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Assessing calves as carriers of *Cryptosporidium* and *Giardia* with zoonotic potential on dairy and beef farms within a water catchment area by mutation scanning

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Abbreviations: *gp60*, 60 kDa glycoprotein gene; *SSU*, small subunit of nuclear ribosomal RNA gene; *tpi*, triose phosphate isomerase gene; *pgp60*, partial *gp60*; *pSSU*, partial *SSU*; *ptpi*, partial *tpi*; *SSCP*, single-strand conformation polymorphism analysis; **REF**, restriction endonuclease fingerprinting; **BI**, Bayesian inference; **pp**, posterior probabilities.

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[Summary]

In the present study, we undertook a molecular epidemiological survey of *Cryptosporidium* and *Giardia* in calves on three dairy and two beef farms within an open drinking water catchment area (Melbourne, Australia). Faecal samples (n = 474) were collected from calves at two time points (five months apart) and tested using a PCR-based mutation scanning-targeted sequencing-phylogenetic approach, employing regions within the genes of small subunit of ribosomal RNA (designated pSSU), 60 kDa glycoprotein (*pgp60*) and triose phosphate isomerase (*tpi*) as genetic markers. Using pSSU, the *C. bovis*, *C. parvum*, *C. ryanae* and a new genotype of *Cryptosporidium* were characterised from totals of 74 (15.6%), 35 (7.3%), 37 (7.8%), 9 (1.9%) samples, respectively. Using *pgp60*, *C. parvum* genotype IIa subgenotype A18G3R1 was detected in 29 samples. Using *tpi*, *G. duodenalis* assemblage A and E were detected in totals of 10 (2.1%) and 130 (27.4%) samples, respectively. The present study showed that a considerable proportion of dairy and beef calves in this open water catchment region excreted *Cryptosporidium* (*i.e.* subgenotype IIaA18G3R1) and *Giardia* (*e.g.*, assemblage A) that are consistent with those infecting humans, inferring that they are of zoonotic importance. Future work should focus on exploring, in a temporal and spatial way, whether these parasite occur in the environment and water of the catchment reservoir.

Keywords:

Cryptosporidium / *Giardia* / SSCP analysis / Restriction endonuclease fingerprinting

1 Introduction

Waterborne, zoonotic infections caused by enteric pathogens represent a significant risk to human health worldwide. Various pathogens that can be transmitted by contaminated water include bacteria, viruses and parasites. Among the parasitic agents are species of *Cryptosporidium* and *Giardia*, which are common gastrointestinal pathogens of a wide range of vertebrates, including humans, and significant causes of diarrhoeal disease [1-4]. Parasites of both of these genera produce infective stages (oocysts or cysts), which are released in the faeces from the infected host and are transmitted *via* contaminated food or water sources, or by direct contact [4, 5].

These protozoa have been increasingly recognised as significant waterborne pathogens due to the number of epidemics linked to drinking and recreational water in developed countries over past few decades [6-8]. Significant morbidity and mortality have also been reported in infants and older children in developing countries due to poor sanitation and inadequate access to clean and safe drinking water [9]. As a result, determining the sources of water contamination and maintaining water quality remains a crucial issue for water authorities throughout the world [1, 2, 10].

Cryptosporidium and *Giardia* are usually recalcitrant to common approaches and disinfectants used for the treatment of drinking water, which makes them a major risk to the water industry [11]. Common strategies used to reduce the risk of waterborne transmission are water treatment by sedimentation, flocculation and/or routine monitoring of these parasites as well as restricting access of humans as well as wild and domesticated animals (which may harbour the parasites) to catchments. The latter aspect is challenging particularly in vast, natural and forested catchment areas.

