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Genetic diversity and shedding profiles for *Cryptosporidium parvum* in adult cattle and their calves

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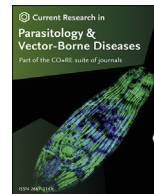
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Current Research in Parasitology & Vector-Borne Diseases

journal homepage: www.editorialmanager.com/crpvbd/default.aspxGenetic diversity and shedding profiles for *Cryptosporidium parvum* in adult cattle and their calvesHannah Jade Shaw^{a,b,*}, Claire Armstrong^a, Kirsty Uttley^a, Liam J. Morrison^c, Elisabeth A. Innes^a, Frank Katzer^{a,**}^a Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, EH26 0PZ, UK^b Harper Adams University, Edgmond, Newport, TF10 8NB, UK^c Roslin Institute, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Midlothian, EH25 9RG, UK

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ABSTRACT

Cryptosporidiosis is an important disease in neonatal calves, causing watery diarrhoea, loss of appetite, and production losses. Dehydration from diarrhoea often results in the calf requiring rehydration or veterinary treatment to prevent calf mortality. Transmission of *Cryptosporidium* to calves still has some major knowledge gaps, such as the initial source of oocysts ingested by calves and how these oocysts can persist between calving periods. Some studies have examined the role of adult cattle in the transmission of *Cryptosporidium* oocysts, although these have yielded inconclusive results. In this study, highly sensitive oocyst extraction from faeces and detection techniques, sensitive to 5 oocysts per gram using a 50 g sample, were used to genotype faecal samples from adult cattle and their calves to determine if adult cattle could be a source of *Cryptosporidium* infection for their calves. On a dairy farm, faecal samples from adult cattle were collected twice per week for 0–3 weeks before calving and from their calves three times per week until they reached 3 weeks of age followed by twice per week until they reached 6 weeks of age. On a beef farm, samples were collected from both adults and calves at a single time point. Faecal samples were examined to compare species and multilocus genotypes of *Cryptosporidium parvum*. Results show that *C. parvum* was the most prevalent species on both the dairy and beef farms. The calves within each herd appear to have one predominant single multilocus genotype, whereas adult cattle have multiple distinct genotypes. Adult cattle on the dairy farm, tested before calving, in the majority of cases had a multilocus genotype that is different from that detected in their calves. On the beef farm, where samples were taken at the same time, the majority of adult cattle matched the multilocus genotype of their calves. This study shows that adult cattle display a higher diversity of *C. parvum* genotypes on both farms compared to the calves. The data also represent a detailed longitudinal prevalence study of the shedding profiles and genotype of *Cryptosporidium* parasites detected in dairy calves from birth to 6 weeks of age.

1. Introduction

Cryptosporidiosis is caused by the protozoan parasites of the genus *Cryptosporidium* and is an important disease on many farms worldwide (Ryan et al., 2016). Despite there being over 44 recognised species to date (Holubová et al., 2020), *Cryptosporidium parvum* is the only species that is known to cause clinical disease in naturally infected calves (Thomson et al., 2017) although some evidence exists detailing reduced milk yield in adult cattle shedding *Cryptosporidium andersoni* (see Esteban & Anderson, 1995; Anderson, 1998). The symptoms of

cryptosporidiosis include watery diarrhoea, loss of appetite, depression, and dehydration, which can become severe enough to cause calf mortality (Thomson et al., 2017). Oocyst shedding starts around 2–6 days of age and lasts between 7 and 15 days (Tzipori et al., 1983; Uga et al., 2000). During this time, each infected calf will shed millions of oocysts, ranging from 3.89×10^{10} in a 6–12 day-old calf to 3.8×10^7 in a 50–56 day-old calf (Nydham et al., 2001). This results in huge environmental contamination and a risk to other young calves vulnerable to transmission and infection from these oocysts (Nydham et al., 2001).

* Corresponding author. Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, EH26 0PZ, UK

** Corresponding author.

E-mail addresses: hshaw@harper-adams.ac.uk (H.J. Shaw), frank.katzer@moredun.ac.uk (F. Katzer).<https://doi.org/10.1016/j.crpvbd.2021.100027>

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Cryptosporidiosis has been reported as being one of the leading causes of neonatal calf diarrhoea worldwide (Thomson et al., 2017). Young calves between 7 and 14 days post-infection have a significant reduction in growth rate (Klein et al., 2008), and this reduction is maintained over six months (Shaw et al., 2020). It has been shown that severe clinical cryptosporidiosis early in life results in an average difference in weight gain of 34 kg at six months of age, potentially costing 130 GBP per infected calf (Shaw et al., 2020). Cryptosporidiosis is difficult to tackle on-farm as the parasite oocyst possesses a strong outer shell, rendering it resistant to many of the common disinfectants used on farms. It requires a very low dose of oocysts to infect and cause disease in neonatal livestock (de Graaf et al., 1999; Zambriski et al., 2013).

Cryptosporidium is transmitted via the faecal-oral route, and the potential of adult cattle as a source of oocysts to infect naïve calves is disputed in the literature. Young calves tend to show clinical signs of infection in the second week of life (Faubert & Litvinsky, 2000) and so this would suggest that infection occurs very soon after birth, due to the 5–7 day incubation period of the parasite (Tzipori et al., 1983). Prevalence of *C. parvum* in adult cattle ranges from 0 to 86.2% (Lorenzo et al., 1993; Atwill & Pereira, 2003; Wells et al., 2015; Thomson et al., 2019) but different detection methods were used, with only the two most recent studies using a very sensitive method developed to detect *Cryptosporidium* oocysts in 50 g of faeces (Wells, et al., 2015; Thomson, et al., 2019). The consensus from the older papers is that adult cattle do not play a significant role in transmission to other cattle or environmental contamination due to the low number of *C. parvum*-positive samples obtained. However, these studies did not use the most sensitive techniques for oocyst detection and so prevalence may have been under-reported. Not only this but it has been documented that as few as 17 oocysts are required to infect a calf (Zambriski et al., 2013) and so even excretion in low concentrations could pose a risk, especially as adult cattle produce much larger faecal pats compared to the calves and therefore the total number of oocysts shed into the environment by adult cattle should be considered when looking at sources of transmission.

