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(i) Shedding of *Cryptosporidium* in calves and dams – evidence of re-infection and shedding of different *gp60* subtypes

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1 Abstract

One of the most common causes of calf diarrhoea is the parasite *Cryptosporidium parvum.* Two longitudinal studies were carried out on a dairy farm Scotland to determine the prevalence of *Cryptosporidium* species and subtypes in a group of calves and to determine whether dams were a possible source of calfhood infection. Faecal samples were collected from 25 calves from birth to 12 months in the first year. In the second year, faecal samples were collected from pregnant cows (n=29) and their calves (n=30) from birth to 6 months. The samples were tested for *Cryptosporidium* and speciated. *Cryptosporidium parvum* positive samples were subtyped by GP60 fragment analysis. All calves in both studies shed *Cryptosporidium* during the study period. *Cryptosporidium parvum* was the predominant species detected in calves ≤ 6 weeks of age and at 6 months of age, *C. bovis* and *C. ryanae* were detected in calves older than 4 weeks of age but ≤ 6 months of age. The prevalence of *Cryptosporidium* was higher in younger animals than in older animals. GP60 subtyping revealed two subtypes in calves on this farm (IIaA15G2R1 and IIaA19G2R1) that differed in frequency by age. Adult cattle also shed *C. parvum,* of four *gp60* genotypes.

Keywords: *C. parvum*, GP60 subtype, dam-calf transmission,

Key Findings

- Dams unlikely source of *C. parvum* infection for young calves
- Multiple *gp60* subtypes identified on one farm
- The same calves shed different *gp60* subtypes at different ages

2 Introduction

Cryptosporidiosis is the disease caused by the protozoan parasite *Cryptosporidium.* It is an important zoonotic pathogen which affects many species including humans (Chalmers and Davies, 2010; Chalmers and Katzer, 2013; Fayer, 2010). In the UK cryptosporidiosis is a common disease in livestock (APHA and SRUC, 2014), although clinical disease is usually limited to neonatal livestock and is caused by *Cryptosporidium parvum* (Tzipori et al., 1983); older animals are believed to mostly be infected with other species of the parasite (*C. andersoni* in adults and *C. ryanae* and *C. bovis* in calves of several months old) and tend not to show clinical signs (Anderson, 1987; Esteban and Anderson, 1995; Fayer et al., 2008; Santin et al., 2008). In livestock and humans, the disease usually causes self-limiting watery diarrhoea, loss of appetite and abdominal pain (Klein et al., 2008; Tzipori and Ward, 2002). Farm animals that have recovered from cryptosporidiosis have in some cases been shown to have slower growth rates than uninfected animals (de Graaf et al., 1999; Sweeny et al., 2011a; Sweeny et al., 2011b). The economic losses associated with *Cryptosporidium* have not been estimated but costs attributable to diarrhoeal disease in calves have been estimated to be at least £34 per affected calf (Gunn and Stott, 1998).

Cryptosporidium oocysts are transmitted between hosts via the faecal-oral route. The oocysts can survive for several months in the environment (Jenkins et al., 2002; Robertson et al., 1992) and are highly resistant to commonly used disinfectants, making them difficult to eradicate (Carpenter et al., 1999; Weir et al., 2002). Infected hosts can shed huge numbers of oocysts per day (over $10E^{10}$), which are immediately infective to other humans or animals (Nydam et al., 2001). There are currently over 30 recognised species of *Cryptosporidium* and many more genotypes (Chalmers and Katzer, 2013; Fayer, 2010) but the most common species in humans and calves is *C. parvum* (Brook et al., 2008; Chalmers and Giles, 2010; Chalmers et al., 2011a; Chalmers et al., 2011b; Gormley et al., 2011; Xiao and Feng, 2008). It is known that young calves become infected with *C. parvum* very soon after birth but it is not known if they pick up the infection from their dams, the environment or via another route.

At present, the only preventative or therapeutic products licensed in the UK for *Cryptosporidium* in cattle are Halocur® (halofuginone lactate (MSD Animal Health)) and Parofor® crypto (paromomycin (Huvupharma). Halocur® must be administered carefully as it is toxic at only twice the recommended dose and contra-indicated in dehydrated animals (MSD Animal Health Data Sheet, 2012). Parofor® crypto must only be given to animals with a confirmed diagnosis of *Cryptosporidium* and should not be given after the onset of diarrhoea (Huvepharma Data Sheet). Both should be given for 7 days consecutively. Due to the lack of suitable therapeutics or prophylaxis to control cryptosporidiosis in calves, it is important to understand transmission routes in order to develop better management strategies.

The studies described here aimed to determine the species and subtypes of *Cryptosporidium* present in calves and the variation in occurrence throughout the first year of life. Although some studies have previously been carried out to determine the prevalence of *Cryptosporidium* in calves in the UK these have mostly used less sensitive techniques and were unable to speciate or subtype the parasites. The present longitudinal study is the first to examine a population of calves from birth to sexual maturity on a farm in the UK and to investigate dams as a possible source of infection for calves.

3 Materials and Methods

3.1 Sample Collection

3.1.1 Calves

Faecal samples were collected from 25 calves (Study-1) and 30 calves born to cows and heifers (Study-2) from the day of birth until six weeks of age, three times per week (Monday, Wednesday and Friday). The calves were kept under the normal working conditions of this farm regarding housing, feeding and veterinary treatment. The calves were kept with their mothers for one or two days in a straw-bedded pen with other dams and new-born calves then moved to a small group of 5 to 8 calves in a straw-bedded pen adjacent to the calving pens for one week. All calves in both studies were seen to suckle their dams within the first few hours of life. Following removal from their dams calves were fed on calf milk replacer by an automatic feeder and housed in larger indoor straw-bedded pens with approximately 20 calves in each pen for the remaining five weeks, calves 1-14 were kept in one pen

and calves 15-25 were kept in another pen (Study-1). Both pens contained other calves that were not included in this study. All calves wore a transponder to enable the recording of daily feed intake, so that it was easy for stock-workers to determine if a calf was inappetant and to intervene accordingly. Calves also had *ad lib* access to hay and pelleted calf feed. Calves older than 6 weeks were moved and housed in a separate straw-bedded pen in larger groups where they had *ad lib* access to water, silage and pelleted feed. Samples were collected from the same calves again at three, six, nine and twelve months of age (Study-1) and at 6 months from calves in Study-2. The samples were aliquoted into 7 ml tubes and stored at -20°C for further processing.

3.1.2 Adult Cattle (Study-2)

Faecal samples were collected from 29 in-calf adult dairy cattle (heifers and cows) three times per week for up to ten weeks pre-calving, in total 209 samples were collected. The cows were kept under the normal working conditions of this farm and as such were housed in cubicle sheds before calving, in straw courts at calving and in slatted cubicle sheds after calving when they returned to the milking herd. 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 125 ml sample pot and stored at 4°C.

3.2 Faecal Consistency

The consistency of the collected samples was assessed (Study-1 only) and assigned one of three possible scores: normal – forms a pat (1), soft – forms a puddle (2), diarrhoeic – entirely liquid, flows freely (3). For statistical analysis as a binary outcome, scores 1 and 2 were combined and contrasted with score 3.

3.3 Sample Processing and DNA Extraction

Parasite DNA was extracted from the faeces using a Macherery-Nagal NucleoSpin® Tissue Kit according to the manufacturer's recommendations. Except that 10 × freeze-thaw cycles in liquid

nitrogen were added prior to the overnight incubation. The DNA was eluted in 100 µl dH_2O and stored at -20°C.

Samples from older calves (> 6 months) and adults were concentrated prior to DNA extraction.

3.3.1 Sample Concentration (Older calves)

Briefly, 3 g faeces were resuspended in 25 ml dH_2O and left to settle for approximately 5 mins. Once large particles had settled the supernatant was removed and added to another tube. This step was repeated and the tube containing the supernatant was centrifuged at 1000 x *g* for 5 mins, the supernatant was poured off and the resultant pellet used for DNA extraction (Brook et al., 2008).

3.3.2 Sample Concentration (Adults)

Samples were processed to concentrate *Cryptosporidium* oocysts from a 50 g starting sample by acid flocculation and salt flotation as described by Wells et al. (2016). This resulted in a faecal pellet which was used for DNA isolation.

3.4 PCR amplification of the 18S rRNA gene

A previously described nested PCR protocol (Xiao et al., 1999) was used to amplify a ~840 bp fragment of the 18S rRNA gene. Each 25 µl reaction contained 10 × PCR buffer (45 mM Tris-HCl pH 8.8, 11 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 4.4 µM EDTA, 113 µg ml-1 BSA, 1 mM each of the four deoxyribonucleotide triphosphates) (Burrells et al., 2013), 0.5 units BioTaq (Bioline, UK), 10 µM of forward and reverse primers ((AL1687 (EF) and AL1691 (ER)) and (AL1598 (IF) and AL3032 (IR)) and 3 µl DNA in the primary round and 1 µl primary PCR product in the secondary round. The total volume was made up to 25 µl with dH_2O. In each PCR run, one set of positive control DNA and negative controls consisting of dH_2O were included. All reactions were carried out in triplicate.

