Prevalence and molecular typing of *Cryptosporidium* in dairy cattle in England and Wales and examination of potential on-farm transmission routes

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ABSTRACT

An average of 70 samples were collected from 80 dairy farms in England and Wales, from cattle, co-grazed sheep, wildlife and farm wastes, to investigate prevalence, potential sources and transmission routes of *Cryptosporidium*. At least one positive sample was detected on 74 of the farms (92.5%) by IFAT microscopy. The prevalence in cattle was 10.2% (95% CI 9.4–11.1%), with greater prevalences detected in calf samples, especially from those under 1 month (45.1%). Young calves were also more likely to be shedding *Cryptosporidium parvum* and larger concentrations of oocysts, whereas older calves and adult cattle were more likely to be shedding *Cryptosporidium bovis* and *Cryptosporidium andersoni*, respectively. The *C. parvum* subtypes detected were predominantly from types commonly identified in UK cattle (67% were either IIaA15G2R1 or IIaA17G1R1). A novel subtype, IIaA17G1R2, was identified from one cattle sample.

The prevalence in co-grazed sheep was low (4%). Birds and rodents may represent significant reservoirs of *Cryptosporidium* due to high prevalence, large oocyst concentrations, and the detection of a *C. parvum* subtype known to be present in human populations, identified in samples from these wildlife. *Cryptosporidium* were detected in dirty water and manure, and also from pasture samples where slurry had been spread.

On 64% of the farms, identical *Cryptosporidium* species were detected (mainly *C. parvum* or *C. bovis*) from different cattle groups on the farms, although no direct or indirect contact between the groups were recorded, apart from sharing staff. The same *Cryptosporidium* species were found in cattle, farm wastes and bird samples on the same farms, but rarely, or not at all, present in sheep or rodent samples. The matching of species/subtypes was also related to the proximity of the different sample sources which may indicate a potential transmission route.

1. Introduction

Cryptosporidium infection is common in farm animals, especially young cattle, but it is often subclinical (De Graaf et al., 1999). *Cryptosporidium* has been shown to be the most commonly detected (53.7%) of the four major enteropathogens (rotavirus, bovine coronavirus, enterotoxigenic *Escherichia coli*) that cause neonatal diarrhoea found in young diarrhoeic calves (Uhde et al., 2008). Evidence that farm animal and environmental contact contributes to zoonotic transmission routes was evidenced through a 40% reduction in confirmed human cryptosporidiosis cases in England and Wales during a period of Foot and Mouth Disease control measures, which included

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public access restrictions to the countryside (Sopwith et al., 2005). However, a combined effect with improvements in drinking water quality has been considered. The importance of understanding and controlling *Cryptosporidium* is highlighted as there is also growing anecdotal evidence of *Cryptosporidium* infections as a problem in the beef industry, in both calves and adults, within the UK and particularly in Scotland (Hateley, pers. comm.). There have been no large scale on-farm *Cryptosporidium* prevalence studies of cattle in the United Kingdom and this was deemed an important gap in the understanding of this parasite and the risk to people coming into contact via food or environmental contact.

As the tools for the molecular differentiation of *Cryptosporidium* have advanced, so has the number of different species associated with public health and the differentiation of species of veterinary importance. Species of *Cryptosporidium* have varying host species, with *Cryptosporidium parvum* being particularly important to epidemiologists as it infects a wide range of animals and also people. The zoonotic transmission of *C. parvum* via direct animal contact has been demonstrated and reported (Casemore, 1990; Elwin et al., 2001), but the risk of disease through indirect contact with animal faeces or from environmental exposure is unmeasured. Previous evidence for the existence of different subtypes of *Cryptosporidium* species, along with their zoonotic capabilities and potential reservoir host range, has been demonstrated (Xiao et al., 2001; Pritchard et al., 2007; Chalmers and Giles, 2010; Smith et al., 2010).

The potential for the circulation of *Cryptosporidium* species between different animal species on a farm has been shown by a previous study, where identical *Cryptosporidium* species and subtypes were detected from different animal species on the same farm (Smith et al., 2010). This may indicate that different animal species are susceptible to the same transmission routes on the farm or that infection is transmitted between animal groups.

The study described here sought to determine the prevalence of *Cryptosporidium* in the cattle population in England and Wales and to investigate and identify potential environmental reservoirs/sources of public and veterinary infections, whether direct, environmental or food/waterborne. The contributions of wild animal and bird populations to the environmental load were also investigated. Molecular tools were used, capable of determining all species of *Cryptosporidium*, to determine the extent of the presence of different species on the farms, and subtyping tools were used to further discriminate those of primary zoonotic importance and assess the potential transmission risk to people and farm animals. The identification of *Cryptosporidium* species of veterinary importance was considered essential as, although some may not be a risk to public health, infection in calves adversely affects farm productivity. This study aimed to inform the development of interventions that would be of use to the farmer in disrupting transmission routes or potential reservoirs of infection, while also helping to determine areas of greatest risk to the public entering farm land for recreational purposes.

2. Materials and methods

2.1. Enrolment of farms

A sample size calculation (Winepiscope, Thursfield et al., 2001) was used to determine that 75 farms would be required to estimate prevalence with 95% confidence and precision of 10%, when the assumed prevalence of infected herds was 66% (Brook et al., 2008). Additional farms were also selected to ensure that sampling was completed across all seasons.

The majority of the enrolled farms were also used for a case–control study, not reported here, which was designed to investigate antimicrobial resistance in commensal *E. coli*. A quarter of the farms (20) were 'case' farms for this antimicrobial resistance study, selected due to cattle movement links with an index farm, but the remainder were controls. The controls were randomly selected from herds in the Animal Health Agency's Vetnet database of livestock premises that met the following eligibility criteria: Dairy farm and 30 or more youngstock (cattle aged 0 – 24 months) present on the holding, as listed by the Cattle Tracing scheme population data taken from January 2009. These farms were supplemented by 13 more farms, enrolled solely for the purpose of this study and selected in the same manner as the controls. The farm selection for this study was limited to a geographical area covering the counties which could be conveniently sampled from the four AHVLA regional offices at Aberystwyth, Shrewsbury, Preston and Penrith. The farms were all located in 7 counties in the West Midlands, North-West England or in Northern Wales. In Wales, 7 farms were located in the county of Clwyd, 9 in Gwynedd and 1 in Powys. In England, 12 farms were from Cheshire, 24 from Cumbria, 25 from Lancashire and 2 from Shropshire.