The city of Melbourne in Victoria, Australia, is one of the largest developed cities in the world that receives largely unfiltered drinking water from protected wilderness catchment areas [12, 13],

which are pivotal to the commitment of Melbourne Water Corporation (MWC) to provide low cost, safe drinking water. MWC owns and manages ten main catchments [13, 14], and relies mainly on long water retention times and minimal water treatment, rather than intensive methods (*e.g.*, using ultraviolet irradiation, ozonation or filtration). MWC's strategy requires a rigorous program of testing and monitoring of water and of animals in catchments for pathogens of zoonotic potential. Because only a relatively small number of species/genotypes of *Cryptosporidium* and *Giardia* are recognized to infect humans, and traditional methods of detection do not allow the accurate identification of these zoonotic agents [15, 16], PCR-based methods have been used to support MWC's management strategy.

In an ongoing program with MWC, we have been monitoring animals in some natural catchment areas (including Cardinia, Greenvale, Maroondah, O'Shannassy, Silvan, Sugarloaf, Thomson, Upper Yarra and Yan Yean) for *Cryptosporidium* and *Giardia* using our PCR-based tools [13]. We have shown a relatively low prevalence of these protozoa, particularly zoonotic genotypes, but some localized hot-spots have been identified [13, 17]. This low prevalence in these closed catchments likely relates to limited access of humans and other key reservoir animals (*e.g.*, cattle) for zoonotic genotypes of these protozoa. However, there is one open catchment (called Tarago) in which cattle are farmed extensively, but in which there has been no study of *Cryptosporidium* and *Giardia*. As various studies have shown that cattle, particularly young calves, can represent major reservoirs of zoonotic *Cryptosporidium* and *Giardia* [18, 19], it was pertinent to explore the nature and extent of these pathogens in calves on selected farms located in relative close proximity to the Tarago water reservoir. Therefore, in the present study, we undertook an epidemiological survey of these parasites in calves on three dairy and two beef farms within this catchment area using molecular diagnostic and analytical tools. We employed a PCR-based mutation scanning approach, combined with targeted DNA sequencing, for their specific and genotypic identification.

2 Materials and methods

2.1 Sample collection, calves and management practices

Faecal samples (n = 474) were collected from calves (mixed sexes), including 293 and 181 samples from calves of ≤ 3 and 3-9 months of age), on dairy farms A-C and beef farms D and E located within the Tarago catchment in November 2011 and April 2012, corresponding to two consecutive calving seasons (spring and autumn). At least 50 calves were present on each of the five farms at each visit. Freshly deposited faecal samples were collected from paddocks or enclosures in which calves were reared. On dairy farms A-C, calves were maintained together and fed whole milk, whereas on beef farms D and E, calves were reared with/by their dams. Calves were born *in situ* on farms A, B, D and E, whereas calves on farm C were introduced, at 3 months of age, from various dairy farms in Gippsland, Victoria. Calves on farm B were kept in community pens until the age of eight weeks, whereas calves on farms A, C, D and E were raised and maintained on paddocks from birth. Calf pens on farm B were bedded with saw dust and were located near the calving area. Calves born on this farm were separated from their dam within 6-24 h.

2.2 DNA isolation, PCR amplification and sequencing

Genomic DNA was extracted from individual faecal samples using the PowerSoil DNA isolation kit (MoBIO, USA), as described by Abeywardena et al. [20], and then frozen at -20 °C until molecular testing. Each genomic DNA sample was tested specifically for the presence of *Cryptosporidium* and *Giardia* DNA, employing genetic markers (designated pSSU, *pgp60* and *ptpi*) in the small subunit (SSU) nuclear ribosomal RNA gene, 60 kDa glycoprotein (*gp60*) and