Cycling conditions were 3 min at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at 55°C and 1 min at 72°C. The final extension was 7 min at 72°C. Secondary round amplification products (~3 µl) were visualised following electrophoresis on a 1.5% agarose gel stained with GelRed™ (Biotium, UK) on an AlphaImager 2000.

3.5 Species identification by nssm-PCR (Nested Species Specific Multiplex-PCR)

For the differentiation of *Cryptosporidium* species, the primary PCR products from the 18S rRNA nested PCR were amplified using a nested species-specific multiplex PCR (nssm-PCR) (Thomson et al., 2016) which can distinguish the four most commonly detected bovine species of *Cryptosporidium*.

The primary PCR products were diluted with 50 µl dH_2O and 1 µl of the dilution used as template in the secondary round. Secondary PCR reactions contained 2.5 µl 10 × PCR buffer, 0.5 units BioTaq (Bioline, UK), 10 µM of each primer (AL1598, AL3032, CaF, CrF, CphF and CbF) and 1 µl template.

Cycling conditions were 3 min at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at 56°C and 1 min at 72°C. The final extension was 7 min at 72°C. Secondary PCR products (~2 µl) were visualised following electrophoresis on a 1.5% agarose gel stained with GelRed™ (Biotium, UK) on an AlphaImager 2000.

3.6 PCR amplification of the GP60 gene for *C. parvum* subtyping

A nested PCR designed to amplify a 450 bp fragment of the GP60 gene (Brook et al., 2009) was used on a selection of *C. parvum* positive samples to identify their subtypes. Each 25 µl reaction contained 10 × PCR buffer, 0.5 units BioTaq (Bioline, UK), 10 µM of forward and reverse primers (GP60 1F and GP60 1R) and (GP60 2F and GP60 2R) with 3 µl DNA in the primary round and 1 µl primary PCR product in the secondary round. Cycling conditions were 3 min at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at 55°C and 1 min at 72°C. The final extension was 7 min at 72°C. Secondary PCR products (~2 µl) were visualised following electrophoresis on a 1.5% agarose gel stained with GelRed™ (Biotium, UK) on an AlphaImager 2000.

3.7 Sequencing of GP60 positive samples

GP60 positive samples were sequenced in the forward and reverse orientation. DNA sequencing was carried out by GATC Biotech (Köln, Germany). The sequences were analysed using ChromasLite (version 2.01) and the subtypes named as according to (Sulaiman et al., 2005).

3.8 Statistical Analysis

Statistical analyses were carried out using R (R Core Team 2016). Response variables (diarrhoea/not diarrhoea, *C. parvum* positive/negative) were coded as binary data. Generalized linear models (GLM) with a binomial error structure and a logit link function were used. Initially, a maximal model was constructed with all variables and interaction terms were first fitted for the effects examined in each study. These models were reduced by successively dropping out non-significant interaction terms. None of the interaction terms were significant, so additive models were used. To more accurately model the effect of time on the response variables, generalized additive models (GAM) were constructed using a smoothed term for days. The GAM and GLM were compared according to the Akaike information criterion (AIC) and the percentage of deviance explained. In all cases, the GAM provided a lower (and hence better) AIC. For Study-1, when the response variable was *C. parvum* positivity, the factors included in the model were date of birth (DOB), pen and days after birth. In Study-1, when the response variable was diarrhoea, effects were days postpartum and *C. parvum* positivity of the sample. In Study-2 (calves), the factors were DOB and days postpartum. In Study-2 (adults), the only factor was the number of days prepartum. For the calf data in Study-1 and Study-2, to determine the effects of the factors of interest, GAMs were run on full data-sets including all time-points, however, for clarity of presentation, GAM figures are presented for only the first 6 weeks (Study-1) or 7 weeks (Study-2).

4 Results

4.1 *Cryptosporidium* prevalence in calves

In total 384 faecal samples were collected from 25 calves in Study-1; 351 samples from the first 6 weeks, 17 from calves at 3 months of age, 16 from calves at 6 months of age. Three hundred and thirty-one samples were collected in total from 30 calves in Study-2; 315 from the initial collection period and 16 from the same calves at 6 months of age. All 715 samples were screened for the presence of *Cryptosporidium* parasite DNA using the 18S PCR and overall the percentage of positive samples was 64.6% (n=248) in Study-1 and 57.4% (n=190) in Study-2. The age of earliest detection of *Cryptosporidium* parasite DNA was one day old, and the longest interval from birth to onset of

247 shedding was twelve days. Figure 1 shows that by Day-6, the majority of the calves in both studies
248 had begun shedding. The GAM from Study-1 for the effects of date of birth, pen, days postpartum
249 explained 23% of the deviance in the probability of *C. parvum* positivity and is shown in Table 1.
250
251 Neither pen nor date of birth significantly affected the period from birth to the onset of shedding of
252 *Cryptosporidium* ($p = 0.17$ and 0.32 respectively). However, days postpartum had a highly significant
253 effect ($p < 0.0001$). Figure 2 shows the predicted probability of any sample being positive for *C.*
254 *parvum* for the entire study period and below shows the GAM for the first 40 days postpartum. The
255 GAM from Study-2 (Figure 3) is very similar to that from Study-1, explains 24% of the deviance and is
256 shown in Table 2. Date of birth did not significantly affect the probability of shedding on any day ($p =$
257 0.43) but day after birth was highly significant ($p < 0.0001$).
258
259
260

4.2 Prevalence of *Cryptosporidium* in adult cattle

Most samples were collected from adult cows during the four-week period prior to calving. All 209 samples were tested for the presence of *Cryptosporidium* parasite DNA by 18S PCR following oocyst concentration. Overall 27.3% (n=57) of samples tested positive for *Cryptosporidium*. Only samples from four cows did not test positive at any point throughout the study, the remaining 24 (82.2%) cows tested positive on at least one occasion. The GAM for the probability of shedding *C. parvum* is shown in Table 3. The model explained only 5% of the deviance and there was no significant effect of time on the probability of adult cattle shedding *C. parvum* in the 100 days prior to calving (p = 0.104) (Figure 4).

4.3 Speciation of *Cryptosporidium* by nssm-PCR

All *Cryptosporidium* positive samples were speciated using a nssm-PCR (Thomson et al., 2016) and four distinct species were identified (*C. parvum, C. bovis, C. ryanae* and *C. andersoni*).

In Study-1 *C. parvum* was identified in 217 samples, *C. bovis* in 10 samples and *C. ryanae* in 6 samples from calves ≤ 6 weeks. Nine of these were as mixed infections; *C. parvum* and *C. bovis* (n=6) and *C. bovis* and *C. ryanae* (n=3). At three and nine months only *C. ryanae* and *C. bovis* were detected in two samples each at three months and as either single (*C. ryanae* (n=2), *C. bovis* (n=3)) or mixed infections (n=2) at nine months. However, at six months of age, the predominant species detected was *C. parvum,* with 7/10 of the positive samples being identified as this species.

Two calves shed *C. parvum* at 12 months of age, one as a mixed infection with *C. bovis*. *Cryptosporidium andersoni* was not detected at all and for three of the samples it was not possible to determine the species by either nssm-PCR or sequence analysis of the 18S rRNA gene.
In Study-2, only *C. parvum* (n=178) was detected in calves ≤ 6 weeks old while mixed infections of *C. bovis* and *C. parvum* (n=4) and *C. bovis* alone (n=1) were detected in calves at 6 months of age.

All four species were identified in samples from adult cattle from Study-2, *C. parvum* alone was detected in 52 (91.2%) samples, *C. ryanae* (mixed with *C. parvum*) in two samples, *C. bovis* (mixed

with *C. ryanae*) in one sample, *C. andersoni* (mixed with *C. parvum*) in one sample and one sample containing *C. parvum*, *C. ryanae* and *C. bovis* (Figure 5).

In both studies, *C. parvum* was the predominant species detected in faecal samples from calves ≤ 6 weeks old, with seven calves from Study-1 showing evidence of mixed infections. Mixed infections of *C. parvum* and *C. bovis* were detected in calves between three and five weeks of age and *C. bovis* and *C. ryanae* were detected together only in six-week old animals. No mixed infections were detected in calves from Study-2 (Figure 6). *Cryptosporidium parvum* was also the most commonly detected species at 6 months of age in both studies with seven and five calves shedding this species in Study-1 and Study-2 respectively (Figure 6).