All farms interested in participating were required to have at least 30 calves (<6 months) on the farm during the period of sampling visits to ensure that enough calves were available to be sampled; this was checked at the time of enrolment and those with less than 30 calves were omitted. A visit was arranged to each participating farm during which samples were collected along with details of each sampled location, as well as a sketched farm layout map annotated with the location of animal groups and sampling locations.

2.2. Sample collection

To determine within-herd prevalence, a sample size calculation showed that 27 animals were required to be sampled to detect prevalence with 95% confidence and 5% accuracy, assuming the group size was 30 animals and that 10 animals were infected.

At each visit, 55 fresh faecal samples (roughly 10 g) were collected from cattle groups. These included 30 individual calf pat samples from fields/accommodation occupied on the day of the visit. Sampling from calves less than 3 months old was prioritised but otherwise the next available youngest age group was sampled. The remaining 25 samples were from adult milking cows. These individual pat samples were collected broadly in proportion to the numbers of animals in each group or enclosure, and were taken

from points distributed across the whole of the accommodation/grazing field that was accessible in order to ensure the sample was representative of the group of animals.

Up to 25 samples were collected from other areas according to the following protocol: (1) Wildlife faeces (maximum 5 samples). Samples represented the risk of infection from a particular wildlife species, such as birds, foxes, deer and rodents (counting birds as a single species) in a particular house or field where calf samples had been collected. As much faecal material as possible, pooled from the same location and the same species, was collected in each sample pot. Pre-moistened, sterile Readiwipes were used to collect bird samples. (2) Co-grazing sheep (maximum. 10 samples). Fresh (voided) faeces were collected from sheep that were co-grazed with cattle. If sheep were co-grazed on more than one field, then the 10 samples were divided between the fields roughly in proportion to the size of the sheep groups in each. If no co-grazed sheep were present then no samples were collected. (3) Manure (maximum. 2 pooled samples). Samples were taken from two composting/stacked manure heaps to which calves and youngstock had most recently contributed. At four separate locations on the heap, surface weathered manure was pulled away and then material removed to a depth of 10–20 cm. Two "pinches" (approx. 4 g) of manure were collected per hole, with a final pinch of surface material. If the farm had only one heap, a second pooled sample was not required. (4) Dirty water (maximum 3 samples). Sampling areas were the milking parlour drain, outside drain that contributes to the farm's dirty water and drains in areas of animal accommodation or collecting yard. The liquid in the drain was mixed well using a plastic rod. The rod was wiped with 70% ethanol and thoroughly rinsed with water between successive samples and a new rod used for each farm. Up to 1 L of drain liquid was collected. (5) Spread slurry (maximum. 5 samples). Five sampling areas were identified (e.g. top left, top right, bottom left, bottom right and centre) in a pasture field used for grazing where slurry had been spread in the last month. If more than one pasture field had been spread in the past month, then the most recently spread field was selected. The areas of the pasture field selected for sampling were preferably damp, shaded areas where slurry had been visibly spread. From each of the 5 areas, a 2 m × 2 m section was selected and a sample (approximately 5 g) of residual manure or surface material (topsoil or visually contaminated grass) was collected from 10 points in the section, by scraping to a depth of no more than 2.5 cm. The pooled sample from the 10 positions was mixed. If no slurry has been spread in the past month then no samples were collected.

2.3. Sample testing

Samples were tested by immunofluorescence (IFAT) microscopy using a commercially available kit (TCS Biosciences). The antibody used has been shown to bind to all *Cryptosporidium* oocyst species, with a known lesser affinity to *Cryptosporidium felis* (Huw Smith, pers. comm. 2003). Samples were deemed positive where any *Cryptosporidium* oocysts were seen. Semi-quantitative counting techniques were used from a scale of 1+ ($<1 \times 10^3$ oocysts per gram)

to 5+ ($>2 \times 10^6$ oocysts per gram) as described by Webster et al. (1996) and defined in Table 3.

A subset of *Cryptosporidium* positive samples, identified by the researchers to be representative of the sampled population (sample types and animal age groups), was selected for further testing. The published PCR-RFLP on the 18srRNA gene (Xiao et al., 2000) was used to speciate the *Cryptosporidium* oocysts demonstrated as present by microscopy. The RFLP enzymes SSPI and VSPI were used, along with MboII (Feng et al., 2007). A large proportion (>50%) of samples, including non-*C. parvum* species as determined by RFLP, all those with weak amplification (making RFLP impossible) or an unclear pattern, and a random selection of 10% of the *C. parvum* samples, were also confirmed by ABI sequencing.

The sequences obtained were compared to other published sequences on the Genbank™ database and sequences of interest were uploaded onto Genbank™. Nucleotide sequence data reported in this paper are available under the accession numbers: **KJ486151, KJ486152, KJ499993** and **KJ499994**.

The GP60 microsatellite region of the *C. parvum* and *Cryptosporidium hominis* genome was used to identify the different subtypes of those species (Strong et al., 2000; Sulaiman et al., 2005). Unusual or rare subtypes were tested twice for confirmation.