The main issue with previous studies is the difficulty in analysing faecal samples from adult cattle for *Cryptosporidium* oocysts. The larger sample volume, reduced oocyst number, and fibrous nature of the faeces makes laboratory detection from adult faecal samples difficult (Wells et al., 2016). A recent report described a highly sensitive technique for detecting *Cryptosporidium* oocysts, using 50 g of faeces in an acid flocculation and salt flotation technique, which effectively deals with extraction from adult faeces. This method has a sensitivity of 5 oocysts per gram and allows for 50 g to be analysed rather than the 2–4 g used in previous techniques (Wells et al., 2016). Higher prevalences of *C. parvum* in adult cattle were found in the studies using this technique compared to those which used alternative techniques (Lorenzo et al., 1993; Atwill & Pereira, 2003). Two studies conducted using this sensitive extraction technique found that the predominant species in adult cattle was *C. parvum* (Wells et al., 2015; Thomson et al., 2019), and not *C. andersoni* as reported by older studies using previous detection methods. Previous work described in all of the aforementioned studies either lacked direct pairings between the dam and calf or did not genotype at multiple loci.

This study aims to examine multilocus genotypes of both adult cattle and their calves to further explore the genetic diversity of *C. parvum*. This information will be vital in understanding potential sources of infection for calves. At present, very few studies describe the shedding profile of individual calves on a longitudinal basis and only one of these has been conducted in Scotland (Thomson et al., 2019). This information is valuable when it comes to controlling this parasite on the farm. This study provides detailed information on the species and genotypes of *Cryptosporidium* parasites shed over six weeks in calves from birth on a Scottish dairy farm.

2. Materials and methods

2.1. Sample collection

In Study 1 (Dairy farm), adult cattle faecal samples were mixed and 125 ml collected using a plastic collection pot following observation of defecation. To reduce environmental contamination, only the freshly deposited faeces were collected, avoiding any straw bedding. Samples were collected from 79 adult Holstein cattle twice a week between 0 and 3 weeks before calving. These were all the adult cattle that were due to calf during the study period. Due to the nature of this collection method, it was not always possible to collect a sample from each individual at every time point. Samples were stored at 4 °C until processed.

Once the adults had given birth, female calves were removed from their mothers within 12 h, placed in individual pens, and fed from pooled colostrum. Male calves were not kept on-farm and so could not be included in the study. Faecal samples were collected from the female calves three times per week ($n = 38$), for 3 weeks, followed by twice per week for weeks 4–6. The entire motion was collected following observation of each individual, inside a plastic bag. It was not always possible to gain a sample from all calves at every sampling point.

In Study 2 (Beef farm), adult cattle faecal samples were mixed and 125 ml collected using a plastic collection pot following observation of defecation. These samples were collected at a single time point from all adult Belgian Blue female cattle ($n = 27$) and 23 of their Belgian Blue × Limousin, mixed-gender calves, which were all housed together and allowed to suckle colostrum and milk from their mothers. These calves ages ranged from 0 to 4 weeks. The entire motion was collected following observation of each individual, inside a plastic bag, avoiding bedding material. Five of the calves died during the study. All were positive for cryptosporidiosis although other factors were involved. Therefore not every adult has a matched calf sample for comparison.

2.2. Sample processing

All faecal samples from adult cattle were processed according to the protocol described in Wells et al. (2016), which had a detection limit of 5 oocysts per gram of faeces. This included using 50 g of starting material in an acid flocculation, followed by a salt flotation on the pellet. Faecal samples from calves were thoroughly mixed before 3 g of faeces was removed and added to 1 ml TE buffer (10 mM Tris-HCl, 0.5 mM EDTA). Both adult and calf samples underwent 10× freeze/thaw cycles in liquid nitrogen before DNA was extracted using a modified Macherey Nagal tissue kit protocol (NZ740952250, Macherey-Nagel, Duren, Germany), which is described in Wells et al. (2016).

2.3. 18S nsm PCR

The species of *Cryptosporidium* present was determined using a multiplex nested species-specific PCR, which amplified the 18S region (Thomson et al., 2019) and allowed the identification of the common cattle species *C. parvum*, *Cryptosporidium bovis*, *Cryptosporidium ryanae*, and *C. andersoni*. Modifications to this PCR protocol were applied during the second round of PCR, where first-round PCR products were not diluted as a template for the second round PCR when testing the adult cattle samples. Calf DNA samples underwent the PCR as previously described (Thomson et al., 2019). All DNA samples were run in triplicate with a negative control, a DNA extraction control, and a positive control. 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 were visualized following electrophoresis on a 1.5% agarose gel stained with GelRed™ (Biotium) on an AlphaImager 2000. This multiplex PCR method was tested against known positive samples for the four common cattle species during its development (Thomson et al., 2019). A proportion of positive samples from this study

was also confirmed *via* sequencing by submitting positive samples to MWG Eurofins.

2.4. *gp60* PCR

The 450 bp fragment of the *gp60* gene was amplified from all *C. parvum*-positive samples using a previously described nested PCR (Brook et al., 2009) and sequenced by GATC Biotech (Köln, Germany). Genotype for the *gp60* was assigned according to Sulaiman et al. (2005).

2.5. Multi-locus genotyping

Four microsatellite markers (MM5, MM18, MM19 and TP14) described previously (Mallon et al., 2003; Morrison et al., 2008), were used to determine amplicon length for each allele at each locus and therefore assign a multilocus genotype (MLG) across the four markers to each sample. Amplicon lengths and locus assignments are shown in Table 1. This reaction included 10× PCR buffer (45 mM Tris-HCl pH 8.8, 11 mM (NH₄)₂SO₄, 4.5 mM MgCl₂, 4.4 μM EDTA, 113 μg/ml BSA, 1 mM each of the four deoxyribonucleotide triphosphates) (Burrells et al., 2013), 0.5 units of Bionline BioTaq, and 10 μM of forward and reverse primers. The PCR protocol was modified to the one described by removing the 1:100 dilution of the primary product for adult cattle samples. A positive control for each fragment size of all four loci was included in the PCR, as was a negative (no template) control.

PCR products underwent fragment analysis (Applied Biosystems; University of Dundee, UK) using Genescan ROX500 and the results were analysed using STRand (<https://www.vgl.ucdavis.edu/informatics/strand.php>). MLG visualisation was achieved using Phyloviz (<https://www.phyloviz.net>) in which the allele sizes for loci *gp60*, MM5, MM18, MM19, and TP14 were inputted.