4.4 Subtyping of *C. parvum* by GP60 PCR

To determine the *gp60* subtypes, 363 samples that were positive for *C. parvum* from both studies were subjected to a nested PCR to amplify a fragment of the GP60 gene. Three hundred and six of these were positive at the GP60 locus, a subset (n=111) from calves in both studies and 17 positive samples from adult cattle were sent for sequence analysis.

In calves from both studies, two distinct *gp60* subtypes were identified. In all of the samples from the calves ≤ 6 weeks the subtype was IIaA19G2R1 (n=101) and in the samples from the same calves at six months the subtype was IIaA15G2R1 (n=10). Four different *gp60* subtypes were identified in adult cattle; these were IIaA15R1 (n=2), IIaA15G2R1 (n=11), IIaA18G2R1 (n=3) and IIaA19G2R1 (n=1).

4.5 Faecal Consistency

All samples collected from calves in Study-1 were scored for faecal consistency (1= normal, 2= soft, 3= diarrhoeic). In the first 6 weeks of life, 21.4% (n=75) of the samples collected were scored as diarrhoeic. The majority of these (n=69) occurred in the first 3 weeks of life, with a peak in diarrhoeic samples (n=17, 89.5%) at 12-14 days of age. By week 6 none of the samples were scored as diarrhoeic or soft. All of the samples that were collected at 3, 6, 9 or 12 months of age were scored as normal; no soft or diarrhoeic samples were seen. *Cryptosporidium bovis* and *C. ryanae* were not isolated from any soft or diarrhoeic samples, although some samples that were negative for

Cryptosporidium were scored as either diarrhoeic or soft and *C. parvum* was detected in normal faeces. The GLM for diarrhoea indicated a significant effect of day and of *C. parvum* positivity on the probability of diarrhoea (p = 0.00013), but only explained 18% of the deviance in probability. However, the GAM (Table 4), which allows for smoothed, non-linear effects of time, provided a significantly better model, explaining 30% of the deviance and showed a highly significant effect of day on the probability of diarrhoea ($p < 0.0001$) but only a weak, non-significant effect of *C. parvum* positivity (p = 0.11). The use of the GAM thus enabled the confounding effects of day and *C. parvum* positivity to be separated. Faecal scores were not recorded for samples collected from calves or adults from Study-2.

5 Discussion

Though there are many species of *Cryptosporidium* described only four are generally found in cattle, in this study all four of the common cattle adapted species were identified. *Cryptosporidium andersoni* is one of the largest of the *Cryptosporidium* species and usually infects the abomasum of adult cattle, this species does not typically cause diarrhoea but has been associated with reduced milk yield (Anderson, 1987). *Cryptosporidium ryanae* and *C. bovis* are similar in size to *C. parvum* and are smaller than the oocysts of *C. andersoni*, these species, as with *C. parvum* tend to infect the small intestine of susceptible hosts. Only *C. ryanae* has not been reported in humans (Zahedi et al, 2016).

In the UK, and worldwide, cryptosporidiosis is frequently reported as a major cause of neonatal calf diarrhoea (APHA and SRUC, 2014), which may have long-term impacts on the health of infected calves (Gunn and Stott, 1998; Thomson et al., 2017). It is well documented that the zoonotic species *C. parvum* is the most commonly detected species in young calves (Rieux et al., 2013b; Santin et al., 2008; Santin et al., 2004) except in a few reports from Sweden (Silverlas and Blanco-Penedo, 2013) and China (Cai et al., 2017) in which *C. bovis* was identified as the most common species in pre-weaned (≤ 8 weeks old) calves. In this study *C. parvum* was detected in 59.3% (n=395) samples from calves six weeks old or younger, *C. bovis* and *C. ryanae* were also detected in calves of this age but at much lower levels (1.9%, n=13) and only in calves from Study 1. In general, only *C. parvum* is associated with diarrhoea, this finding was confirmed again in this study.

Previous reports suggest an age-related distribution of *Cryptosporidium* species in cattle that *C. parvum* is most frequent in pre-weaned calves, that the most common species in calves older than 2 months are *C. bovis* and *C. ryanae* (Langkjaer et al., 2007; Santin et al., 2008; Santin et al., 2004), and that adult cattle are usually infected with *C. andersoni*. Consistent with this, pre-weaned calves in the present study were infected with *C. parvum*, those of three and nine months of age were infected with *C. bovis* and *C. ryanae* (Study-1), however, at six months of age (Study-1 and Study-2) the calves were mostly infected with *C. parvum*. This inconsistent pattern of infection is very unusual and *C. parvum* in older calves has only been reported in three previous studies (Follet et al., 2011; Huetink et al., 2001; Santin et al., 2004) although in the study by Follet et al (2011) the calves examined were between 31 days and 6 months of age and it is not stated in exactly which age group *C. parvum* was detected. Most other studies examining calves in this age group have reported *C. bovis* and *C. ryanae* (Follet et al., 2011; Langkjaer et al., 2007; Silverlas et al., 2010), occasionally *C. andersoni* (Santin et al., 2004), *C. ubiquitum* (Follet et al., 2011) and *C. suis*-like (Langkjaer et al., 2007).

It is well known that young calves can become infected with, and begin shedding, *C. parvum* very soon after birth (Rieux et al., 2013b; Santin et al., 2008; Silverlas and Blanco-Penedo, 2013; Silverlas et al., 2010). In these studies, the earliest detection of *C. parvum* DNA in faeces was at 1 day of age. At this point it is unlikely that this animal was actively infected with the parasite as the prepatent period of *C. parvum* is 2-7 days (Tzipori et al., 1983) however, it does demonstrate that calves can ingest the parasite very early in life.

The reported prevalence of *Cryptosporidium* in adult cattle varies markedly between studies from 0-80% (Atwill et al., 1998; Atwill and Pereira, 2003; Lorenzo Lorenzo et al., 1993; Silverlas and Blanco-Penedo, 2013; Smith et al., 2014; Wells et al., 2015) but many of these studies did not use the most sensitive techniques for oocyst detection and so prevalence, in some studies, may have been under-reported. Many of the previous studies report that the most common species of *Cryptosporidium* in adult cattle is *C. andersoni* (Fayer et al., 2010; Feng et al., 2007; Ralston et al., 2010; Santin et al., 2008). This species is only very rarely detected in young calves (Kvac et al., 2011; Gong et al., 2017) suggesting that adult cattle are likely not a source of infection for neonatal calves. However, in

addition to the present study, other recent work has shown that adult cattle can shed *C. parvum* (Wells et al., 2015) and that a large percentage of adults may be shedding this species. In the present study, 86.2% (n=25) of the cows tested positive for *C. parvum* and the work by Wells et al (2015) showed that 80.0% (n=24) of adult cattle from four different farms were shedding *Cryptosporidium* with 96.0% of *Cryptosporidium* positive samples being identified as *C. parvum*.

One possible source of infection for calves which has been proposed is transmission at birth from their mother. This theory has been examined previously but most of the studies were carried out prior to the development of molecular typing tools which allow the identification of subtypes of *C. parvum*. Therefore, it is important to revisit adult cattle as a source of infection for neonatal calves. Subtype analysis of *C. parvum* positive samples from calves and adults in this study revealed that, on this farm, there seems to be an age-related separation in subtypes in cattle of different ages. In young calves (≤ 6 weeks of age) the only *gp60* subtype to be identified was IIaA19G2R1 while in samples from the same calves at 6 months of age the only subtype detected was IIaA15G2R1 in both Study-1 and Study-2. Adult cattle shed four subtypes, including both of the subtypes identified in pre-weaned and 6-month-old calves. The most prevalent subtype in the adult cattle was IIaA15G2R1, which is the most frequently reported *gp60* subtype in animals and humans worldwide (Imre and Darabus, 2011), but this subtype was not identified in any calves ≤ 6 weeks of age. The subtype found in calves aged 6 months was only identified in one sample from an adult cow, and is much less frequently reported in cattle than IIaA15G2R1 (Couto et al., 2013; Hijjawi et al., 2016; Rieux et al., 2013a). Subtype IIaA19G2R1 has also been detected in pre-weaned calves on the farm where these studies were carried out for several years and was the only one we identified in young calves, despite the fact that only one adult cow showed evidence of this subtype. It is quite possible that young calves were infected with multiple *gp60* subtypes but that the methods used in the present study were not sensitive enough to detect them. Grinberg et al. (2013) demonstrated that when using next generation sequencing (NGS) methods compared with Sanger sequencing, up to ten distinct *gp60* subtypes were identified in samples in which Sanger sequencing had only identified one. In the present study, two samples were sequenced from five cows each and three of these cows were shedding different *gp60* subtypes at each sample point. This may indicate that many of the other animals in this study could have been shedding multiple subtypes which were simply not detected. It also highlights the

importance of carrying out longitudinal studies rather than point-prevalence studies in order to gain a true and clear picture of the *Cryptosporidium* situation on a farm.