triose phosphate isomerase (*tpi*) genes, respectively. For the amplification of pSSU, nested PCR was performed in a 50 µl volume containing 2.0 mM of MgCl₂, 200 µM of each deoxynucleotide triphosphate (dNTP), 25 pmol of each oligonucleotide primer and 1.25 U of *MangoTaq* polymerase in a standard PCR buffer (Bioline, USA). In the primary reaction of SSU for *Cryptosporidium*, primers XF2 (forward: 5'-GGAAGGGTTGTATTTATTAGATAAAG-3') and XR2 (reverse: 5'-AAGGAGTAAGGAACAACCTCCA-3') [21] were employed, and primers pssu-f (forward: 5'-AAAGCTCGTAGTTGGATTTCTGTT-3') and pssu-r (reverse: 5'-ACCTCTGACTGTAAATACRAATGC-3') were utilised for the secondary reaction to amplify pSSU (~ 240 bp) [22]. The cycling protocol for the primary amplification included an initial cycle of 94 °C/5 min (initial denaturation), followed by 30 cycles of 94 °C/45 s (denaturation), 45 °C/2 min (annealing), and 72 °C/1.5 min (extension) and a final extension of 72 °C/10 min. From 1 µl of primary amplicon, the nested amplification was performed using a cycling protocol of 94 °C/5 min (initial denaturation), followed by 35 cycles of 94 °C/30 s (denaturation), 55 °C/30 s (annealing) and 72 °C/30 s (extension), followed by a final extension of 72 °C/10 min.

For the amplification of *pgp60* and *ptpi*, nested PCR was performed in a 50 µl volume containing 3.0 mM of MgCl₂, 200 µM of each deoxynucleotide triphosphate (dNTP), 25 pmol of each oligonucleotide primer and 1.25 U of *GoTaq* polymerase in standard PCR buffer (Promega, USA). In the primary reaction of *gp60* for *Cryptosporidium*, primers gp15-ATG (forward: 5'-ATGAGATTGTGCGCTCATTATC-3') and gp15-STOP (reverse: 5'-TTACAACACGAATAAGGCTGC-3') [23] were employed, and primers gp15-15A (forward: 5'-GCCGTTCCACTCAGAGGAAC-3') and gp15-15E (reverse: 5'-CCACATTACAAATGAAGTGCCGC-3') [24] were utilised for the secondary reaction to amplify *pgp60* (350-450 bp). The cycling protocol for the primary amplification included an initial cycle of 94 °C/5 min (initial denaturation), followed by 35 cycles of 94 °C/30 s (denaturation), 55 °C/45 s (annealing), and 72 °C/60 s (extension) and a final extension of 72 °C/10 min. From 1 µl of primary

amplicon, *pgp60* was amplified using a cycling protocol of 94 °C/5 min (initial denaturation), followed by 30 cycles of 94 °C/30 s (denaturation), 55 °C/30 s (annealing) and 72 °C/30 s (extension), followed by a final extension of 72 °C/10 min. In the primary reaction for *tpi* of *Giardia*, primers AL3543 (forward: 5'-AAATTATGCCTGCTCGTCG-3') and AL3546 (reverse: 5'-CAAACCTTTTCCGCAAACC-3') were employed, whereas primers AL3544 (forward: 5'-CCCTTCATCGGIGGTAACCTT-3') and AL3545 (reverse: 5'-GTGGCCACCACTCCCGTGCC-3') were utilised in the secondary reaction to amplify *tpi* (530 bp) (*cf.* Nolan et al.; ref. [25]). The cycling protocol for primary amplification included a cycle of 94°C/5 min (initial denaturation), followed by 35 cycles of 94 °C/45 s (denaturation), 50 °C/45 s (annealing) and 72 °C/60 s (extension), followed by a final extension of 72 °C/10 min. The secondary PCR was identical, except that the annealing temperature was 60 °C.

2.3 Mutation scanning, direct sequencing and analyses

For all amplicons of SSU and *gp60*, single-strand conformation polymorphism (SSCP) analysis [26] was carried out as described previously [27]. For *tpi* amplicons, restriction endonuclease fingerprinting (REF) was employed using the enzyme *RsaI* (Promega) [25]. This enzyme was selected because of its potential ability to cleave the *tpi* sequences of *G. duodenalis* assemblages A to G. Amplicons representing each banding profile were selected and treated with exonuclease I and shrimp alkaline phosphatase (Fermentas), according to the manufacturer's instructions, and then sequenced in both directions by direct, automated sequencing (BigDye Terminator v.3.1 chemistry, Applied Biosystems, USA), using the same primers employed in secondary PCR. The quality of each sequence was assessed based on the corresponding electropherogram using the program BioEdit [28], and the sequences determined were compared with known reference

sequences using the Basic Local Alignment Search Tool (BLAST; <http://www.ncbi.nlm.nih.gov/BLAST>).