2.4. Data analysis

Data collected from the farm visits and the test results were entered into a purpose-built Microsoft Access (Microsoft corp. 2003. Redmond, WA) database, wherein a 20% data entry error check and a search for improbable values were completed. Farm-level and within-herd prevalence estimates were calculated by conventional methods with 95% confidence intervals, and tabular summaries produced in Microsoft Excel (Microsoft corp. 2010. Redmond, WA). The survey (svy) command in Stata (StataCorp. 2007. Stata Statistical Software: Release 10. College Station, TX: StataCorp LP) was also used to produce corrected confidence interval estimates around the prevalence, adjusting for farm level strata. Analyses, using Chi-Squared tests, were made to compare the sample results at each category of a number of variables, such as season of sampling. Yates' correction and Fisher's exact *P*-values were used when at least one cell of the 2xn table had an expected count smaller than 5.

3. Results

A total of 80 farms were tested for *Cryptosporidium* presence. These farms consisted of 67 farms visited, as part of the *E. coli* case–control study, between November 2009 and March 2010, and 13 farms visited between May and September 2010 specifically for this project. No significant difference was found between the test results or main management structures of the case and control farms. No significant differences were found between the participating farms and those that declined participation in the study with respect to the main farm details, such as herdsize and farm location.

Table 1
Percentage of samples IFAT microscopy positive for *Cryptosporidium*, with confidence intervals (CI), by species of animal sampled or sampling area.

Sample	No. of farms submitting samples	No. of samples positive	No. of samples collected	% positive	Conventional 95% CI		Adjusted 95% CI	
Bird	46	14	121	11.6	5.7	17.4	5.9	17.3
Cattle	80	508	4974	10.2	9.4	11.1	9.4	11.0
Deer	1	0	2	0.0	–	–	–	–
Dirty water	63	11	99	11.1	4.9	17.3	4.9	17.3
Foxes	2	0	2	0.0	–	–	–	–
Manure	64	11	85	12.9	5.8	20.1	5.8	20.1
Mice	10	3	13	23.1	0.0	46.9	0.1	46.0
Rats	13	5	19	26.3	6.0	46.7	6.5	46.1
Sheep	13	5	121	4.1	0.6	7.7	0.6	7.7
Spread slurry	34	3	158	1.9	0.0	4.0	0.0	4.0

An average of 70 samples were tested from each farm (range 38–110), with a total of 5594 tested overall. At least one positive sample was detected on 74 of the 80 farms (92.5%, 95% CI 86.7–98.3%). The planned 55 cattle samples were not collected from all farms: five farms were missing between one and four samples due to a lack of faecal material in animal areas, and a further four farms had fewer youngstock or adult cattle present on the farm than expected so the sampling represented the smaller number of animals. *Cryptosporidium* prevalence was highest (20.6%) in the 553 samples collected in the spring months (March–May) rather than in the other seasons (8.8%) (odds ratio (OR_x^2) 2.7, $P < 0.001$).

The preliminary results for the presence of any species of *Cryptosporidium* from the IFAT microscopy testing showed that a prevalence of 10.2% (95% CI 9.4–11.1%) was detected from cattle samples collected from each farm, with little change between the confidence limits calculated by conventional or adjusted methods (Table 1). The results also indicate that a relatively high percentage of positive samples (>10%) were detected from rats, mice and bird samples, collected from the cattle enclosures (Table 1). Dirty water samples and manure samples were found to have prevalences similar to that detected from the cattle, but the number of positive test results from samples collected from grazing fields where slurry had been spread in the preceding 30 days, was found to be low. A low prevalence (4.1%) was detected from the 121 sheep samples and no positive samples were detected from deer and fox samples, although only 2 samples were collected of each.

Cryptosporidium prevalence appeared to be related to the age of cattle present in the group. As the samples were collected from groups of animals, the exact age of the animals was unknown. However, the highest prevalence (45.1%) was detected in groups where the oldest age category was less than one month (Table 2).

The semi-quantitative test results for mice and rats had a greater percentage of samples with high *Cryptosporidium* concentrations than other sample types (Table 3). Mice had 7.7% of samples with an estimated 3+ concentration and rats had 5.3% at that concentration and 5.3% at 5+, in comparison with an average from all samples of 0.5% at 3+ and 0.8% at 5+. However, there was no statistical evidence that rodents had a higher probability of a result above 2+ (OR_x^2 3.3 $P = 0.11$). The semi-quantitative results broken down by the age categories in the cattle groups indicated only calf groups (aged 0–6 months) had concentrations of 4+ and 5+ (Table 4). Calf groups containing only calves under 1 month of age, were significantly more likely ($P < 0.001$) to have concentrations of 5+ (6.6%) than those that contained calves up to 3 or 6 months of age (1.8% and 0.2%, respectively).

3.1. PCR analysis

A total of 422 IFAT positive samples from 52 farms were tested by 18s rRNA PCR. However, the samples from four farms only provided 'unclear' results. The number of farms supplying PCR-RFLP results for each sample type is shown in Table 5. Each of the 52 farms had an average of 8 samples tested by PCR-RFLP, with only one farm having only one sample tested (range 1–27). The most common patterns of *Cryptosporidium* species detected on these farms were *Cryptosporidium bovis*, *C. parvum* and unclear pattern (10 farms), *C. bovis* and *C. parvum* (7 farms), and *C. parvum* and unclear pattern (6 farms). Eight farms had only one species result detected (including the farm which had one sample tested). One farm had five *Cryptosporidium* species detected: *Cryptosporidium bovis* (from weaned and new born calves); *C. parvum* (new born calves); *Cryptosporidium ryanae* (new born calves); *Cryptosporidium ubiquitum* (rats); and unclear pattern (new born and weaned calves).

Table 2
Cryptosporidium prevalence, determined by IFAT microscopy, with confidence intervals (CI), by the oldest age category in each cattle group.

Oldest age category in group	No. of samples positive	No. of samples collected	% positive	Conventional 95% CI		Adjusted 95% CI	
<1 month	144	319	45.1	39.7	50.6	39.8	50.5
1–3 months	247	1046	23.6	21.0	26.2	21.1	26.2
4–6 months	55	628	8.8	6.5	11.0	6.6	10.9
7–12 months	11	244	4.5	1.9	7.1	1.9	7.1
13–24 months	0	68	0.0	–	–	–	–
>24 months	51	2669	1.9	1.4	2.4	1.4	2.4

Table 3

Semi-quantitative *Cryptosporidium* results, determined by IFAT microscopy, from samples collected from dairy farms.