3. Results

3.1. Species present in adult cattle in Study 1

Adult dairy cattle in Study 1 had a *C. parvum* prevalence of 67.1% (±10.4%). This includes cattle shedding *C. parvum* only (*n* = 26) and *C. parvum* as a mixed infection with other species (*n* = 27). Twenty-six of these mixed infections were with *C. andersoni* and one with *C. ryanae*. The only other species detected in the dairy cattle as a single infection was *C. andersoni* (8.9% (±6.3%)). The rest of the cattle, 24.1% (±9.5%) were negative for the four most common species of *Cryptosporidium* found in cattle (Fig. 1).

3.2. Species present in adult cattle in Study 2

Of the 27 samples collected from adult beef cattle, 59.3% (±18.6%) contained *C. parvum*. One of these was a mixed infection with *C. andersoni*, *C. bovis*, and *C. ryanae*. The only other species detected in adult beef cattle as a single species was *C. andersoni*, detectable in 7.4% (±7.4%) of cattle. No detectable *Cryptosporidium* species were present in 33.3% (±17.7%) of cattle (Fig. 1).

Table 1

Amplicon length (bp) for each of the MLG alleles described

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

3.3. Species present in calves in Study 1

Female calves on the dairy farm (*n* = 38) were tested for *Cryptosporidium* species from birth until they reached 6 weeks of age (Fig. 2). A positive *C. parvum* result from any of the samples that week was counted as *C. parvum*-positive. If multiple species were found during that week, either in a single sample or between samples within a week, it was counted as a mixed infection.

In total, there were 15 sampling points for each calf which totalled 400 faecal samples. The average number of samples obtained was 10.4 per calf but this ranged between 6 and 15 samples. Of these 400 samples, 180 were positive for *C. parvum* giving a sample prevalence of 45% (±4.9%). For individual calves, the prevalence of *C. parvum* in all collected faecal samples ranged from 8.3 to 90.9%. The lowest *C. parvum* prevalence was observed in the final sampling week at 8.3% and the highest observed at sampling timepoint three which occurred in the first week of life at 68.2%.

Results also detail longitudinal species prevalence data for the 38 individual calves, which are shown in Fig. 2. Of the 38 calves, 25 showed intermittent shedding of *C. parvum* oocysts. In the majority of these cases, the gap was only observed in a single sample (*n* = 15) but one calf had four gaps in *C. parvum* shedding. Two calves had four negative samples between *C. parvum*-positive samples. No calves remained negative for the full 6-week period, with every calf testing positive for *C. parvum* at some point over the sampling period (Fig. 2). Calves tended to be positive for *C. parvum* in the first two weeks of life, although *C. parvum* was detected at all sampling time points, whereas *C. bovis* and *C. ryanae* tended to be detected in weeks 3–6. In two instances (calves 9 and 17), *C. parvum* was only detected once early in infection and no *Cryptosporidium* was detected thereafter. This could suggest that infection in these calves was effectively controlled. While the overall pattern also indicates control of *C. parvum*, several calves (e.g. calf 16, 18 and 26) were shedding *Cryptosporidium* from week 1 to week 6. While a few calves were positive at all sampling time points during a week (e.g. calves 30–33 in week 2), in the majority of cases there was variable detection of parasites in samples taken during a week and between weeks, indicating variable and intermittent shedding of oocysts, for all three *Cryptosporidium* species. For most calves, a parasite species detected once was also detected on a further occasion, indicating infection establishment and persistence and providing reassurance that the experimental setup was robust.

Cryptosporidium species present in dairy calves from birth over a 6-week period are summarised in Fig. 3. In week 1, 73% (±14.3%) were positive for *C. parvum*, with the remainder having no detectable *Cryptosporidium*. In week 2, 62.9% (±16%) were positive for *C. parvum* with two calves having a *C. parvum* + *C. ryanae* mixed infection. In week 3, 61% (±15.5%) were positive for *C. parvum* with two calves showing a *C. parvum* + *C. bovis* mixed infection and three showing a *C. parvum* + *C. ryanae* mixed infection. One calf was positive for *C. bovis* only. In week 4, 27.8% (±14.6%) were positive for *C. parvum*. One calf was positive for *C. bovis* and three calves for *C. ryanae*. One calf was positive for *C. ryanae* + *C. bovis* as a mixed infection, four calves had a *C. parvum* + *C. bovis* mixed infection, and one a *C. parvum* + *C. ryanae* mixed infection. At 5 weeks, 42.1% (±15.7%) were positive for *C. parvum*, one for *C. bovis*, and two calves for *C. ryanae*. Four calves had a *C. parvum* and *C. bovis* mixed infection and two calves a *C. parvum* + *C. ryanae* mixed infection. In week 6, 18.4% (±12.3%) were positive for *C. parvum*, two for *C. bovis*, and four for *C. ryanae*. There was also one *C. parvum* + *C. bovis* mixed infection. No samples were able to be collected for one calf in week 1, three calves in week 2 and two calves in week 4.

3.4. Species present in calves in Study 2

On the beef farm, 23 calves were tested, and all of them were positive for *C. parvum*. No other species was found either alone or as a mixed infection.

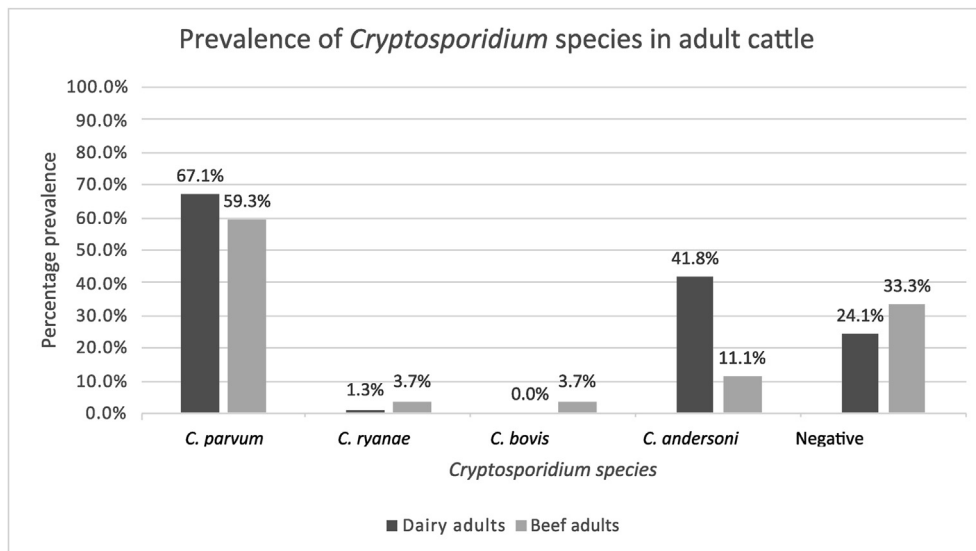


Fig. 1 Prevalence of *Cryptosporidium* species in adult cattle on both a dairy (Study 1) and a beef (Study 2) farm. Mixed infections are accounted for by including them in each of the species categories, therefore the total will exceed 100%