If adult cattle are not the source from which new-born calves become infected (because they mostly shed different genotypes) then other sources of infection, including other calves, require investigation. A study by Smith et al. (2014) showed that wildlife (small rodents and birds) living in and around farm buildings are often infected with *C. parvum*. In their study the subtypes found in some of the wildlife samples were the same as those found in the cattle. It is possible that calves are first infected by environmental contamination or other host species rather than from their dams. Wells et al (2015) also examined some wildlife (deer) and found that they shed the same *gp60* subtypes found in cattle; however, more work is probably required to confirm this.

6 Conclusion

The studies described here confirm that all calves shed *Cryptosporidium* at some point in their life and that younger animals are more susceptible to the parasite, peak shedding occurs at around three weeks of age and the most predominant species at this time is *C. parvum.* The species which cattle are infected with varies with age; and it is possible for calves to become re-infected with, and shed, different *C. parvum* subtypes later in life. In these studies, we have also demonstrated that adult cattle are an unlikely source of infection as only very few dams in these studies shed the *C. parvum* genotype identified in young calves. Although, adult cattle may shed large numbers of oocysts into the environment due to the larger volume of faeces and so cannot be completely ruled out.

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9 Conflict of Interest

None

10 References

Anderson, BC (1987) Abomasal cryptosporidiosis in cattle. *Veterinary Pathology* **24**, 235-238

APHA and SRUC (2014) Veterinary Investigational Diagnosis Analysis (VIDA) report

Atwill, ER and Pereira, MG (2003) Lack of detectable shedding of *Cryptosporidium parvum* oocysts by periparturient dairy cattle. *Journal of Parasitology* **89**, 1234-1236. https://doi.org/10.1645/GE-3192RN

Atwill, ER, Harp, JA, Jones, T, Jardon, PW, Checel, S and Zylstra, M (1998) Evaluation of periparturient dairy cows and contact surfaces as a reservoir of *Cryptosporidium parvum* for calfhood infection. *American Journal of Veterinary Research* **59**, 1116-1121

Brook, EJ, Christley, RM, French, NP and Hart, CA (2008) Detection of *Cryptosporidium* oocysts in fresh and frozen cattle faeces: comparison of three methods*. Letters in Applied Microbiology* **46**, 26-31. https://doi.org/10.1111/j.1472-765X.2007.02257.x

Brook, EJ, Anthony, HC, French, NP and Christley, RM (2009) Molecular epidemiology of *Cryptosporidium* subtypes in cattle in England. *Veterinary Journal* **179**, 378-382. https://doi.org/10.1016/j.tvjl.2007.10.023

Cai, M, Guo, Y, Pan, B, Li, N, Wang, X, Tang, C, Feng, Y and Xiao, L (2017) Longitudinal monitoring of *Cryptosporidium* species in pre-weaned dairy calves on five farms in Shanghai, China. *Veterinary Parasitology* **241**, 14-19. https://doi.org/10.1016/j.vetpar.2017.05.005

Carpenter, C, Fayer, R, Trout, J and Beach, MJ (1999) Chlorine Disinfection of Recreational Water for *Cryptosporidium parvum*. *Emerging Infectious Diseases*. **5**, 579-584. doi.org/10.3201/eid0504.990425

Chalmers, RM and Davies, AP (2010) Minireview: clinical cryptosporidiosis. *Experimental Parasitology* **124**, 138-146. https://doi.org/10.1016/j.exppara.2009.02.003

Chalmers, RM and Giles, M (2010) Zoonotic cryptosporidiosis in the UK – challenges for control. *Journal of Applied Microbiology* **109**, 1487 – 1497. https://doi.org/10.1111/j.1365-2672.2010.04764.x

Chalmers, RM and Katzer, F (2013) Looking for *Cryptosporidium*: the application of advances in detection and diagnosis. *Trends in Parasitology* **29**, 237-251. https://doi.org/10.1016/j.pt.2013.03.001

Chalmers, RM, Smith, RP, Hadfield, SJ, Elwin, K and Giles, M (2011a) Zoonotic linkage and variation in *Cryptosporidium parvum* from patients in the United Kingdom. *Parasitology Research* **108**, 1321. https://doi.org/10.1007/s00436-010-2199-x

Chalmers, RM, Smith, R, Elwin, K, Clifton-Hadley, FA, and Giles, M (2011b) Epidemiology of anthroponotic and zoonotic human cryptosporidiosis in England and Wales, 2004–2006. *Epidemiology and Infection* **139**, 700–712. doi:10.1017/S0950268810001688.

Couto, MC, Lima, MD and Bomfim, TC (2013) New *Cryptosporidium parvum* subtypes of IIa subfamily in dairy calves from Brazil. *Acta Tropica* **130C**, 117-122. https://doi.org/10.1016/j.actatropica.2013.11.002

De Graaf DC, Vanopdenbosch E, Ortega-Mora LM, Abbassi H and Peeters JE (1999) A review of the importance of cryptosporidiosis in farm animals. *International Journal for Parasitology* **29**, 1269-1287.

Esteban, E and Anderson, BC (1995) *Cryptosporidium muris*: prevalence, persistency, and detrimental effect on milk production in a drylot dairy. *Journal of Dairy Science* **78**, 1068-1072. https://doi.org/10.3168/jds.S0022-0302(95)76723-6

Fayer, R, Santin, M and Trout, JM (2008) *Cryptosporidium ryanae* n. sp. (Apicomplexa: Cryptosporidiidae) in cattle (*Bos taurus*). *Veterinary Parasitology* **156**, 191-198. https://doi.org/10.1016/j.vetpar.2008.05.024

Fayer, R, Santin, M and Dargatz, D (2010) Species of *Cryptosporidium* detected in weaned cattle on cow-calf operations in the United States. *Veterinary Parasitology* **170**, 187-192. https://doi.org/10.1016/j.vetpar.2010.02.040

Fayer, R (2010) Taxonomy and species delimitation in *Cryptosporidium*. *Experimental Parasitology* **124**, 90-97. https://doi.org/10.1016/j.exppara.2009.03.005

Feng, Y, Alderisio, KA, Yang, W, Blancero, LA, Kuhne, WG, Nadareski, CA, Reid, M and Xiao, L (2007) *Cryptosporidium* genotypes in wildlife from a New York watershed. *Applied Environmental Microbiology* **73**, 6475-6483. https://doi.org/10.1128/AEM.01034-07

Follet, J, Guyot, K, Leruste, H, Follet-Dumoulin, A, Hammouma-Ghelboun, O, Certad, G, Dei-Cas, E and Halama, P (2011) *Cryptosporidium* infection in a veal calf cohort in France: molecular characterization of species in a longitudinal study. *Veterinary Research* **42**, 116. https://doi.org/10.1186/1297-9716-42-116

Gait, R, Soutar, RH, Hanson, M, Fraser, C and Chalmers, R (2008) Outbreak of cryptosporidiosis among veterinary students. *Veterinary Record* **162**, 843-845.

Grinberg, A, Biggs, PJ, Dukkipati, VS and George, TT (2013) Extensive intra-host genetic diversity uncovered in *Cryptosporidium parvum* using Next Generation Sequencing. *Infection, Genetics and Evolution* **15**, 18-24. https://doi.org/10.1016/j.meegid.2012.08.017

Gunn, GJ and Stott, AW (1998) A comparison of economic losses due to calf enteritis and calf pneumonia in Scottish beef herds. In: *XX World Buiatrics Congress* pp. 357 - 360.

Gong, C, Cao, XF, Deng, L, Li, W, Huang, XM, Lan, JC, Xiao, QC, Zhang, ZJ, Feng, F, Zhang, Y, Wang, WB, Guo, P, Wu, KJ and Peng, GN (2017) Epidemiology of *Cryptosporidium* infection in cattle in China: a review. *Parasite* **24**, 1. doi.org/10.1051/parasite/2017001

Gormley, FJ, Little, CL, Chalmers, RM, Rawal, N and Adak GK (2011) Zoonotic Cryptosporidiosis from Petting Farms, England and Wales, 1992–2009*. Emerging Infectious Diseases* **17**, 151-152. doi:10.3201/eid1701.100902.