For each *Cryptosporidium* and *Giardia*, the sequences determined herein were compared with selected, publicly available sequences. Sequences were aligned using the program BioEdit, and the resultant alignments were improved manually. Phylogenetic analysis of sequence data was performed using the Bayesian Inference (BI) tree building method in MrBayes 3.1.2 [29, 30]. Posterior probabilities (pp) were calculated *via* 1 000 000 (pSSU) or 2 000 000 generations (*ptpi*), utilizing four simultaneous tree-building chains, with every 100th tree being saved. At this point, the standard deviation of split frequencies was < 0.01, and the potential scale reduction factor (PSRF) approached one. A 50% majority rule consensus tree for each analysis was constructed based on the final 75% of trees generated by BI. Categorical data were analysed using the Fisher's exact test in the program WINPEPI [31]. Odds ratios (OR) and their 95% confidence intervals (CIs) were calculated to test for associations between the likelihood that samples tested positive and all variables for which data were available, by constructing a multivariable logistic regression model in the SPSS statistical package (<http://www-01.ibm.com/software/analytics/spss/>).

2.4 Cloning of PCR products and sequencing

Selected pSSU amplicons were cloned and then sequenced, as described previously [32]. In brief, amplicons were purified using minicolumns (Wizard PCR Preps DNA Purification System, Promega) and ligated into the pGEM-T-Easy vector (Promega) and *Escherichia coli* (α -select chemically competent cells, Bioline). Then, the cells were plated on to agar plates and incubated overnight at 37 °C. Ten colonies were picked from each plate and grown at 37 °C overnight for plasmid purification (using Wizard Plus SV Minipreps DNA Purification System, Promega). Thereafter, inserts were amplified using the primers pssu-f and pssu-r (sub-section 2.2), and

amplicons subjected to SSCP analysis. Multiple amplicons representing each of the profiles displayed by SSCP were sequenced as described in sub-section 2.3.

3 Results

3.1 *Cryptosporidium*

All 474 genomic DNA samples were subjected to genetic analysis for *Cryptosporidium*. For pSSU, amplicons were produced from 155 of these samples. SSCP analysis of all amplicons displayed five profiles (Fig. 1). In total, 45 amplicons representing four of the five banding profiles were sequenced directly. These sequences (240 bp in length) were compared with reference sequences (GenBank accession nos. EF493324, AY741305, EU410344 and AF108861 for *C. parvum*, *C. bovis*, *C. ryanae* and *C. suis*, respectively). Based on this comparison, *C. parvum* was detected in 35 (7.3%), *C. bovis* in 74 (15.6%), *C. ryanae* in 37 (7.8%) and a new genotype of *Cryptosporidium* in nine (1.9%) of the 155 samples tested. The pSSU sequence (accession no. KC778530) of this new genotype was 99% similar to that of *C. suis* (accession no. AF108861) (Table 1). An attempt was made to directly sequence eight of the 22 amplicons representing the fifth SSCP profile. However, complex chromatograms indicated the presence of multiple types of sequence within each of the eight amplicons. Therefore, three representative pSSU amplicons were cloned and sequenced. For each amplicon, 10 clones were selected for PCR-based SSCP analysis to display all of the sequence variation among all 30 clones. Three distinct sequence types (accession nos. KC778529, KC778534 and KC778535) represented all three amplicons (*cf.* Fig. 1; lanes 6-8). These sequence types were identical to three publicly available reference sequences (accession nos.

EU410344, GU124622 and GU124627) representing *C. ryanae*. Phylogenetic analysis supported the classification of the species and genotypes of *Cryptosporidium* (Fig. 2).