3.5. *gp60* subtyping in Study 1

The adult cattle samples that were positive for *C. parvum* underwent sequence typing at the *gp60* gene. The genotypes present were IIAA15R1 ($n = 26$), IIAA15G2R1 ($n = 22$), IICAA5G3 ($n = 2$), and IIAA17G1R1 ($n = 1$). We were unable to sequence two samples at the *gp60* gene. The first sample containing *C. parvum* oocysts from each of the 38 calves was chosen for *gp60* subtyping. All of the 38 *C. parvum*-positive calves had the *gp60* genotype IIAA15G2R1.

3.6. *gp60* subtyping in Study 2

In Study 2, 16 adult cattle and 23 calves gave a positive test for *C. parvum* on the beef farm and these underwent *gp60* genotyping. All except for one adult cow were positive on amplification of this gene. The predominant *gp60* genotype was IIAA17G1R1, which was present in 63% (10/16) of the adult cattle and 100% (23/23) of the *C. parvum*-positive calves. IIAA15R1, IIAA19G2R1, IIAA16G3R1 were found in 19% (3/16), 6% (1/16), and 6% (1/16) of the adult cattle respectively.

3.7. Multilocus genotyping

3.7.1. Study 1

Those adult cattle that were the mothers of the 38 calves in this study were chosen for microsatellite analysis. The sample selected for adult cattle was the *C. parvum*-positive sample collected closest to the time of calving, as this was the most likely genotype to be shed at birth. The calf sample chosen was the first detected *C. parvum*-positive in the first two weeks of life to characterise the primary infection source to the calf. Of the 38 adults, 28 were positive for *C. parvum* and underwent further genotyping. Amplification at all five alleles was successful for 19 out of 28 adults. Of the 38 calves, 34 were positive for *C. parvum* in the first two weeks of life. For the calf samples, 32 out of 34 were successfully assigned an MLG by amplifying at all five loci.

Results show that 28 calves and 2 adult cattle had MLG 1, although only one of these was a matched dam and calf pair. A single calf had MLG 2. One calf had MLG 3 which was shared by four of the adult cattle, none of which was the dam of that calf. One calf and one adult had MLG 4 and they were also unrelated. One calf was shedding MLG 5 and the majority of adult cattle ($n = 12$) were shedding MLG 9. The multilocus genotype results for adult cattle and their matched calves are provided in Table 2. The relationship between these MLGs as a whole dataset is visualised in

Fig. 4 showing the relatedness between the MLGs. Those MLGs which are joined by a line share 4 out of the 5 alleles and so there is a smaller difference between these two multilocus genotypes compared to those which are not joined by a line. This information is vital as it shows how related the genotypes are.

3.7.2. Study 2

All *C. parvum*-positive cows and calves (16 and 23, respectively), underwent microsatellite analysis. Of the 16 positive adult cattle samples, 13 were amplified at all 5 alleles and were assigned an MLG. Positive calf samples (22 out of 23) were successfully amplified at all 5 alleles and were assigned an MLG. Results show that 21 calves and 7 adult cattle had MLG 10, and all 7 adult cattle shedding MLG 10 had the same MLG as their calves. A single calf had MLG 11, one adult was shedding MLG 15, and another MLG 14, which is different from MLG 10 only by a single allele. One adult was shedding MLG 12 and another adult was shedding MLG 13. The matched pairs between calves and their mothers are shown in Table 3 and the relationship between these MLGs as a whole dataset is visualised in Fig. 5.

4. Discussion

Cryptosporidium is a parasite of great importance on farms as it is one of the leading causes of neonatal calf diarrhoea. The role that adult cattle play in potentially being a source of *C. parvum* oocysts to their calves is currently unknown due to the difficulty of concentrating and detecting oocysts in adult faecal samples (Wells et al., 2016). Overall, *C. parvum* was the most prevalent species found in pre-weaned calves on both farms in this study, which is consistent with previous research conducted in Scotland, England, and Wales (Smith et al., 2014; Wells et al., 2015; Thomson et al., 2019). *Cryptosporidium* species of cattle tend to follow an infection pattern that depends on the age of the host animal, with neonatal and pre-weaned calves mostly shedding *C. parvum*, post-weaned calves with *C. bovis* and *C. ryanae*, and adult cattle with *C. andersoni* (Robertson et al., 2014; Thomson et al., 2019). *Cryptosporidium ryanae* was detected in one calf in the second week of life. Although uncommon, this species has previously been found in pre-weaned calves (Murakoshi et al., 2013; Åberg et al., 2020).

Longitudinal sampling results from birth to six weeks (Fig. 2) show that calves intermittently shed *C. parvum* oocysts over multiple sampling periods. Despite difficulties in collecting a sample at every time point for every calf, the data indicate that calves can shed *C. parvum* in their faeces