Hijjawi, N, Mukbel, R, Yang, R and Ryan, U (2016) Genetic characterization of *Cryptosporidium* in animal and human isolates from Jordan. *Veterinary Parasitology* **228**, 116-120. https://doi.org/10.1016/j.vetpar.2016.08.015

Hotchkiss, EJ, Gilray, JA, Brennan, ML, Christley, RM, Morrison, LJ, Jonsson, NN, Innes, EA, and Katzer, F (2015) Development of a framework for genotyping bovine-derived *Cryptosporidium parvum*, using a multilocus fragment typing tool. *Parasites & vectors* **8**, 500. https://doi.org/10.1186/s13071-015-1107-8

Huetink, RE, van der Giessen, JW, Noordhuizen, JP and Ploeger, HW (2001) Epidemiology of *Cryptosporidium spp*. and *Giardia duodenalis* on a dairy farm. *Veterinary Parasitology* **102**, 53-67. https://doi.org/10.1016/S0304-4017(01)00514-3

Imre, K and Darabus, G (2011) Distribution of *Cryptosporidium* species, genotypes and *C. parvum* subtypes in cattle in European countries. *Scientia Parasitologica* **12**, 1-9.

Jenkins, M, Trout, J, Higgins, J, Dorsch, M, Veal, D and Fayer, R (2002) Comparison of tests for viable and infectious *Cryptosporidium parvum* oocysts. *Parasitology Research* **89**, 1-5. https://doi.org/10.1007/s00436-002-0720-6

519 **Klein, P, Kleinova, T, Volek, Z and Simunek, J** (2008) Effect of *Cryptosporidium parvum* infection
520 on the absorptive capacity and paracellular permeability of the small intestine in neonatal calves.
521 *Veterinary Parasitology* **152**, 53-59. https://doi.org/10.1016/j.vetpar.2007.11.020
522 **Kvac, M, Hromadova, N, Kvetonova, D, Rost, M and Sak, B** (2011) Molecular characterization of
523 *Cryptosporidium* spp. in pre-weaned dairy calves in the Czech Republic: absence of *C. ryanae* and
524 management-associated distribution of *C. andersoni*, *C. bovis* and *C. parvum* subtypes. *Veterinary*
525 *Parasitology* **177**, 378-382. https://doi.org/10.1016/j.vetpar.2010.11.048
526 **Langkjaer, RB, Vigre, H, Enemark, HL and Maddox-Hyttel, C** (2007) Molecular and phylogenetic
527 characterization of *Cryptosporidium* and *Giardia* from pigs and cattle in Denmark. *Parasitology* **134**,
528 339-350. https://doi.org/10.1017/S0031182006001533
529 **Lorenzo Lorenzo, MJ, Ares-Mazas, E and Villacorta Martinez, dM, I** (1993) Detection of oocysts
530 and IgG antibodies to *Cryptosporidium parvum* in asymptomatic adult cattle. *Veterinary Parasitology*
531 **47**, 9-15. https://doi.org/10.1016/0304-4017(93)90171-I
532 **Nydam, DV, Wade, SE, Schaaf, SL and Mohammed, HO** (2001) Number of *Cryptosporidium*
533 *parvum* oocysts or *Giardia* spp cysts shed by dairy calves after natural infection. *American Journal of*
534 *Veterinary Research* **62**, 1612-1615
535 **Ralston, B, Thompson, RC, Pethick, D, McAllister, TA and Olson, ME** (2010) *Cryptosporidium*
536 *andersoni* in Western Australian feedlot cattle. *Australian Veterinary Journal* **88**, 458-460.
537 **Rieux, A, Chartier, C, Pors, I, Delafosse, A and Paraud, C** (2013a) Molecular characterization of
538 *Cryptosporidium* isolates from high-excreting young dairy calves in dairy cattle herds in Western
539 France. *Parasitology Research* **112**, 3423-3431. https://doi.org/10.1007/s00436-013-3520-2
540 **Rieux, A, Paraud, C, Pors, I and Chartier, C** (2013b) Molecular characterization of *Cryptosporidium*
541 isolates from pre-weaned calves in western France in relation to age. *Veterinary Parasitology* **197**, 7-
542 12. https://doi.org/10.1016/j.vetpar.2013.05.001
543 **Robertson, LJ, Campbell, A and Smith, HV** (1992) Survival of *Cryptosporidium parvum* oocysts
544 under various environmental pressures. *Applied Environmental Microbiology* **58**, 3494-500.
545 **Santin, M, Trout, JM, Xiao, L, Zhou, L, Greiner, E and Fayer, R** (2004) Prevalence and age-related
546 variation of *Cryptosporidium* species and genotypes in dairy calves. *Veterinary Parasitology* **122**, 103-
547 117. https://doi.org/10.1016/j.vetpar.2004.03.020

548 **Santin, M, Trout, JM and Fayer, R** (2008) A longitudinal study of cryptosporidiosis in dairy cattle
549 from birth to 2 years of age. *Veterinary Parasitology* **155**, 15-23.
550 https://doi.org/10.1016/j.vetpar.2008.04.018
551 **Silverlas, C and Blanco-Penedo, I** (2013) *Cryptosporidium spp*. in calves and cows from organic
552 and conventional dairy herds. *Epidemiology and Infection* **141**, 529-539.
553 https://doi.org/10.1017/S0950268812000830
554 **Silverlas, C, Naslund, K, Bjorkman, C and Mattsson, JG** (2010) Molecular characterisation of
555 *Cryptosporidium* isolates from Swedish dairy cattle in relation to age, diarrhoea and region. *Veterinary*
556 *Parasitology* **169**, 289-295. https://doi.org/10.1016/j.vetpar.2010.01.003
557 **Smith, RP, Clifton-Hadley, FA, Cheney, T and Giles, M** (2014) Prevalence and molecular typing of
558 *Cryptosporidium* in dairy cattle in England and Wales and examination of potential on-farm
559 transmission routes. *Veterinary Parasitology* **204**, 111-119.
560 https://doi.org/10.1016/j.vetpar.2014.05.022
561 **Sulaiman, IM, Hira, PR, Zhou, L, Al-Ali, FM, Al-Shelahi, FA, Shweiki, HM, Iqbal, J, Khalid, N and**
562 **Xiao, L** (2005) Unique endemicity of cryptosporidiosis in children in Kuwait. *Journal of Clinical*
563 *Microbiology* **43**, 2805-2809. https://doi.org/10.1128/JCM.43.6.2805-2809.2005
564 **Sweeny, JPA, Ryan, UM, Robertson, ID, Yang, R, Bell K and Jacobson C** (2011a) Longitudinal
565 investigation of protozoan parasites in meat lamb farms in southern Western Australia. *Preventive*
566 *Veterinary Medicine* **101**, 192-203. https://doi.org/10.1016/j.prevetmed.2011.05.016
567 **Sweeny, JPA, Ryan, UM, Robertson, ID and Jacobson C** (2011b) *Cryptosporidium* and *Giardia*
568 associated with reduced lamb carcase productivity. *Veterinary Parasitology* **182**, 127-139.
569 https://doi.org/10.1016/j.vetpar.2011.05.050
570 **Thomson, S, Innes, EA, Jonsson, NN and Katzer, F** (2016) A multiplex PCR test to identify four
571 common cattle-adapted *Cryptosporidium* species. *Parasitology Open* **2**, 9.
572 https://doi.org/10.1017/pao.2016.2
573 **Thomson, S, Hamilton, CA, Hope, JC, Katzer, F, Mabbott, NA, Morrison, LJ and Innes, EA**
574 (2017) Bovine cryptosporidiosis: impact, host-parasite interaction and control strategies. *Veterinary*
575 *Research* **48**, 42. https://doi.org/10.1186/s13567-017-0447-0
576 **Tzipori, S and Ward, H** (2002) Cryptosporidiosis: biology, pathogenesis and disease. *Microbes and*
577 *Infection* **4**, 1047-1058.

Tzipori, S, Smith, M, Halpin, C, Angus, KW, Sherwood, D and Campbell, I (1983) Experimental cryptosporidiosis in calves: clinical manifestations and pathological findings. *Veterinary Record* **112**, 116-120.

Weir, SC, Pokorny, NJ, Carreno, RA, Trevors, JT and Lee H (2002) Efficacy of common laboratory disinfectants on the infectivity of *Cryptosporidium parvum* oocysts in cell culture. *Applied Environmental Microbiology* **68**, 2576-9.

Wells, B, Thomson, S, Ensor, H, Innes, EA and Katzer, F (2016) Development of a sensitive method to extract and detect low numbers of *Cryptosporidium* oocysts from adult cattle faecal samples. *Veterinary Parasitology* **227**, 26-29. https://doi.org/10.1016/j.vetpar.2016.07.018

Wells, B, Shaw, H, Hotchkiss, E, Gilray, J, Ayton, R, Green, J, Katzer, F, Wells, A and Innes, E (2015) Prevalence, species identification and genotyping *Cryptosporidium* from livestock and deer in a catchment in the Cairngorms with a history of a contaminated public water supply. Parasites and Vectors **8**, 66. https://dx.doi.org/10.1186%2Fs13071-015-0684-x

Xiao, L, Escalante, L, Yang, C, Sulaiman, I, Escalante, AA, Montali, RJ, Fayer, R and Lal, AA (1999) Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene locus. *Applied and environmental microbiology* **65**, 1578-1583.