Based on these findings, *C. bovis* was detected in 74 of the 474 samples from all five farms, followed by *C. ryanae* (n = 37) from farms A, B, C and E, *C. parvum* (35) from farms B, D and E, and the new genotype of *Cryptosporidium* with similarity to *C. suis* (9) on farm C (Table 1). *C. parvum* was detected exclusively in samples from calves of ≤ 3 months of age. As species and genotypes of *Cryptosporidium* known to infect humans can be characterised further to the sub-genotypic level based on their *gp60* sequence, *pgp60* amplicons produced from the 29 samples from farms B, D and E were subjected to SSCP analysis. No profile variation was detected, and two amplicons were sequenced. This analysis revealed *C. parvum* IIaA18G3R1 based on a perfect sequence match to a publicly available reference sequence (accession no. AF203016). Phylogenetic analysis supported the classification of samples as this sub-genotype (Fig. 3).

These results were then put into the epidemiological context (Table 2). *Cryptosporidium* was detected in 93 and 62 of 155 samples tested at the first (spring) and the second (autumn) samplings, but there was no significant difference in numbers of test-positive between the samplings. *Cryptosporidium* was detected in at least one sample on all five farms at both collections, and in 35% and 29% of samples at the first and second samplings, respectively. *Cryptosporidium* was detected least (11% and 5%) in samples from beef farm D (beef) and most (72% and 80%) in samples from dairy farm B at the first and second samplings, respectively. Samples collected from dairy farms were 4.33 times (95% CI: 2.72, 7.00) more likely to have *Cryptosporidium* than samples collected from beef farms in the same geographical region.

3.2 *Giardia*

All 474 faecal genomic DNA samples were subjected to genetic analysis for *Giardia*. SSCP analysis of all 140 *ptpi* amplicons revealed 27 distinct profiles. Sequencing of 41 amplicons representing all of these profiles defined 27 distinct sequences (GenBank accession nos. KC778536-KC778562). There was no nucleotide variation among multiple amplicons representing each profile. Twenty six of the 27 sequences were consistent (94-97% similar) with a reference sequence (accession no. AF069559) representing *G. duodenalis* assemblage E (Table 3). The one other sequence was identical to a reference sequence (accession no. L02120) for *G. duodenalis* assemblage A. Phylogenetic analysis supported the classification of samples as *G. duodenalis* assemblage E or A (Fig. 4).

These results were then put into the epidemiological context (Table 2). *G. duodenalis* assemblage E was detected in 130 of the 474 (27.4%) samples from all five farms, and assemblage A in 10 samples from farms A, B, C and E. *Giardia* was detected in 97 and 43 of 140 samples tested at the first (spring) and the second (autumn) samplings, respectively. The number of samples test-positive for *Giardia* was significantly higher at the first time point than the second (OR: 2.5; 95% CI: 1.587, 4.108). *Giardia* was identified in at least one sample on all five farms at both samplings. *Giardia* was detected in 37% and 20% of samples at the first and second samplings, respectively. The highest percentages of test-positive samples were detected on dairy farm B (52%) and beef farm D (33%) at the first and second samplings, respectively. The odds of samples testing positive for *Giardia* from dairy farms was 49% higher than samples from beef farms in the same region (OR: 1.49; 95% CI: 0.97, 2.29).

4 Discussion

Cryptosporidium and *Giardia* were detected in 32.9 % (95% CI: 28.8, 37.2) and 29.5 % (95 % CI: 25.5, 33.7), respectively, of 474 faecal samples collected at two time points, five months apart,