Sample type	IFAT result					
	Negative	1+	2+	3+	4+	5+
Bird	107 (88%)	11 (9%)	1 (1%)	1 (1%)	1 (1%)	
Cattle	4466 (90%)	380 (8%)	48 (1%)	23 (1%)	16 (0%)	41 (1%)
Deer	2 (100%)					
Dirty water	88 (89%)	7 (7%)	2 (2%)	2 (2%)		
Foxes	2 (100%)					
Manure	74 (87%)	9 (11%)	2 (2%)			
Mice	10 (77%)	2 (15%)		1 (8%)		
Rats	14 (74%)	3 (16%)		1 (5%)		1 (5%)
Sheep	116 (96%)	4 (3%)	1 (1%)			
Spread slurry	155 (98%)	2 (1%)	1 (1%)			
Total	5034 (90%)	418 (7%)	55 (1%)	28 (1%)	17 (0%)	42 (1%)

Negative, $<1 \times 10^3$; 1+, $1 \times 10^3 – 1 \times 10^4$; 2+, $>1 \times 10^4 – 1.5 \times 10^5$; 3+, $5 \times 10^5 – 7 \times 10^5$; 4+, $>7 \times 10^5 – 2 \times 10^6$; 5+, $>2 \times 10^6$ oocysts per gram of faeces.

Table 4

Semi-quantitative *Cryptosporidium* results, determined by IFAT microscopy, from samples from cattle groups.

Oldest age category in cattle group	IFAT result					
	Negative	1+	2+	3+	4+	5+
<1 month	175 (55%)	90 (28%)	19 (6%)	7 (2%)	7 (2%)	21 (7%)
1–3 months	799 (76%)	184 (18%)	21 (2%)	14 (1%)	9 (1%)	19 (2%)
4–6 months	573 (91%)	51 (8%)	3 (1%)			1 (0%)
7–12 months	233 (95%)	11 (5%)				
13–24 months	68 (100%)					
>24 months	2618 (98%)	44 (2%)	5 (0%)	2 (0%)		

Negative, $<1 \times 10^3$; 1+, $1 \times 10^3 – 1 \times 10^4$; 2+, $>1 \times 10^4 – 1.5 \times 10^5$; 3+, $5 \times 10^5 – 7 \times 10^5$; 4+, $>7 \times 10^5 – 2 \times 10^6$; 5+, $>2 \times 10^6$ oocysts per gram of faeces.

A *Cryptosporidium baileyi* was detected in a sample from a calf group and confirmed by sequencing. However, the novel detection of this species in cattle could not be verified, as the sample was from floor faeces and could have been contaminated by another source.

A comparison between the semi-quantitative IFAT result and the *Cryptosporidium* species indicated *C. parvum* was significantly more likely to be detected in samples with larger concentrations ($\geq$3+) than the other *Cryptosporidium* species (OR_x^2 19.0 $P < 0.001$). Only 60 (44%) *C. parvum* samples had concentrations of 1+, 24 (18%) of 2+, and 52 (38%) of $\geq$3+, whereas the other *Cryptosporidium* species had 90% of samples with concentrations of 1+, 7% with 2+ and 3% with $\geq$3+.

Of those with positive results, faecal samples collected from cattle groups were most likely to have been either *C. parvum* or *C. bovis*, and sheep were mainly *C. bovis* (Table 6). Waste sources (manure, dirty water or spread slurry) contained mainly *Cryptosporidium andersoni* (4 samples) or *C.*

parvum (9 samples). The three 'positive' results from birds were all *C. parvum*, whereas the mice and rat samples had identified species rarely found in cattle apart from one sample from mice with *C. andersoni*.

Of the 382 cattle samples tested by PCR, samples from groups containing calves aged under a month (175 samples) were more likely to have *C. parvum* detected (52.6%), whereas *C. bovis* (46.3%) were mainly detected in samples from groups of cattle aged between 1 and 6 months (160 samples). The 11 samples from groups of cattle aged over 7 months were predominantly unclear pattern results (81.8%) and samples from groups of only adults aged over 24 months (36 samples) mostly had *C. andersoni* (61.1%).

Most PCR tested cattle groups were housed, with only 11 samples tested from pasture. The samples from grazing cattle were 3 *C. bovis* (2 from <1 month old groups and 1 from a >24 months old group), 1 *C. ryanae* (1–3 month olds) and 7 unclear. The season of sampling was associated with the *Cryptosporidium* species detected, as significantly more

Table 5

Number of farms providing PCR-RFLP *Cryptosporidium* species results by sample type.

Sample type	No. of farms providing samples	No. of farms with *Cryptosporidium* detected by IFAT	No. of farms with PCR-RFLP results[a]	No. of farms with PCR-RFLP speciation results
Dirty water	43	8	5	4
Cattle	80	72	51	46
Manure	63	10	9	7
Parlour	44	1	1	0
Pest faeces	54	15	11	8
Sheep	13	4	4	3
Spread slurry	34	3	2	1

[a] Including 'unclear pattern' results.

Table 6
Cryptosporidium species detected by RFLP, for each sample type. A proportion (>50%) of samples, including non-*C. parvum* species as determined by RFLP, all those with weak amplification or an unclear pattern, and a random selection of 10% of the *C. parvum* samples, were confirmed by ABI sequencing.