Zahedi, A, Paparini, A, Jian, F, Robertson, I, Ryan, U (2016) Public health significance of zoonotic *Cryptosporidium* species in wildlife: Critical insights into better drinking water management. International Journal for Parasitology: Parasites and Wildlife **5,** 88-109 https://dx.doi.org/10.1016%2Fj.ijppaw.2015.12.001

599 **Table 1: Primer sequences and size of amplicon when used with primer pair for the 18S rRNA gene.**

Primer	Sequence 5'-3'	Fragment Size (bp)
AL1687 (EF)	TTCTAGAGCTAATACATGCG	1370
AL1691 (ER)	CCCATTTCTTCGAAACAGGA	
AL1598 (IF)	GGAAGGGTTGTATTTATTAGATAAAG	840
AL3032 (IR)	AAGGAGTAAGGAACAACCTCCA	

600

601

602 **Table 2: Primer sequences and size of amplicon for nssm-PCR.**

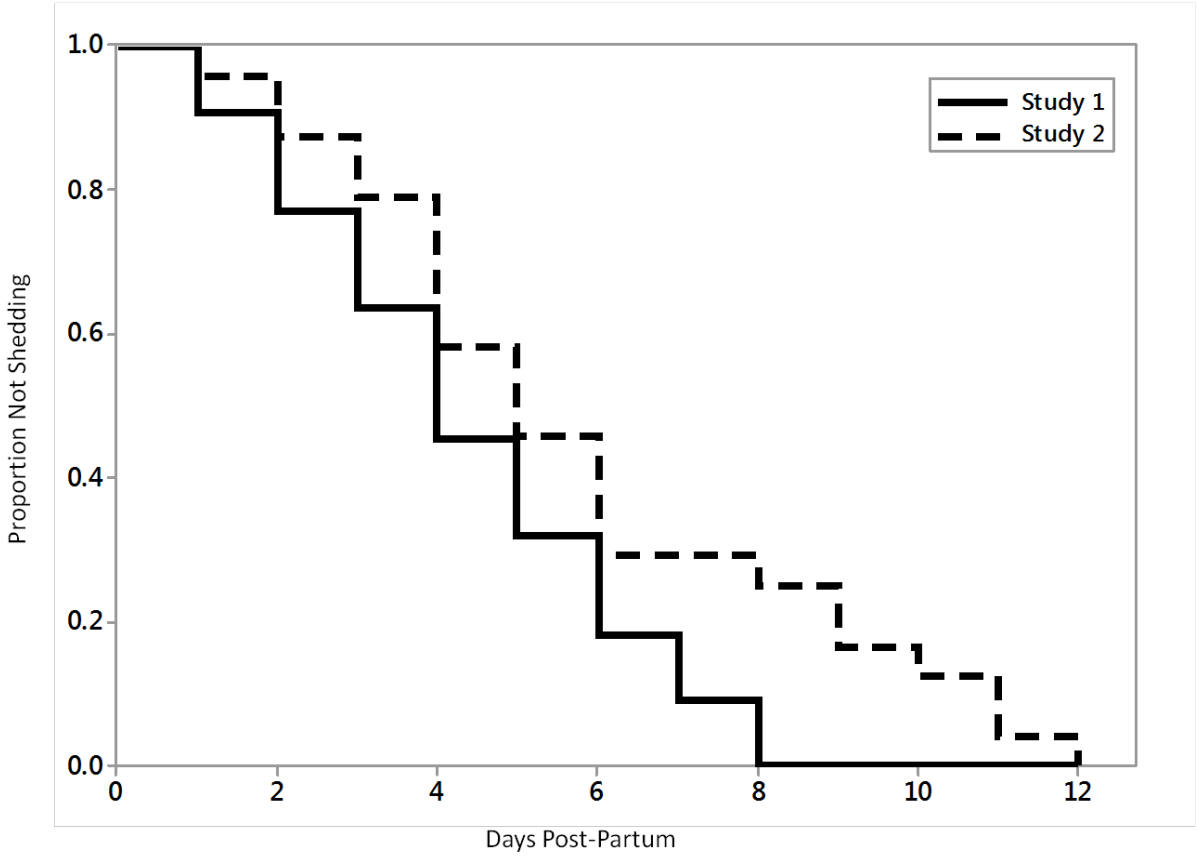
603 The *C. ryanae* specific primer contains a degenerate base which is shown in bold and underlined. The amplicon

604 size is shown when used the species specific forward primer is used with the internal reverse primer (AL3032).

Primer	Sequence 5'-3'	Fragment Size (bp)
AL1598 (IF)	GGAAGGGTTGTATTTATTAGATAAAG	840
AL3032 (IR)	AAGGAGTAAGGAACAACCTCCA	
CaF	GCAAATTACCCAATCCTGAC	625
CrF	TGTTAATTTTTATATACAAT**R**CTACGG	415
CphF	AGAGTGCTTAAAGCAGGCATA	241
CbF	CTTCTTATTCCTTCTAGAATAAAAATG	305