from calves on five farms in the Tarago catchment area. Overall, *C. bovis* was detected most frequently (47.7%), and was more common in calves of > 3 months of age than younger calves, consistent with previous studies from other countries around the world, including Denmark, France, Nigeria, the UK and the USA [33-37]. *C. ryanae* was detected in 37 of 155 *Cryptosporidium* test-positive faecal samples. Most (n = 33) of these samples were from calves of ≤ 3 months of age, whereas four were from older calves (> 3 months). Although 15 samples contained *C. ryanae* whose pSSU sequence matched perfectly the sequence with GeneBank accession no. EU410344, population variants of *C. ryanae* were detected within 22 faecal samples. Genetic variation within *C. ryanae* has been recorded previously [38-40], but multiple variants of *C. ryanae* within individual samples have not been reported previously. Interestingly, a new genotype of *Cryptosporidium* with 99% similarity in pSSU sequence to that of *C. suis* (GeneBank accession no. AF108861) was identified in nine of 474 faecal samples. While the zoonotic potential of this new genotype is unknown in Australia, it has been identified previously in faecal samples from sporadic human cases from Canada (accession nos. AY030084 and AY030085; ref. [41] and the UK (accession no. HQ822146; ref. [42]) as well as from calves in Denmark (accession no. DQ182599; ref. [33]), India (accession no. GQ345008; ref. [43]) and adult cattle in the UK (accession no. HQ822134; ref. [42]). The validity of this genetic variant is also supported by sequence data for the heat shock protein 70 (*hsp70*) and actin genes (*cf.* [42]).

C. parvum was detected exclusively in calves of ≤ 3 months of age, which is also consistent with previous findings of studies of both dairy and beef calves around the world [37, 42, 44-46]. Here, at both samplings, most (82.9% of 35) *C. parvum* cases related to dairy farm B, and 14.3% and 2.9% the beef farms D and E, respectively. Importantly, the dominant subgenotype, IIaA18G3R1, is commonly found in both cattle and humans, and is thus considered of major zoonotic significance [47, 48]. The odds of detecting *C. parvum* in samples from dairy farm B were 7.57 times higher (95% CI: 3.69, 15.5) than those in the reference group (farm E) (Table 2). This finding likely

relates to the difference in husbandry on farm B compared with other farms; indeed, calves on this farm were reared at relatively high density in pens with saw dust bedding up to eight weeks of age, and were kept in paddocks thereafter. In contrast, calves on farms A and C were held in paddocks (0.5-2 ha), and beef calves were maintained with their dams in much larger paddocks (2-4 ha). The crowding factor and contaminated bedding are likely to have contributed significantly to increased cycling of *C. parvum* among calves on farm B, consistent with previous observations [49-51].

Although most (92.8%) *Giardia* test-positive faecal samples contained *G. duodenalis* assemblage E, in agreement with the studies conducted in dairy and beef cattle in other states of Australia [52, 53] and around the world [54-56], *G. duodenalis* assemblage A was identified in the minority of test-positive samples. All of the sequences representing assemblage A in this study were identical to one another and were also the same as a publicly available *ptpi* sequence (GenBank accession no. L02120) representing this assemblage. Of the 10 samples test-positive for this assemblage, eight were from calves on dairy and two from calves on beef farms. Surprisingly, this is the first molecular characterization of *Giardia* in cattle in the state of Victoria, Australia. For both samplings, the number of samples test-positive for *Cryptosporidium* was observed to be higher on dairy farms than beef cattle farms, consistent with results from various previous studies [44, 50, 57]. The higher occurrence in dairy calves is most likely explained by environmental and/or host factors. Environmental factors can include crowding (high population density) of dairy calves in enclosed pens or paddocks and the associated accumulation of oocyst/cyst contamination from infected calves. An important host factor might include a lack of passive immunity in some calves. Dairy calves are quite often prematurely separated from their mother, so that they are unable to access colostrum within the first critical hours after birth [58]; in contrast, beef calves usually have immediate and ready access to colostrum, thus giving them adequate passive immunity to resist infections early in life [59]. In addition, the proportions of samples test-positive for *Giardia* were higher in the first (spring) sampling than in the second (autumn) sampling. This

difference may be due to confounding by age in that most calves (72.5% of 262) in the first cohort were 2-3 months of age, whereas most (75% of 212) in the second cohort were < 1 month of age. According to current literature [52, 60, 61], *Giardia* infection is most prevalent in calves of 5-10 weeks of age.