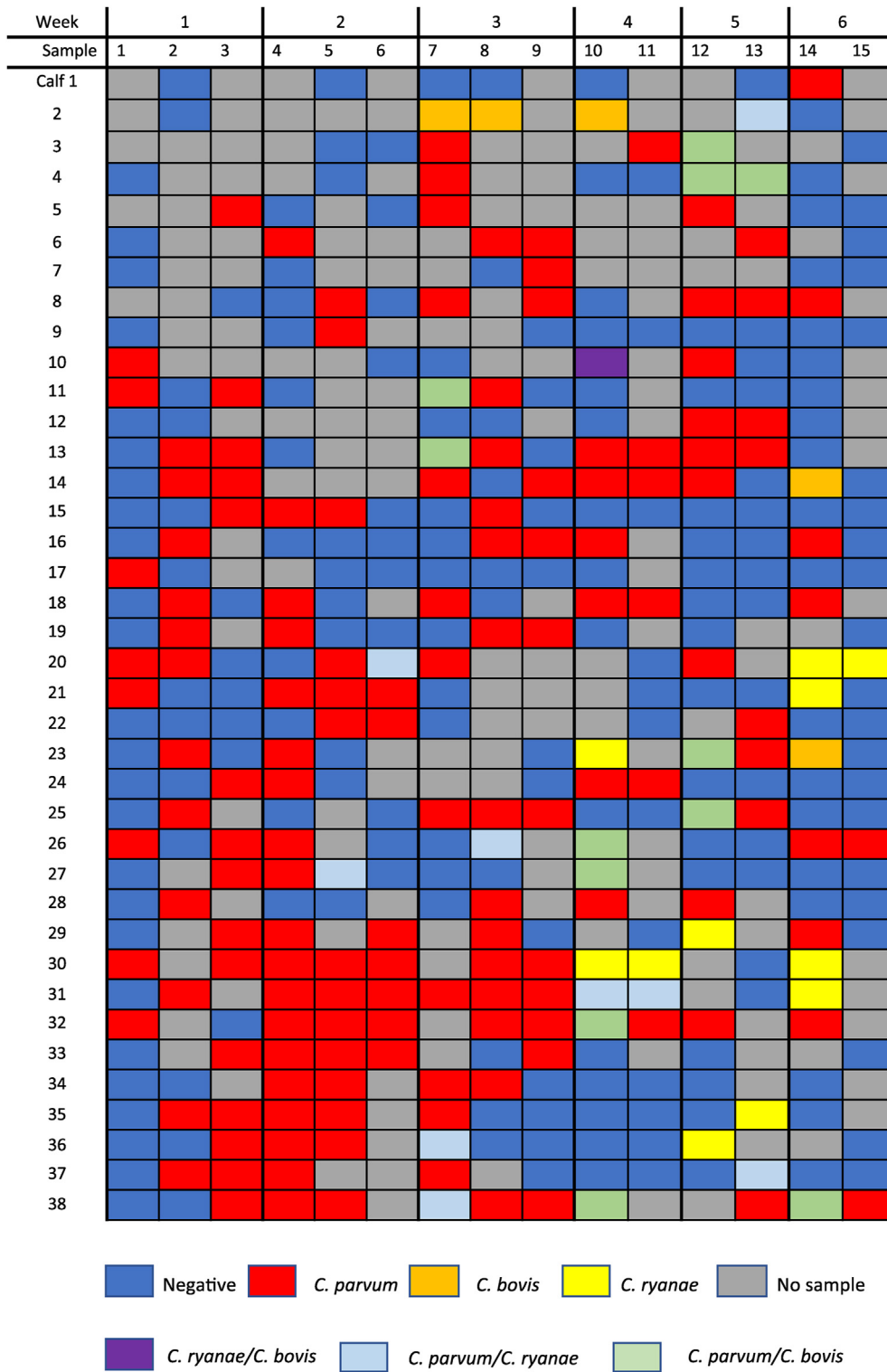


Fig. 2 Longitudinal prevalence data for *Cryptosporidium* species in dairy calves

(or the other *Cryptosporidium* species) on two occasions with negative results in-between. There are very few studies looking at longitudinal shedding profiles of calves and so these data provides evidence that calves shedding *Cryptosporidium* oocysts can do so intermittently during the pre-weaned stage. Existing studies in calves state that shedding increases, peaks, and then decreases rather than being shed intermittently (Nydham et al., 2001; Tiranti et al., 2011; Zambriski et al., 2013). However, two of these only examined the calves for 21 days (Nydham et al.,

2001; Zambriski et al., 2013) and none of them reported calf data on an individual basis. A study by Thomson et al. (2019) did collect data from calves on an individual basis although these data was not shared in the publication. Results from that study showed intermittent shedding in individual calves and this has also been seen in experimentally infected animals (F. Katzer, unpublished observations). To the best of our knowledge, this is the first study to report *Cryptosporidium* shedding for calves on an individual basis from birth to six weeks. Intermittent

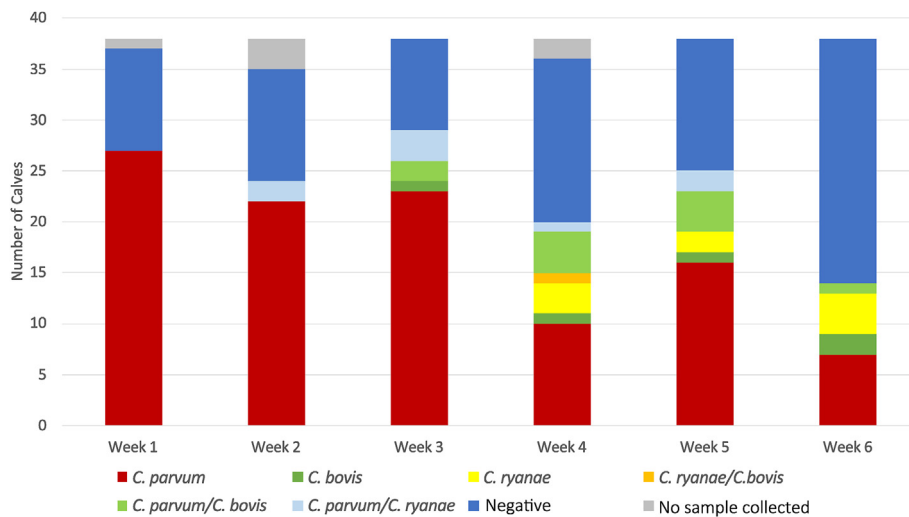


Fig. 3 Species of *Cryptosporidium* present in dairy calves (Study 1) in the first six weeks of life

Table 2
Multilocus genotypes between matched calves and their mothers for *C. parvum* on a dairy farm (Study 1)