605

606



607

608 Fig 1

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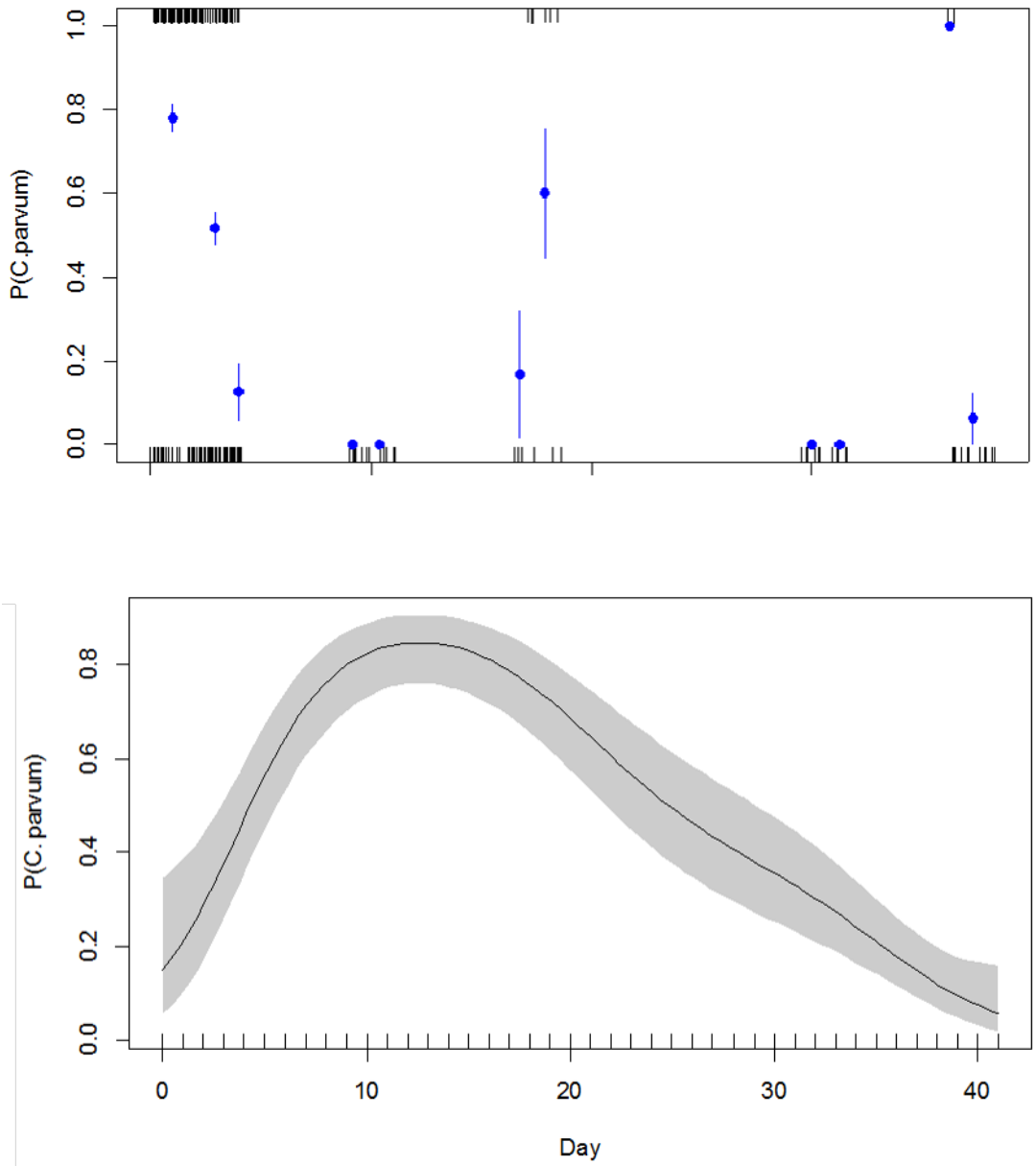
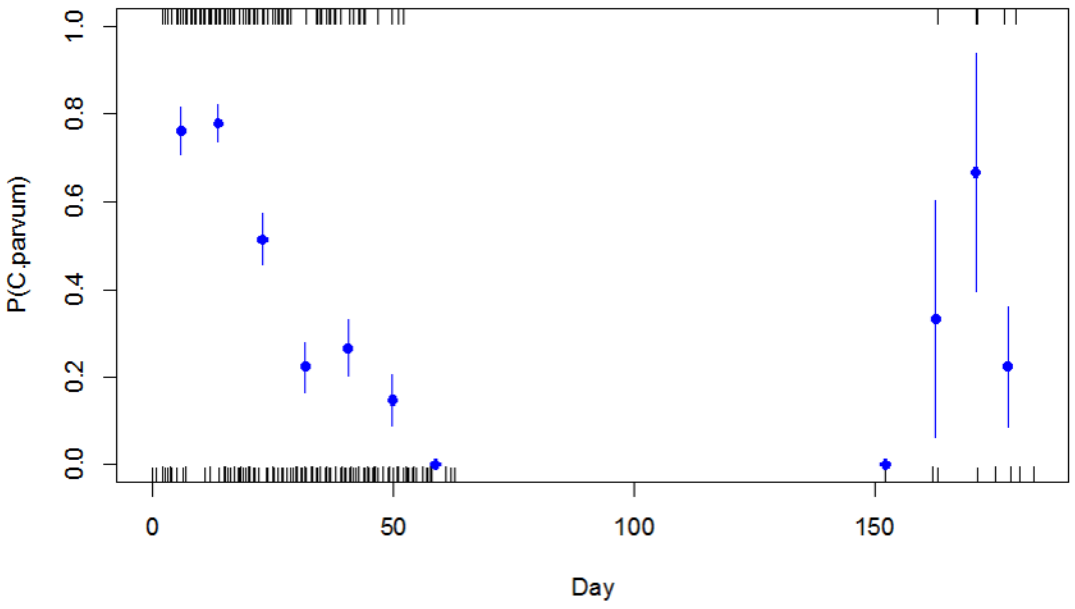
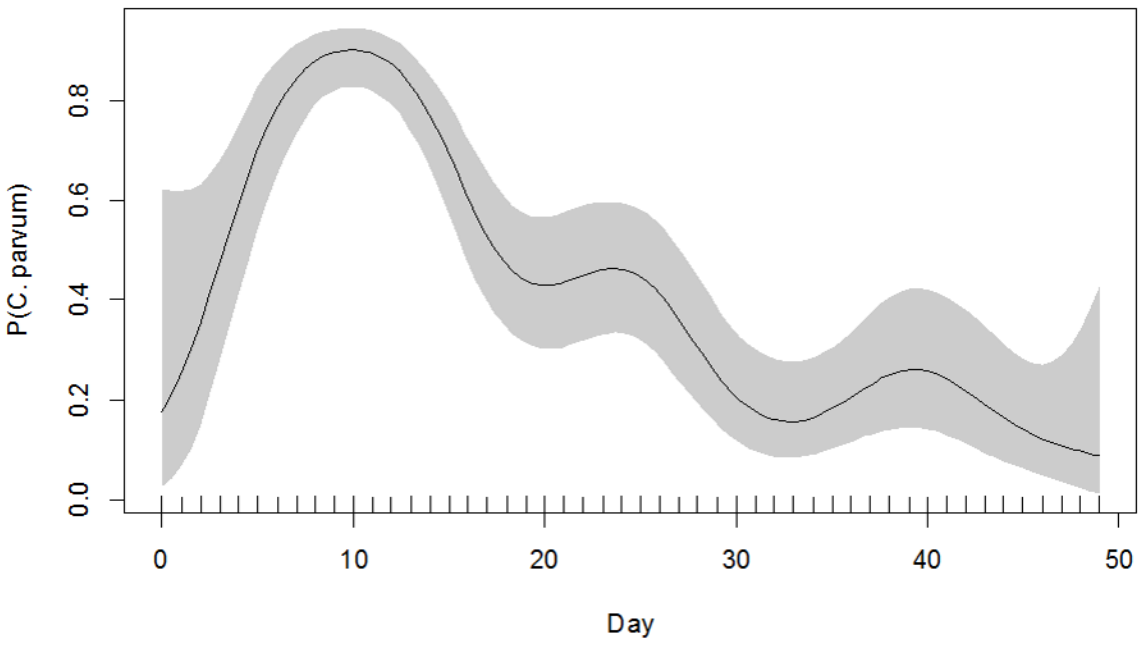


611

612 Fig 2

613





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615 Fig 3

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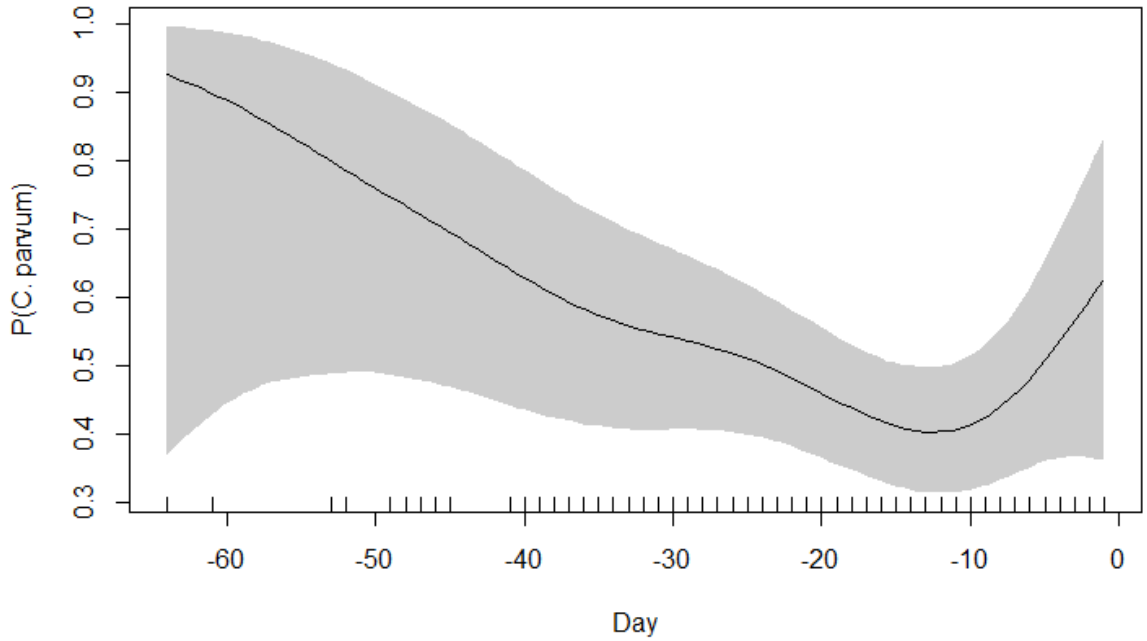

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619 Fig 4

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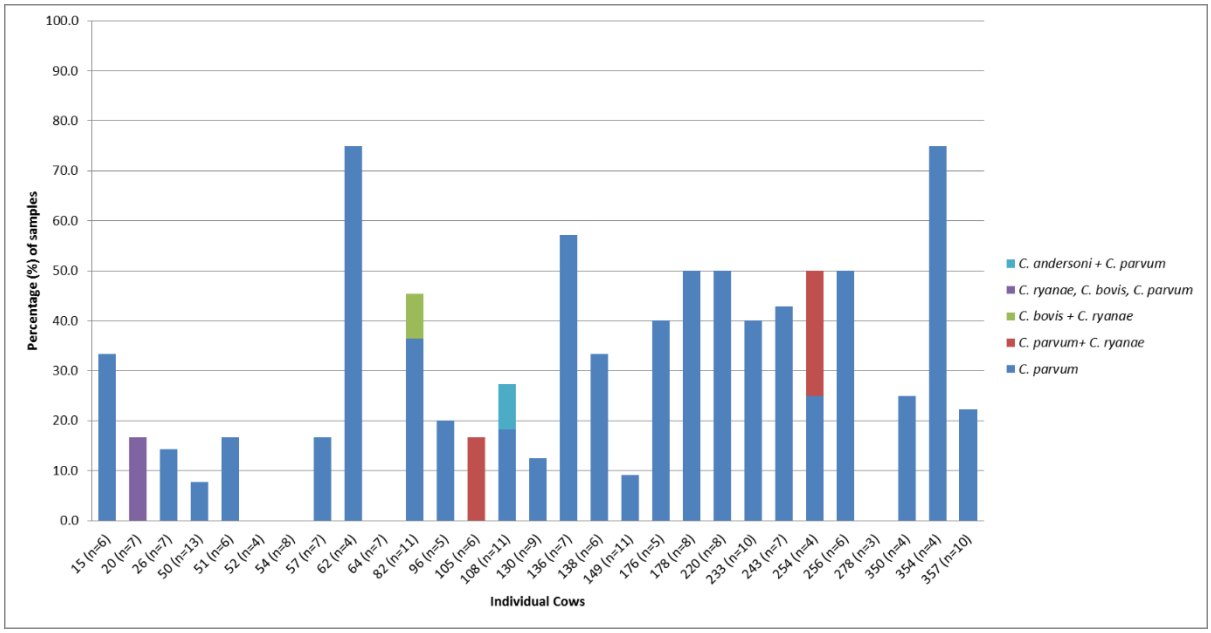