In conclusion, the present study showed that a considerable proportion of dairy and beef calves living in this open water catchment region (Tarago) did harbour, at particular time points, genetic types of *Cryptosporidium* (subgenotype IIaA18G3R1) and *Giardia* (assemblage A) that are consistent with those infecting humans, inferring that they are of zoonotic importance. This finding emphasizes the need for further work on assessing levels of oocyst/cyst contamination in areas surrounding calf enclosures/paddocks, in waste water run-off or creeks/water ways flowing from the farms to water reservoirs. Moreover, it will be particularly important to investigate, in a temporal and spatial way, the presence of the vegetative stages of these parasites in water from the Tarago catchment and to establish (if present) whether they match genetically those found in calves on the farms studied here.

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Table 1. Details on numbers of faecal samples collected from calves raised on three dairy (A-C) and two beef (D-E) farms (samplings 1 and 2) in consecutive calving seasons.

Farm	Sampling	Total	<i>Cryptosporidium</i> species (accession nos.)					<i>Giardia duodenalis</i> assemblage
			<i>C. parvum</i> (KC778531)	<i>C. bovis</i> (KC778533 ^a & KC778532 ^b)	<i>C. ryanae</i> (KC778529)	<i>C. ryanae</i> & two variants (KC778529, KC778534 & KC778535)	New genotype (KC778530)	A (KC778562)
A	1 (spring)	50	0	5	6	16	0	0
	2 (autumn)	27	0	2	0	0	0	3
B	1	50	0	28	2	6	0	0
	2	36	29	0	0	0	0	2
C	1	57	0	13	1	0	0	0
	2	53	0	6	0	0	9	3
D	1	65	1	6	0	0	0	0
	2	39	0	2	0	0	0	0
E	1	40	0	9	0	0	0	0
	2	57	5	3	6	0	0	2
Total		474	35	74	15	22 ^c	9	10

Cryptosporidium species/genotypes and *Giardia duodenalis* assemblages in these samples were classified by scanning and targeted sequencing of the loci pSSU (*Cryptosporidium*) and ptpi (*Giardia*). GenBank accession numbers are in parentheses.

^a *C. bovis* sequence type 1 (detected in 73 amplicons originating from all five farms)

^b *C. bovis* sequence type 2 (detected in one amplicon originating from farm C)

^c Mixed populations of *C. ryanae* and two variants of this species (see Table 3) inferred in all 22 samples.

^d cf. Table 3

Table 2. Results of multivariable logistic regression analyses to test for associations between the presence of *Cryptosporidium* or *Giardia*, calf age, sampling time point and farm.

Variable	Category	<i>Cryptosporidium</i> species			<i>Giardia duodenalis</i>		
		Odds ratio	95% CI	<i>P</i> -value	Odds ratio	95% CI	<i>P</i> -value
Age category							
	≤ 3 months	1.14	0.51, 2.57	0.745	1.70	0.84, 3.47	0.143
	> 3 months	1			1		
Calving period							
Sampling	1 (spring)	1.35	0.81, 2.24	0.247	2.55	1.59, 4.11	<0.001
	2 (autumn)	1			1		
Farm				<0.001			0.348
	A (dairy)	1.59	0.73, 3.46		1.71	0.79, 3.70	
	B (dairy)	7.57	3.69, 15.5		1.31	0.64, 2.70	
	C (dairy)	1.12	0.52, 2.41		1.44	0.69, 3.04	
	D (beef)	0.25	0.11, 0.61		0.96	0.47, 1.94	
	E (beef)	1			1		

Table 3. The numbers of samples from individual farms (at each sampling) with particular sequence types (GenBank accession nos. indicated in round parentheses). Twenty six different *ptpi* sequences representing assemblage E of *Giardia duodenalis* (cf. Table 1) detected in faecal samples from calves from three dairy (A-C) and two beef (D and E) farms collected on two occasions (samplings 1 and 2) in consecutive calving seasons.