Sample type	C. andersoni	C. baileyi	C. bovis	Mixed C. bovis/C. parvum	Mouse genotype 1	C. parvum	C. ryanae	C. ubiquitum	Unclear pattern
Dirty water	0	0	0	0	0	6	0	0	1
Cattle	30	1	104	1	0	124	15	1	106
Manure	3	0	1	0	0	3	0	0	2
Parlour	0	0	0	0	0	0	0	0	1
Sheep	0	0	4	0	0	0	0	0	1
Spread slurry	1	0	0	0	0	0	0	0	1
Bird	0	0	0	0	0	3	0	0	6
Mice	1	0	0	0	1	0	0	0	1
Rats	0	0	0	0	0	0	0	3	1

C. parvum were detected in winter (38.6%) than in other seasons (12.5–25.4%) (OR_x^2 1.6 $P = 0.024$). In contrast, the proportion of *C. bovis* detected was consistent throughout the year (25.0–26.6%).

Comparisons of the PCR results from cattle samples shows a slight regional bias, with farms in Wales more likely to have at least one *C. bovis* sample detected (50.0% compared with 37.0% in Mid & West England and 37.5% in Northern England), although the difference was not significant (OR_x^2 1.7 $P = 0.365$).

Of the 52 farms that provided PCR results, 44 provided PCR results from more than one cattle group or sample type, and on 28 (64%) of these the same *Cryptosporidium* species was detected in more than one group or sample type. The identical species were predominantly *C. parvum* (12 farms) or *C. bovis* (9 farms), but also *C. andersoni* (2 farms), *C. bovis* and *C. parvum* (2 farms), *C. andersoni* and *C. bovis* (1 farm), *C. bovis* and *C. ryanae* (1 farm), *C. andersoni* and *C. parvum* (1 farm). The groups/sample types with matching *Cryptosporidium* species were most likely to be groups of calves (10 farms) or calves and adults (5 farms).

Nine farms had the same species (5 *C. parvum*, 3 *C. andersoni* and 1 *C. bovis*) detected in cattle and farm waste, out of a total of 12 farms that had PCR results from both sources (1/1 slurry, 3/4 dirty water and 5/7 manure). All the farms spread their own farm's manure on grazing fields and spread other animal waste on fields for grazing or for the production of fodder crops for cattle, whereas one farmer also spread manure from another farm which may be a *Cryptosporidium* transmission risk. For the five farms that had PCR results from rodent (rat or mice) and cattle samples, none of the *Cryptosporidium* species detected from rodents and cattle matched.

On three farms with species results for cattle and bird samples, all three had matching species. On two of the three farms where *C. parvum* was detected in both calf and bird samples, the farmer had responded that birds had access to calf and cattle accommodation on the farm, whereas on the other farm no access to birds had been noted. Three farms had species results for cattle and sheep samples. One farm detected *C. bovis* in sheep and cattle. The sheep were recorded as co-grazed together with cattle, with sheep reintroduced to cattle pastures after more than three weeks. On the two farms where the same *Cryptosporidium* species was not detected from the sheep and cattle samples, the particular groups with PCR results

were not co-grazed on the same land, although the sheep were grazed on other cattle pastures.

Where the same species was found in more than one cattle group, there were few specific contacts recorded between the groups by the farmer. Only one farm with matching groups reported that animals shared water/feed troughs and had nose-to-nose contact, while four other farms with matching groups had only nose-to-nose contact. In comparison, 6 farms had groups with nose-to-nose contact, which did not have the same *Cryptosporidium* species. All of the 23 farms that had matching species between groups, shared staff between those groups, whereas only 7 of the 11 farms where none of the groups had matching species shared staff ($P = 0.01$).

3.2. Results of GP60 microsatellite analysis

In total, 89 *C. parvum* samples were subtyped, originating from 33 farms. One sample was subtyped on 22 farms, with the remaining 11 farms having between 2 and 8 samples subtyped. The subtyped samples were from cattle faeces (81), dirty water (6) and bird samples (2), but none of the *C. parvum* manure samples were successfully subtyped. The most common subtype was IIaA15G2R1 which was detected in all three sample types, and the least common were IIaA15G2R0 and IIaA17G1R2, with the latter being a novel subtype not previously reported (Table 7). From the 11 farms that had multiple samples tested, three had more than one subtype detected in cattle: IIaA18G1R1 and IIaA16G3R1; IIaA15G2R0 and IIaA15G2R1; and IIaA15G2R2 and IIaA16G3R1.

On 4 of the 5 farms where more than 1 cattle group was tested, identical subtypes were detected in the different groups. The tested cattle groups for the farm that had non-identical subtypes were located separately, with one present on the main farm and the other on an out-farm. Only 1 farm had both cattle and dirty water tested and both sources had the same *C. parvum* subtype (IIaA15G2R1). One dirty water sample came from the drain outside the cattle group's accommodation, but the other came from a drain outside a cattle group that tested negative for *Cryptosporidium*. On the 2 farms where bird samples were tested, the subtypes (IIaA15G2R1 and IIaA19G1R1) matched the cattle subtypes. On both farms the bird samples were collected from the accommodation of a cattle group with the matching subtype, but the same subtype

Table 7
Summary of the numbers of *C. parvum* GP60 subtypes detected, by sample type.

Subtype	All samples	Cattle faeces samples	Dirty water samples	Bird samples
IIaA15G2R1	47	42	4	1
IIaA17G1R1	13	13	0	0
IIaA19G1R1	5	4	0	1
IIaA18G3R1	4	4	0	0
IIaA17G2R1	4	4	0	0
IIaA13G2R1	4	4	0	0
IIaA18G1R1	3	2	1	0
IIaA16G3R1	3	2	1	0
IIaA20G3R1	2	2	0	0
IIaA15G2R2	2	2	0	0
IIaA17G1R2	1	1	0	0
IIaA15G2R0	1	1	0	0

was also detected in more distant cattle groups on the farm.

