocysts compared to the dose of 1×10^6 given to the younger lambs (*CP1*-1-1w). The difference in the challenge dose may account for the differences in the mean numbers of oocysts shed by individual animals and the significant differences in oocyst shedding between the age groups. This study clearly demonstrates that prior exposure of neonatal animals to *C. parvum* does not protect against a secondary challenge (even a homologous challenge) and large numbers of oocysts are shed after secondary challenge. A recent study in cattle has shown that at six months of age dairy cattle were re-infected with *C. parvum* but with a different *gp60* subtype (Thomson et al., 2019), while Shaw et al. (2021) demonstrated intermittent oocysts shedding in calves throughout their first 6 weeks of life, which could have been due to the animals being re-infected (Shaw et al., 2021). The present experimental infection study has definitively shown that lambs do not develop sterile immunity against re-infection with *C. parvum*, even if they are re-exposed to the same isolate. Although re-infection at age 6 weeks (*CP1*-2-6w) did not result in obvious clinical signs of infection (i.e. diarrhoea) it did result in significant amounts of oocysts being shed in the faeces.

Very little work has been carried out to examine the differences in virulence of *C. parvum* isolates infecting farm livestock. However, one study by Jenkins et al. (2008) showed that distinct isolates of *C. parvum* differ in their fecundity. In the Jenkins et al. (2008) study, naïve dairy calves infected with 1×10^6 *C. parvum* (Beltsville isolate) shed five times as many oocysts as naïve dairy calves infected with 1×10^6 *C. parvum* (IOWA isolate), which is consistent with previous observations the researchers had made. In their study, the increased fecundity of *C. parvum* Beltsville was associated with higher levels of the viral symbiont *Cryptosporidium parvum virus* (CPV) and they hypothesised that perhaps the virus may have an effect on fecundity and virulence of different isolates (Jenkins et al., 2008). In the present study, the levels of CPV in the *C. parvum* isolates were not examined but perhaps it is an avenue that could be explored in the future.

The higher mean total number of oocysts shed by animals infected with *CP1* could be due to oocysts from this isolate excysting more rapidly than *CP2* oocysts. *In vitro* studies have shown that different isolates of *C. parvum* have different excystation efficiencies (Smith et al., 2005) and while some isolates are fully excysted after 30 min others take up to 90 min (Robertson et al., 1993).

As far as we are aware, there are no other experimental studies in farm livestock examining differences in clinical signs caused by different *C. parvum* isolates. However, it has been recognised for many years that cryptosporidiosis manifests differently in infected humans with some experiencing mild or asymptomatic disease, while others are severely affected. Much of this variation in disease expression is likely to be due to host factors such as immune status, age, the presence of co-infections, nutritional status etc., but some of the variation seen may also be due to the parasite isolate. The genetic factors involved in virulence of *Cryptosporidium* spp. are still poorly understood, this is partly due to a lack of availability of genomic sequence data, along with the inability to continuously culture *Cryptosporidium* spp. *in vitro*, making it difficult to test the effects of gene knockout (Bouzid et al., 2013).

Most of the information in the literature regarding differences in virulence or pathogenicity of different isolates of the same species of *Cryptosporidium* is limited to epidemiological studies rather than experimental studies. However, there is some evidence that genetic variation can be linked to phenotypical variation. In human cases of cryptosporidiosis, *C. hominis* subtype Ib is the subtype most commonly associated with water- and food-borne outbreaks in many countries (Xiao, 2010). In particular, subtype IbA10G2 has been found in most *C. hominis*-associated outbreaks worldwide (Li et al., 2013) and has been associated with nausea and vomiting in a longitudinal birth-cohort study carried out in Peru (Cama et al., 2008). *Cryptosporidium hominis* subtype Id has been associated with more severe clinical disease than other subtypes; three separate studies have reported an association of *C. hominis* subtype Id with an increased risk of diarrhoea (Cama et al., 2007; Iqbal et al., 2011; Anejo-Okopi et al., 2016). Information relating to *C. parvum* and disease in livestock is much scarcer. A study carried out in Poland showed that the occurrence of diarrhoea in calves was associated with the presence of the IIaA16G1R1 subtype in the faeces (Kaupke and Rzezutka, 2015) but other studies have contradicted this and found no associations between isolate and disease (Geurden et al., 2007; Rieux et al., 2013). There is also recent evidence from murine models suggesting that unique genomic characteristics (e.g. sequence polymorphism or variations in the copy number of putative virulence genes in the subtelomeric regions) among *C. parvum* isolates could also account for differences in virulence (Audebert et al., 2020; Jia et al., 2022).

Previous exposure to *C. parvum* did not prevent oocyst shedding in secondary challenged animals; however, these secondary challenged animals showed no, or only very mild clinical signs. This is very important as it highlights that previously exposed older animals may pose a high risk to naïve young animals despite showing no signs of disease themselves (Wells et al., 2015). For this reason, it is important to keep different age groups of young ruminant stock separate.

In the naturally infected calves, from which the oocysts were originally isolated, *CP1* was associated with higher oocyst output while *CP2* was associated with more severe clinical disease (diarrhoea). These relationships were maintained in the experimentally infected lambs. Interestingly, disease severity did not correlate with oocysts shedding in lambs.

5. Conclusions

This study revealed a difference in virulence and pathogenicity of two distinct *C. parvum* isolates (*CP1* and *CP2*). Further work is required to understand potential links between parasite genotype and phenotype and to identify factors that may contribute to parasite virulence. A good *in vitro* culture system would be ideal to allow testing of different isolates and may help lead to the identification of these virulence factors in combination with a symptomatic *in vivo* model. This present study also demonstrated that the age of a host has an effect on reducing clinical disease severity, but did not have any effect on the oocyst output of the lambs. Similarly, lambs that had been exposed to the parasite previously, can be re-infected, even with the same parasite isolate, and still shed high numbers of oocysts.

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Ethical approval

All animals were used in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines. The experimental design was approved sought prior to the commencement of the study by the Moredun Research Institute Animal Welfare Ethical Review Board (AWERB) (E11/13).

CRediT authorship contribution statement

Paul Bartley: conceptualisation, methodology, formal analysis, writing - original draft, writing - review & editing, visualisation. **Sarah Thomson:** conceptualisation, methodology, formal analysis, writing - original draft, writing - review & editing, visualisation. **Nicholas Jonsson:** formal analysis, writing - review & editing, funding acquisition. **Alessandra Taroda:** methodology, writing - review & editing. **Elisabeth Innes:** conceptualisation, writing - review & editing, funding acquisition. **Frank Katzer:** conceptualisation, methodology, formal analysis, writing - original draft, writing - review & editing, funding acquisition, visualisation, project administration. All authors read and approved the final manuscript.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Given their role as Co-Editor, Frank Katzer had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Editor-in-Chief Aneta Kostadinova.

Data availability

The data supporting the conclusions of this article are included within the article and its supplementary files.

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Appendix A. Supplementary data

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