Farm	Sampling	Numbers of samples (accession nos. KC778536 –KC778561; 26 distinct sequences)	Subtotal
A	1	13 (KC778536), 7 (KC778537), 2 (KC778541), 1 (KC778540), 1 (KC778554), 1 (KC778546)	25
	2	5 (KC778537)	5
B	1	8 (KC778536), 6 (KC778537), 4 (KC778538), 2 (KC778542), 2 (KC778548), 1 (KC778554), 1 (KC778545), 1 (KC778553), 1 (KC778547)	26
	2	1 (KC778536)	1
C	1	15 (KC778536), 5 (KC778537), 1 (KC778543), 1 (KC778549), 1 (KC778550), 1 (KC778556)	24
	2	1 (KC778537)	1
D	1	6 (KC778537), 4 (KC778536), 2 (KC778557), 1 (KC778539), 1 (KC778556), 1 (KC778561), 1 (KC778552)	16
	2	5 (KC778537), 4 (KC778536), 1 (KC778561), 1 (KC778558), 1 (KC778542), 1 (KC778554)	13
E	1	3 (KC778536), 1 (KC778554), 1 (KC778555), 1 (KC778544)	6
	2	10 (KC778536), 1 (KC778559), 1 (KC778560), 1 (KC778543)	13
Total			130

FIGURES

Figure 1. Representative examples of SSCP analyses of pSSU or pgp60 amplicons (*Cryptosporidium*), and REF analysis of ptpi amplicons (*Giardia*). Lanes 1-5: profiles representing *C. parvum*, *C. bovis*, a new genotype of *Cryptosporidium*, *C. ryanae*, and *C. ryanae* plus a mix of genetic variants of *C. ryanae*, respectively (cf. Table 1). Lanes 6-8: profiles for amplicons from individual clones representing *C. ryanae* and two *C. ryanae* variants (cf. Supplementary Table 1). Lanes 9-11: three profiles representing *C. parvum* genotype IIa subgenotype A18G3R1 (linked to GenBank accession no. KC778528); no evidence of sequence variation. Lanes 12-18: display of sequence variation in ptpi among selected amplicons representing *G. duodenalis* assemblage E (linked to accession nos. KC778536-KC778561, respectively; cf. Table 3).

Figure 2. Phylogenetic relationship among pSSU sequences of *Cryptosporidium* based on Bayesian Inference (BI) analysis. The seven distinct pSSU sequences determined in the present study and 39 reference sequences representing *Cryptosporidium* (Supplementary Table 1) were included in the analysis. *Eimeria acervulina* and *E. maxima* sequences were used as outgroups. Accession numbers of publicly available sequences are indicated. In bold-type are accession numbers of sequences determined in the present study, and numbers of samples with these sequences (in parentheses). Posterior probabilities (pp) are indicated at all major nodes.

Figure 3. Phylogenetic relationship among *pgp60* sequences of *Cryptosporidium parvum* based on Bayesian Inference (BI) analysis. One sequence determined in the present study, and 26 reference sequences representing *C. parvum* genotypes IIa-IIk (Supplementary Table 2) were included in the analysis. A sequence representing *C. hominis* genotype Ib was used as an outgroup. In bold-type are accession numbers of sequences determined in the present study, and numbers of samples with these sequences (in parentheses). Posterior probabilities (pp) are indicated at all major nodes.

Figure 4. Phylogenetic relationships among *ptpi* sequences of *Giardia duodenalis* based on Bayesian Inference (BI) analysis. Twenty six sequences determined in the present study, and 38 reference sequences representing *G. duodenalis* assemblages A to G (Supplementary Table 3) were included in the analysis. Sequences representing *G. ardeae*, *G. muris* and *G. microti* were used as outgroups. In bold-type are accession numbers of sequences determined in the present

study, and numbers of samples with these sequences (in parentheses). Posterior probabilities (pp) are indicated at all major nodes.

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