4. Discussion

Cryptosporidium was found to be ubiquitous on dairy farms, with *Cryptosporidium* detected in almost all (92.5%) of the 80 sampled farms. The prevalence of *Cryptosporidium* in cattle was low overall but was high in young calves, especially those under one month of age. A trend of decreasing prevalence with increasing age of the cattle was observed. Another study in the same area showed a prevalence rate of 28% in young calves at any one time (Brook et al., 2008) which is lower than seen in other EU countries where 47.9% prevalence rates have been reported in beef and dairy calves under 22 days of age (Castro Hermida et al., 2002). The risk to people from young calves is higher as young calves were also more likely to be shedding *C. parvum* which is the species responsible for up to 50% of human cases (Chalmers et al., 2009), and young calves were found to be shedding larger concentrations of oocysts in the faeces samples. However, if the calves are housed indoors then the risk is predominantly to the handlers rather than the general public. Older calves and adult cattle were more likely to be shedding *C. bovis* and *C. andersoni*, respectively, for which the public health/zoonotic risk is low, although there is still a risk from those older calves and cattle shedding *C. parvum*. However, there is a possibility that a proportion of *C. bovis* results may have been *Cryptosporidium Xiao* as only circa 20% were confirmed by sequencing.

Co-grazed sheep were found to have a low prevalence (4%) of *Cryptosporidium* in this study, which was not always the case in other published studies, and *C. parvum* was not detected, which is also in contrast to previous studies (Mueller-Doblies et al., 2008). US and European studies have shown that the cross-sectional prevalence rate in ewes was 25% and in lambs was between 59% and 77% (Causapé et al., 2002; Santín et al., 2007). However, in a longitudinal study on a single UK farm, the rates were shown to be 6.4% in ewes and 12.9% in lambs (Sturdee et al., 2003). When assessing the prevalence rate detected in this study, consideration must be given to the lack of epidemiological data collected for the sheep samples (i.e. no ages were supplied), which may account for the prevalence being lower than expected. The detection of *C. bovis* in sheep was not unexpected, as it has been found in previous studies of both mixed sheep/cattle farms and sheep only farms (Mueller-Doblies et al., 2008).

The wildlife samples indicated that birds and rodents may be significant reservoirs of *Cryptosporidium* and a potential risk to people. The rodent samples had a high prevalence and had large concentrations of oocysts in the samples, although the *Cryptosporidium* species detected were not a known zoonosis risk and so rodent faeces may be of a lower risk to people. The high prevalence agrees with a longitudinal study completed on a single UK farm which showed the prevalence in small wild mammals (mainly rodents) living in and around farm buildings as 32.8% and living in areas of pasture as 29.9% (Sturdee et al., 2003). Mice have also been shown to be potential vectors of *Cryptosporidium* in other studies (Klesius et al., 1986) and the identification of *C. andersoni* in the mouse sample, confirmed by ABI sequencing (100% match to all sequenced *C. andersoni*), may also provide evidence that rodent faeces could be a source of infection to cattle. In the bird samples, a subtype of *C. parvum* known to be detected in human populations was found and birds may help spread infection to different buildings (potentially shown by the subtyping results detected in this study) and to other farms, and areas contaminated with their faeces may also pose a risk to people.

The results from the environmental sources provided further evidence of the survival of *Cryptosporidium* in waste. *Cryptosporidium* was found in dirty water and manure sources, and was also detected in some pasture samples where slurry had been spread. *C. parvum* was detected in dirty water and manure samples, and although *C. parvum* was not detected in a small number of microscopy-positive soil samples, the presence of a *Cryptosporidium* species detected in the cattle on the farm shows that this transmission route may be a risk to people who have contact with pasture. However, we are unable to state whether all *Cryptosporidium* positive waste samples were viable. Due to the larger quantities of farm waste that would generally be produced by adult cattle rather than calves, it is interesting that it was the *Cryptosporidium* species rarely found in adults that was mostly detected in the waste. Soil can be difficult to process using molecular techniques as it may contain inhibitory factors, although it has been shown that up to 17% of 782 soil samples taken from dairy farms in the USA contain *Cryptosporidium* oocysts (Barwick et al., 2003). This environmental risk

is particularly important in England and Wales, where the public have greater access to farm land than in many other countries (Countryside and Rights of Way Act 2000). It is interesting that the number of human cases from contact with recreational water is increasing (Yoder et al., 2012) and this may be related to run off from waste contaminating waterways. It may reasonably be concluded that muck heap management and slurry management prior to spreading can be considered an intervention point to reduce environmental contamination with benefits to both veterinary and public health.

In the UK, the number of human cases with *C. parvum* peaks in the spring, which coincides with the higher total *Cryptosporidium* prevalence detected from these dairy farms, potentially indicating a role in zoonotic transmission to people. However, the highest proportion of *C. parvum* was collected in the winter months, when the animals were kept indoors. The infected animals may pose a risk to staff on the farms, but would present less risk to the general public via contamination of fields and pathways.

The same *Cryptosporidium* species were often detected from multiple groups or sample types on the same farm. On more than half of the farms where this occurred, identical species were detected, which were mainly *C. parvum* or *C. bovis* extracted from calf groups or mixed groups of calves and adults. Interestingly, there was no specific contact (e.g. nose-to-nose contact) recorded that was different between groups with matching species and either non-matching groups on that same farm or on other farms, although sharing staff between groups appeared to increase the risk. This indicates that staff members moving between buildings without taking appropriate biosecurity measures, such as disinfecting boots or using pen-specific equipment, overalls and boots, may spread *Cryptosporidium* between pens.

The transmission analysis also shows that the same species were found in cattle and all farm wastes and bird samples, but rarely or not at all in sheep or rodents. This may indicate which sample types are involved in the transmission on the farm. However, it is not possible to determine the direction of the transmission, i.e. do cattle contaminate the waste sources, which in turn are spread on grazing land and infect grazing cattle. Birds have previously been shown to shed *C. parvum* (Quah et al., 2011), but this is the first time that the subtyping using the microsatellite sequences of the GP60 gene have matched to the circulating subtype on the farm. The matching of *Cryptosporidium* appeared to be related to proximity, with matching cattle and sheep being co-grazed and birds matching to cattle where birds could access the animal buildings.