Calf	Calf <i>gp60</i>	Calf MM5	Calf MM18	Calf MM19	Calf TP14	Calf MLG	Dam <i>gp60</i>	Dam MM5	Dam MM18	Dam MM19	Dam TP14	Dam MLG
1	×	×	×	×	×	×	IlaA15R1/IlaA15G2R1	2	1	3	1	9
2	×	×	×	×	×	×	IlaA15R1/IlaA15G2R1	2	1	3	1	9
3	×	×	×	×	×	×	IlaA15R1	2	1	3	1 + 2	9
4	IlaA15G2R1	2	1	3 + 1	2	4	IlaA15G2R1	2	1	1	2	1
5	IlaA15G2R1	2	2	3 + 1	2	5	×	×	×	×	×	×
6	IlaA15G2R1	2	1	1	1	3	IlaA15R1	2	1	3	1	9
7	IlaA15G2R1	2	2	1	2	2	×	×	×	×	×	×
8	IlaA15G2R1	2	1	1	2	1	IlaA15R1 + IlaA5G3	2	1	3	1	9
9	IlaA15G2R1	2	1	1	2	1	IlaA17G1R1	×	×	1	1	×
10	IlaA15G2R1	2	1	1	2	1	IlaA15R1	2	1	3	1	9
11	IlaA15G2R1	2	1	1	2	1	IlaA15R1	2	1	3	1	9
12	×	×	×	×	×	×	×	×	×	×	×	×
13	IlaA15G2R1	2	1	1	2	1	IlaA15G2R1	2	1	1	1	3
14	IlaA15G2R1	2	1	1	2	1	IlaA15R1	2	1	3	1	9
15	IlaA15G2R1	2	1	1	2	1	×	×	×	×	×	×
16	IlaA15G2R1	2	×	×	2	×	IlaA15G2R1	×	×	×	2	×
17	IlaA15G2R1	×	×	×	×	×	×	×	×	×	×	×
18	IlaA15G2R1	2	1	1	2	1	IlaA15G2R1	×	×	×	×	×
19	IlaA15G2R1	2	1	1	2	1	×	×	×	×	×	×
20	IlaA15G2R1	2	1	1	2	1	IlaA15G2R1	2	1	3	2	4
21	IlaA15G2R1	2	1	1	2	1	×	×	×	×	×	×
22	IlaA15G2R1	2	1	1	2	1	×	×	×	×	×	×
23	IlaA15G2R1	2	1	1	2	1	IlaA15R1/IlaA15G2R1	2	1	3	1	9
24	IlaA15G2R1	2	1	1	2	1	IlaA15G2R1	2	1	1	1	3
25	IlaA15G2R1	2	1	1	2 + 1	1	IlaA15R1	2	1	3	1	9
26	IlaA15G2R1	2	1	1	2	1	IlaA17G2R1	×	×	×	1	×
27	IlaA15G2R1	2	1	1	2 + 1	1	IlaA15G2R1	×	×	×	1	×
28	IlaA15G2R1	2	1	1	2	1	×	×	×	×	×	×
29	IlaA15G2R1	2	1	1	2	1	IlaA16G1R1	×	×	×	1	×
30	IlaA15G2R1	2	1	1	2	1	IlaA15R1	×	×	×	×	×
31	IlaA15G2R1	2	1	1	2 + 1	1	IlaA15R1	×	×	×	1	×
32	IlaA15G2R1	2	1	1	2	1	IlaA15R1	×	×	×	1	×
33	IlaA15G2R1	2	1	1	2	1	IlaA15G2R1/IlaA15R1	2	1	1	1	3
34	IlaA15G2R1	2	1	1	2	1	×	×	×	×	×	×
35	IlaA15G2R1	2	1	1	2	1	IlaA15R1	2	1	3	1	9
36	IlaA15G2R1	2	1	1	2	1	IlaA15G2R1	2	1	1	1	3
37	IlaA15G2R1	2	1	1	2	1	IlaA15R1/IlaA15G2R1	2	1	3	1	9
38	IlaA15G2R1	2	1	1	2	1	IlaA15G2R1/IlaA17G1R1	2	1	1	2	1

Notes: An ‘×’ denotes that a genotype was not obtained at this locus, either because the sample was not *C. parvum*-positive or due to insufficient PCR amplification. Final MLG assignment is presented in bold. Matched calf and dam pairs have their MLG underlined.

shedding has, however, been reported for natural *C. parvum* infection in humans (Chappell et al., 1996). Our data indicate that there is variation in shedding profiles across the calves, with some calves shedding oocysts for longer periods and in consecutive sampling, others being intermittently positive, and a few calves for which oocysts were only detected on

one occasion. Understanding the reasons behind this variation, and the relative contribution of infectious dose size, time of exposure, nutrition, maternal antibodies or genetics, would be of significant interest concerning patterns of disease in calves, and these data highlight the value of longitudinal genotyping studies.

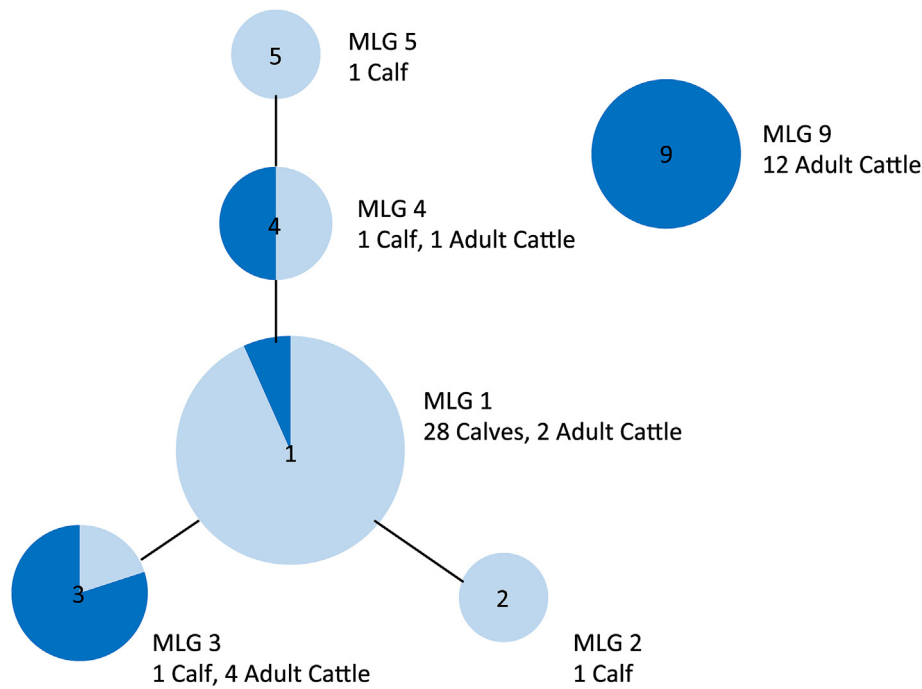


Fig. 4 Multilocus genotypes (MLGs) present in calves and their mothers on a dairy farm (Study 1). Each node represents a single MLG. The size of the node represents the number of animals with that MLG. Nodes are connected by a line if they share 4 out of 5 tested alleles. Dark blue represents adult cattle and light blue represents the calves

Table 3
Multilocus genotypes between matched calves and their mothers for *C. parvum* on a beef farm (Study 2)