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623 Fig 5

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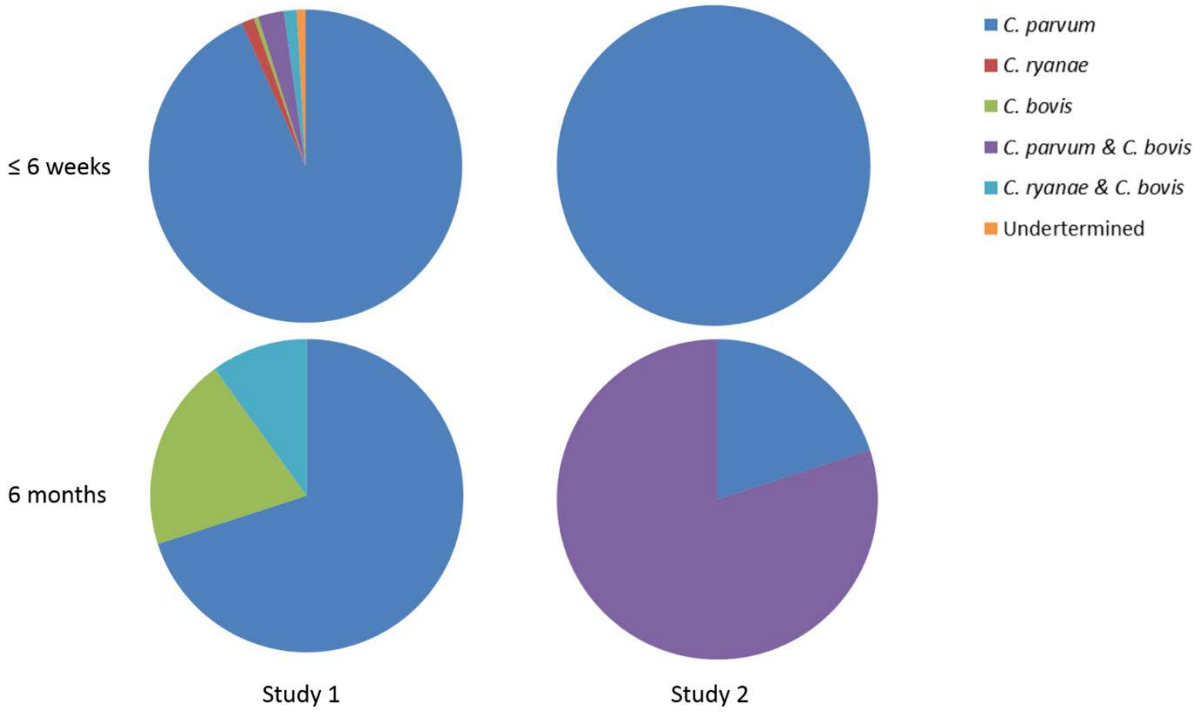

625
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627 Fig 6

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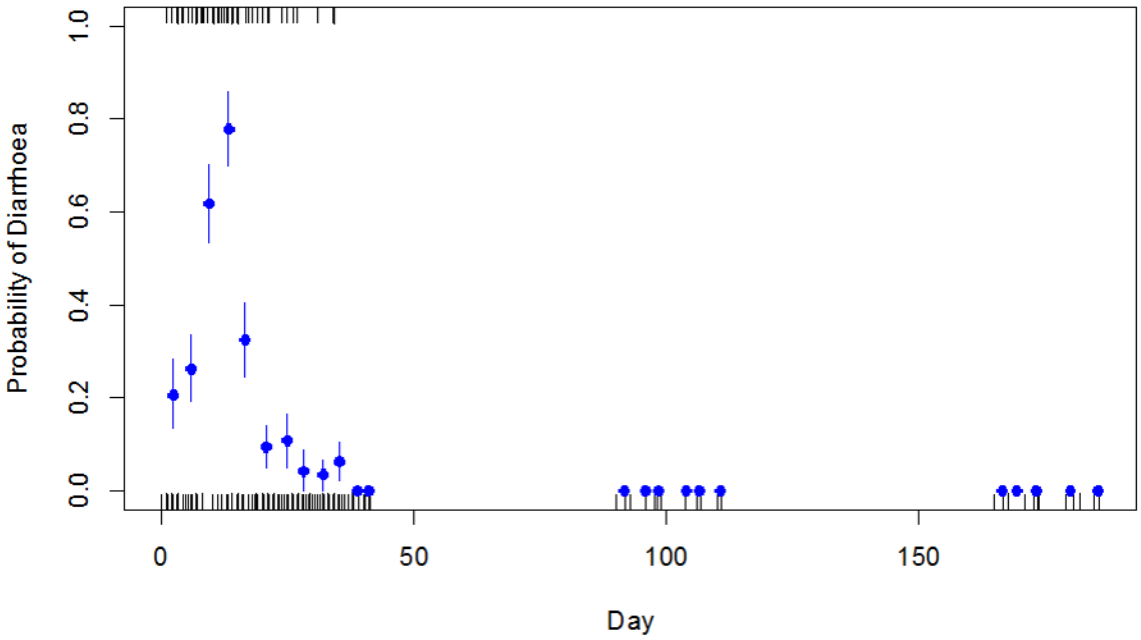
Probability of Diarrhoea
0.0
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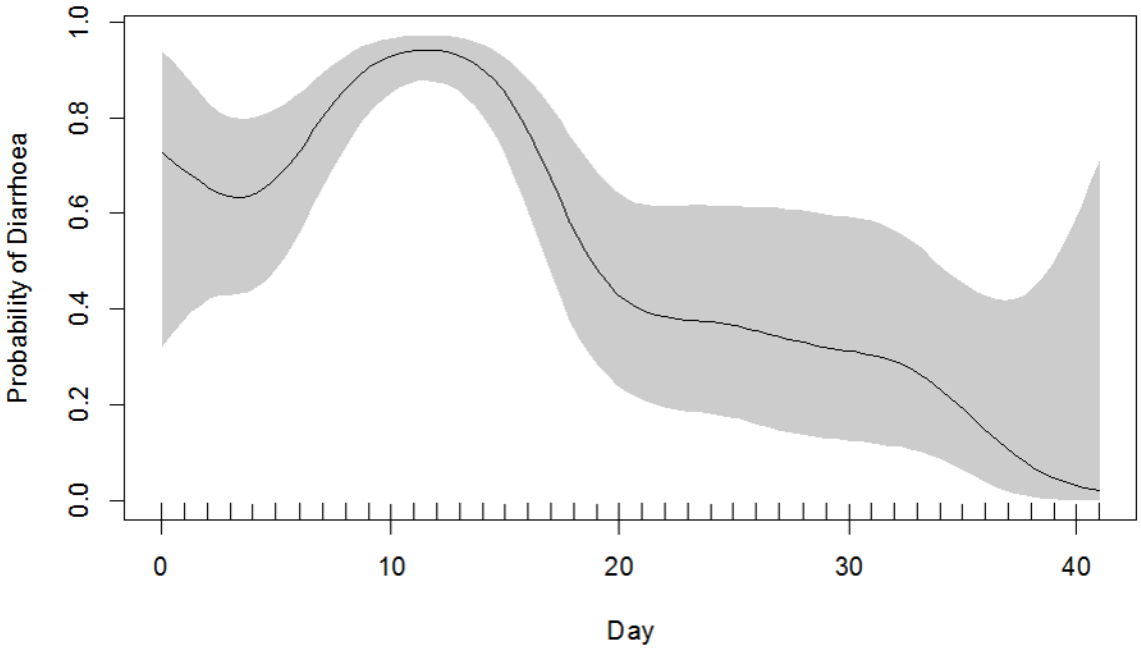
0.4
0.6
0.8
1.0
0
50
100
150
Day


Probability of Diarrhoea
0.0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
Day

629

630 Fig 7