The subtypes of the *C. parvum* shed by the cattle or detected in the environmental and bird samples were examined for similarity to other cattle populations in the UK/World as well as the human population. The top four subtypes detected in this study have been commonly reported in UK cattle populations, with fewer reports of IIaA20G3R1 and IIaA17G2R1 (Brook et al., 2009; Chalmers et al., 2005; Thompson et al., 2007). The subtype IIaA13G2R1 has been reported in cattle in Belgium (Geurden et al., 2007) and Netherlands (Wielinga et al., 2008) and IIaA15G2R2 in US (Xiao et al., 2007).

The main subtypes detected by this study were similar in proportion to those detected in the UK human population, with subtype IIaA15G2R1 the most commonly reported and IIaA17G1R1 also frequently reported (Chalmers et al., 2011). Three of the other subtypes have been detected in people in specific regions of England: IIaA19G1R1 in North West England; IIaA18G1R1 in East and South West England; and IIaA18G3R1 in South West England. The other subtypes detected in this study have not been published in any UK human study.

With the exception of one farm, where the cattle groups were located on distant sites, the same *C. parvum* subtype was found within individual farms. This may indicate a circulating effect of *Cryptosporidium* within farms, possibly with staff indirectly carrying contaminated faeces between buildings and wild birds moving between buildings and contaminating the environment via their faeces, or it could demonstrate persistence on the farm. However, the two farms with birds and cattle with matching subtypes were both IIaA15G2R1, which is the most common subtype found in this study and in the published literature. It should be noted that few human samples have been analysed using GP60 subtyping both in the UK and around the World and so the subtypes not currently reported in human populations should not be regarded as non-zoonotic subtypes, as they may just be rarer or not detected yet.

The use of a number of non-randomly selected farms may have introduced some bias; although the farms were selected for reasons unrelated to *Cryptosporidium* presence and were not significantly different in terms of the main size and management characteristics. However, the majority of enrolled farms were randomly selected from the study areas and no difference was found between participating farms and those that declined participation, and so it is believed that these farms were generally representative of the cattle farm population. Regional differences in climate and possibly management may occur in England and Wales, and although the study covered areas of high cattle herd density, the results may not represent all of the dairy farms in England and Wales. Collecting voided faeces rather than individual rectal samples may have biased the results, with the possibility that faeces from a single animal may be overrepresented from the samples from that pen. However, sampling the freshest faeces from a number of locations within the enclosure should have limited this possibility.

5. Conclusion

The important findings from this study have helped inform the level of risk of infection to people from dairy farms, with young calves being of particular risk due to a high prevalence, large oocyst concentrations and the identification of a proportion of *C. parvum* which commonly causes disease in the public. The results have also highlighted that *Cryptosporidium* contamination by rodents and birds may be a route of transmission to cattle and that *Cryptosporidium* survived well in farm waste and was detected in soil samples where waste had been spread, indicating another route of *Cryptosporidium* transmission between cattle and a possible risk to the general public. These

findings should help inform biosecurity practices on the farm and guide advice to visitors and staff who may have contact with young calves.

Conflict of interest statement

Acknowledgements

References

Barwick, R.S., Mohammed, H.O., White, M.E., Bryant, R.B., 2003. Prevalence of *Giardia* spp. and *Cryptosporidium* spp. of Dairy Farms in Southeastern New York State. Prev. Vet. Med. 59, 1–11.

Brook, E., Hart, C.A., French, N., Christley, R., 2008. Prevalence and risk factors for *Cryptosporidium* spp. infection in young calves. Vet. Parasitol. 152, 46–52.

Brook, E.J., Hart, C.A., French, N.P., Christley, R.M., 2009. Molecular epidemiology of *Cryptosporidium* subtypes in cattle in England. Vet. J. 179, 372–382.

Casemore, D.P., 1990. Epidemiological aspects of human cryptosporidiosis. Epidemiol. Infect. 104, 1–28.

Castro Hermida, J.A., Gonzalez Losada, Y.A., Ares Mazas, E., 2002. Prevalence of and risk factors involved in the spread of neonatal bovine cryptosporidiosis in Galicia (NW Spain). Vet. Parasitol. 106, 1–10.

Causapé, A.C., Quilez, J., Sánchez-Acedo, C., del Cacho, E., López-Bernad, F., 2002. Prevalence and analysis of potential risk factors for *Cryptosporidium parvum* infection in lambs in Zaragoza (northeastern Spain). Vet. Parasitol. 104, 287–298.

Chalmers, R.M., Ferguson, C., Cacció, S.M., Gasser, R.B., Abs El-Osta, Y.G., Heijnen, L., Xiao, L., Elwin, K., Hadfield, S., Sinclair, M., Stevens, M., 2005. Direct comparison of selected methods for genetic categorisation of *Cryptosporidium parvum* and *Cryptosporidium hominis* species. Int. J. Parasitol. 35, 397–410.

Chalmers, R.M., Elwin, K., Thomas, A.L., Guy, E.C., Mason, B., 2009. Long-term *Cryptosporidium* typing reveals the aetiology and species-specific epidemiology of human cryptosporidiosis in England and Wales, 2000 to 2003. Euro Surveill. 14, 2.

Chalmers, R.M., Giles, M., 2010. Zoonotic cryptosporidiosis in the UK – challenges for control. J. Appl. Microbiol. 109, 1487–1497.

Chalmers, R.M., Smith, R.P., Hadfield, S.J., Elwin, K., Giles, M., 2011. Zoonotic linkage and variation in *Cryptosporidium parvum* from patients in the United Kingdom. Parasitol. Res. 108, 1321–1325.

De Graaf, D.C., Vanopdenbosch, E., Ortega-Mora, L.M., Abbassi, H., Peeters, J.E., 1999. A review of the importance of cryptosporidiosis in farm animals. Int. J. Parasitol. 29, 1269–1287.