Calf	Calf <i>gp60</i>	Calf MM5	Calf MM18	Calf MM19	Calf TP14	Calf MLG	Dam <i>gp60</i>	Dam MM5	Dam MM18	Dam MM19	Dam TP14	Dam MLG
1	IlaA17G1R1	2	1	3	1	10	×	×	×	×	×	×
2	IlaA17G1R1	2	1	3	1	10	×	×	×	×	1	×
3	IlaA17G1R1	2	1	3	1	10	IlaA17G1R1	2	1 + 2	3	1	10
4	IlaA17G1R1	2	1	10 + 3	1	11	IlaA15R1	2	1 + 2	8	1	13
5	IlaA17G1R1	2	1	3 + 9	1	10	×	×	×	×	×	×
6	IlaA17G1R1	2	1	3	1	10	×	×	×	×	×	×
7	IlaA17G1R1	2	1	3	1	10	IlaA15R1	2	1	8	1	13
8	IlaA17G1R1	2	1	3	1	10	×	×	×	×	×	×
9	IlaA17G1R1	2	1	3	1	10	IlaA17G1R1	2	1	3	1	10
10	IlaA17G1R1	2	1	3	1	10	IlaA17G1R1	×	×	×	×	×
11	IlaA17G1R1	2	1	3 + 9	1	10	IlaA17G1R1	2	1	3	1	10
12	IlaA17G1R1	2	1	3 + 9	1	10	IlaA17G1R1	2	4	3	1	14
13	IlaA17G1R1	2	1	3	1	10	×	×	×	×	×	×
14	IlaA17G1R1	2	1	3	1	10	IlaA17G1R1	2	1	3	1	10
15	IlaA17G1R1	2	1	3	1	10	IlaA16G3R1	2 + 3	1	3	1	15
16	IlaA17G1R1	2	1	3	1	10	×	×	×	×	×	×
17	IlaA17G1R1	2	1	3 + 9	1	10	IlaA19G2R1	×	×	×	1	×
18	IlaA17G1R1	2	1	3 + 9	1	10	IlaA19G2R1	2	1	8	1	12
19	IlaA17G1R1	2	1	3 + 9	1	10	IlaA17G1R1	2 + 3	1	3	1	10
20	IlaA17G1R1	2	1	3	1	10	IlaA17G1R1	2	1	3	1	10
21	IlaA17G1R1	2	1	3 + 9	1	10	×	×	×	×	×	×
22	IlaA17G1R1	2	1	3 + 9	1	10	IlaA17G1R1	2	1	3	1	10
23	Calf died						IlaA17G1R1	×	×	×	1	×
24	Calf died						IlaA15R1	2	1	8	1	13
25	IlaA17G1R1	×	×	×	×	×	×	×	×	×	×	×

Notes: An ‘×’ denotes that a genotype was not obtained at this locus, either because the sample was not *C. parvum*-positive or due to insufficient PCR amplification. Final MLG assignment is presented in bold. Matched calf and dam pairs have their MLG underlined.

Cryptosporidium parvum was detected as the most prevalent species on both farms in adult cattle, with 67.1% prevalence on the dairy farm (Study 1) and 59.3% on the beef farm (Study 2). The finding that *C. parvum* is the most prevalent species in adult cattle is consistent with previous studies using the same sensitive techniques designed to work with adult cattle faeces and molecular typing tools (Wells et al., 2015; Thomson et al., 2019).

In this study, a high prevalence of *C. parvum* in adult cattle was found both on its own or as a mixed infection with other species. *C. andersoni* was present in 41.8% of the adult cattle either alone or as a mixed infection with *C. parvum* in Study 1 and in 11.1% of the adult cattle in Study 2. When these techniques were used in previous studies, similar results were shown; a study looking at the species of *Cryptosporidium* present in livestock in a catchment in the Cairngorms, Scotland, found

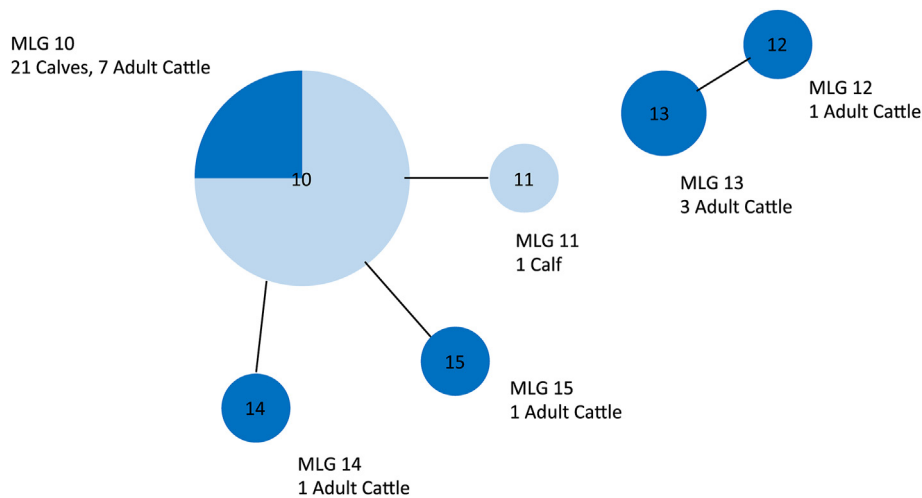


Fig. 5 Multilocus genotypes (MLGs) present in calves and their mothers for a beef farm (Study 2). Each node represents a single MLG. The size of the node represents the number of animals with that MLG. Nodes are connected by a line if they share four out of five tested alleles. Dark blue represents adult cattle and light blue represents the calves

that *C. parvum* was the predominant species in adult cattle, present throughout three sampling time points (Wells et al., 2015). A further study examining the shedding of *Cryptosporidium* in calves and dams found that 86.2% of the adult cattle were shedding *C. parvum* (see Thomson et al., 2019). This present study builds on this work by continuing the research on alternative farms and typing multiple loci and examining paired samples from dams and their calves.

The *gp60* genotype discovered in the calves in Study 1 was exclusively IIAA15G2R1. This appears to be the most common genotype in calves in the UK (Brook et al., 2009; Smith et al., 2014; Wells et al., 2015) and in Europe (Holzhausen et al., 2019). In Study 2, however, the calves had exclusively IIAA17G1R1, which is less common although has been found in the UK, Germany, Hungary, Sweden, and Poland (Plutzer & Karanis, 2007; Silverlås & Blanco-Penedo, 2013; Smith et al., 2014; Kaupke & Rzezutka, 2015; Holzhausen et al., 2019). The studies by Silverlås and Blanco-Penedo (2013) and Smith et al. (2014) were conducted in dairy cattle but unfortunately, the other studies do not state whether these were from dairy or beef calves. *Cryptosporidium parvum* subtype IIAA15G2R1 was not found in any of the animals in Study 2, but IIAA17G1R1 was detected in one adult cow in Study 1. This might suggest that IIAA15G2R1 may be associated with increased pathogenicity when compared to IIAA17G1R1 and indeed the clinical disease in Study 1 was notably higher than in Study 2 when considering the whole group (our unpublished observations). Pathogenicity of different *C. parvum* genotypes has been shown to differ according to the corresponding *gp60* genotype (Sayed et al., 2016), and work looking at the pathogenicity of *C. parvum* showed that different isolates differ in their ability to alter host cell monolayers (Holzhausen et al., 2019). It has been suggested that *C. parvum* with the *gp60* genotype IIAA15G2R1 may be one of the most virulent to infect calves and lambs (Feng et al., 2013).

Species identification was achievable using the present protocols when used for adult cattle samples; however, the protocol had to be adapted when amplifying at the MM5, MM18, MM19, and TP14 gene to remove dilution steps. Multiple PCR repeats were also required to ensure adult cattle samples were amplified at all loci. This could suggest that either there is a reduced number of *C. parvum* oocysts present in adult cattle samples, that the ratio of faeces to oocysts is greater resulting in reduced sensitivity, or the presence of PCR inhibitors has underestimated the prevalence in adult cattle (Moreira et al., 2020). The *18S* target sequence is a multi-copy target while the other alleles used in the study are single-copy targets of the genome. An effective qPCR to quantify the *C. parvum* DNA would be useful although this would also be subject to PCR inhibition. An alternative could be to separate oocysts using

immunomagnetic separation (IMS) and quantify using microscopy. Future work should include improving detection rates in adult cattle faeces by optimising methodology in order to increase the reliability of prevalence data in adult cattle.

Despite previous studies finding *C. parvum* in adult cattle, many of these have not been genotyped or have only been genotyped at the *gp60* locus, making it difficult to conclude if the genotype in the adults matches the genotype in the calves, especially if the IIAA15G2R1 genotype is found as this is the most common genotype in calves and humans within the UK. When further genotyping is done at multiple loci, as in the present study, the results show a greater variety of genotypes present in the adult cattle compared to their calves. In the dairy farm (Study 1), the majority of the adult cattle had a genotype that differs by more than one of the five tested loci. Furthermore, only one matched calf and dam shed the same genotype on the dairy farm. This would suggest that the main genotype found in adult cattle is different from the main genotype found in the calves. However, the genotype which is present in the calves may be present within the adult cattle, but at levels below the detection threshold. This result is mirrored in the multilocus genotypes found in adult cattle and calves in a Scottish catchment, where the majority of adult cattle had an MLG that differed from that of the calves, although calves in this study were not matched pairs (Wells et al., 2015).

In the beef farm (Study 2), the majority of adult cattle did have a multilocus genotype that matches the calves (54%) although it must be noted that these animals were sampled at the same time. When it came to matched calf and dam pairs, there were seven out of a possible 25 matches. This match cannot directly confirm transmission between the two; however, it does suggest that adult cattle may be a potential source of oocysts to their calves. This is not an unexpected result as these adults and calves are housed together in the same shed and therefore this result could reflect also that adult cattle may become infected from oocysts that have originated from the calves. The way these cattle are housed together is likely to account for the difference in genotypes shed in matched dam-calf pairs observed when compared to the dairy farm. The dairy calves were removed from their dams within 12 h of birth whereas the beef calves were housed alongside their dams. Further work is required to determine whether these matched genotypes in the adult cattle could pose a transmission risk to calves. This would involve a detailed look at how oocysts are able to move from the adult cattle, through the environment to then become a source of infection to the calves. Future work should include testing the adult cattle before the birth of the calves, to see if a similar pattern is observed.

Results from the dairy farm (Study 1) show that adult cattle and their calves predominantly have different multilocus genotypes with only one matched pair. The beef farm presented with seven matched pairs which show multilocus genotypes shed by the calves can also be shed by the adult cattle. Therefore adult cattle may potentially be a source of oocysts to calves, but this is more likely on a beef farm where they are housed together than on a dairy farm where they are separated. Overall the difficulty in amplification indicated a low level of oocyst shedding in both the adult dairy and beef cattle, although further method optimisation is required to confirm this. Nevertheless, as little as 17 oocysts have been shown to infect calves and result in diarrhoea and oocyst shedding (Zambriski et al., 2013), and therefore low-level shedding within a larger sample volume in adult cattle may still contribute a substantial number of oocysts and therefore be responsible for maintaining *C. parvum* between calving seasons.

As cattle on the dairy farm calved all year around, it was impossible to determine how genotypes evolve over a calving period. This information would be useful as it would allow for the development of risk forecasting and provide future opportunities to discover if oocysts persist between calving seasons. Therefore future work could involve similar studies on farms, which have set calving periods, to see how genotypes evolve and persist.

5. Conclusions

The results of this study have shown that when using the most sensitive technique available for the processing of adult cattle faecal samples, *C. parvum* is the most prevalent species in both adult cattle and calves on both a dairy and beef farm. The dairy farm study (Study 1) is one of the first studies to examine longitudinal shedding profiles on an individual calf basis from birth to six weeks and it revealed intermittent shedding of *Cryptosporidium* oocysts by the calves. The difficulty in multilocus amplification of *C. parvum* in adult cattle and the requirement to alter the published protocols suggests that further work is required to optimise the detection of oocysts in adult cattle faeces. This study indicates that adult cattle do have the ability to shed the same genotype of *C. parvum* as their calves and so with the low infectious dose and increased sample size, could potentially be a source of oocysts to calves. Further work would be required to confirm their potential role in transmission by analysing oocyst movement and survival in the environment.

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Ethical approval

The study was carried out with the permission of the farmers and all positive animals were experiencing a natural infection with *Cryptosporidium* which was confirmed using 18S nssm PCR. Both farms had a history of clinical cryptosporidiosis.

CRedit author statement

FK, EI, LM and HS conceived and designed the study. HS collected the samples, performed the laboratory analysis, genotyping analysis and drafted the manuscript. CA and KU aided in sample collection and processing. FK, EI, LM edited the manuscript. All authors read and approved the final manuscript.

Declaration of competing interests

The authors declare that they have no competing interests.

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