Elwin, K., Chalmers, R.M., Roberts, R., Guy, E.C., Casemore, D.P., 2001. Modification of a rapid method for the identification of gene-specific polymorphisms in *Cryptosporidium parvum* and its application to clinical and epidemiological investigations. Appl. Environ. Microbiol. 67, 5581–5584.

Feng, Y., Ortega, Y., He, G., Das, P., Xu, M., Zhang, X., Fayer, R., Gatei, W., Cama, V., Xiao, L., 2007. Wide geographic distribution of *Cryptosporidium bovis* and the deer-like genotype in bovines. Vet. Parasitol. 144, 1–9.

Geurden, T., Berkvens, D., Martens, C., Casaert, S., Vercruysse, J., Claerebout, E., 2007. Molecular epidemiology with subtype analysis of *Cryptosporidium* in calves in Belgium. Parasitology 134, 1981–1987.

Klesius, P.H., Haynes, T.B., Malo, L.K., 1986. Infectivity of *Cryptosporidium* sp isolated from wild mice for calves and mice. J. Am. Vet. Med. Assoc. 189, 192–193.

Mueller-Doblies, D., Giles, M., Elwin, K., Smith, R.P., Clifton-Hadley, F.A., Chalmers, R.M., 2008. Distribution of *Cryptosporidium* species in sheep in the UK. Vet. Parasitol. 154, 214–219.

Pritchard, G.C., Marshall, J.A., Giles, M., Chalmers, R.M., Marshall, R.N., 2007. *Cryptosporidium parvum* infection in orphan lambs on a farm open to the public. Vet. Rec. 161, 11–14.

Quah, J.X., Ambu, S., Lim, Y.A., Mahdy, M.A., Mak, J.W., 2011. Molecular identification of Cryptosporidium parvum from avian hosts. Parasitology 138 (5), 573–577 (Epub ahead of print).

Santín, M., Trout, J., Fayer, R., 2007. Prevalence and molecular characterization of *Cryptosporidium* and *Giardia* species and genotypes in sheep in Maryland. Vet. Parasitol. 146, 17–24.

Smith, R.P., Chalmers, R.M., Mueller-Doblies, D., Clifton-Hadley, F.A., Elwin, K., Watkins, J., Paiba, G.A., Hadfield, S.J., Giles, M., 2010. Investigation of farms linked to human patients with cryptosporidiosis in England and Wales. Prev. Vet. Med. 94, 9–17.

Sopwith, W., Osborn, K., Chalmers, R., Regan, M., 2005. The changing epidemiology of cryptosporidiosis in North West England. Epidemiol. Infect. 133 (5), 785–793.

Strong, W.B., Gut, J., Nelson, R.G., 2000. Cloning and sequence analysis of a highly polymorphic *Cryptosporidium parvum* gene encoding a 60-kilodalton glycoprotein and characterization of its 15- and 45-kilodalton zoite surface antigen products. Infect. Immun. 68, 4117–4134.

Sturdee, A.P., Bodley-Tickell, A.T., Archer, A., Chalmers, R.M., 2003. Long-term study of *Cryptosporidium* prevalence on a lowland farm in the United Kingdom. Vet. Parasitol. 116, 97–113.

Sulaiman, I.M., Hiram, P.R., Zhou., L., Al-Ali, Al-Shelahi, F.M., Shweiki, F.A., Iqbal, H.M., Khalid, J., Xiao, N.L., 2005. Unique endemicity of cryptosporidiosis in children in Kuwait. J. Clin. Microbiol. 43, 2805–2809.

Thompson, H.P., Dooley, J.S., Kenny, J., McCoy, M., Lowery, C.J., Moore, J.E., Xiao, L., 2007. Genotypes and subtypes of *Cryptosporidium* spp. in neonatal calves in Northern Ireland. Parasitol. Res. 100, 619–624.

Thursfield, M., Ortega, C., de Blas, I., Noordhuizen, J.P., Frankena, K., 2001. WIN EPISCOPE 2.0: improved epidemiological software for veterinary medicine. Vet. Rec. 148, 567–572.

Uhde, F.L., Kaufmann, T., Sager, H., Albini, S., Zanoni, R., Schelling, E., Meylan, M., 2008. Prevalence of four enteropathogens in the faeces of young diarrhoeic dairy calves in Switzerland. Vet. Rec. 163, 362–366.

Webster, K.A., Green, J., Dawson, C., Giles, M., Catchpole, J., 1996. Diagnostic methods for the detection of *Cryptosporidium parvum* in faeces. J. Protozool. Res. 6, 113–120.

Wielinga, P.R., de Vries, A., van der Goot, T.H., Mank, T., Mars, M.H., Kortbeek, L.M., van der Giessen, J.W., 2008. Molecular epidemiology of *Cryptosporidium* in humans and cattle in The Netherlands. Int. J. Parasitol. 38, 809–817.

Xiao, L., Alderisio, K., Limor, J., Royer, M., Lal, A.A., 2000. Identification of species and sources of *Cryptosporidium* Oocysts in Storm Waters with a small-subunit rRNA-based diagnostic and genotyping tool. Appl. Environ. Microbiol. 66, 5492–5498.

Xiao, L., Singh, A., Limor, J., Graczyk, T.K., Gradus, S., Lal, A., 2001. Molecular characterization of *Cryptosporidium* oocysts in samples of raw surface water and wastewater. Appl. Environ. Microbiol. 67, 1097–1101.

Xiao, L., Zhou, L., Santín, M., Yang, W., Fayer, R., 2007. Distribution of *Cryptosporidium parvum* subtypes in calves in eastern United States. Parasitol. Res. 100, 701–706.

Yoder, J.S., Wallace, R.M., Collier, S.A., Beach, M.J., Hlavsa, M.C., 2012. Cryptosporidiosis Surveillance – United States, 2009–2010. MMWR, CDC Surveillance Summaries 61 (SS05), 1–12 http://www.cdc.gov/mmwr/preview/mmwrhtml/ss6105a1.htm