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ORIGINAL PAPER

Seasonal variation in the prevalence and molecular epidemiology of *Cryptosporidium* infection in dairy cattle in the New York City Watershed

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Abstract We conducted cross-sectional studies in the New York City Watershed to ensure a valid estimate of the risk associated with *Cryptosporidium* infection in dairy herds. Our aims were to obtain species-specific estimates of the prevalence of *Cryptosporidium* in dairy cattle and to investigate seasonal variations in prevalence. We validated our empirical estimates using a Bayesian approach. Samples were collected on 32 study farms, once in each of 3 different seasons using an age-stratified sampling design. The overall prevalence of *Cryptosporidium parvum*-like species and *Cryptosporidium andersoni* among the 1911 animals tested by the flotation method was 5% and 1%, respectively. Among preweaned calves (<65 days of age), the prevalence of *C. parvum*-like species was twice as high in the summer (26%) compared with the winter (11%). Herd prevalence showed the same seasonal trend. Preweaned calves were also shedding *C. andersoni* at an average intensity of 20 oocysts per gram of feces. We did not detect *C. parvum*-like oocysts in cattle older than 5 months. Sequencing of a portion of the 18s rRNA gene revealed that in the summer, 42% of the *C. parvum*-like oocysts shed by preweaned calves were zoonotic, compared with >74% during the rest of the year. Both empirical and stochastic methods revealed a summer peak in the prevalence of *C. parvum*-like oocysts in preweaned calves. Determining whether seasonal variation in the prevalence and proportion of *Cryptosporidium* species shed by preweaned calves is due to management practices or ecological factors will have important implications for effective control of this parasite.

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Introduction

Cryptosporidiosis is an emerging waterborne protozoan infection affecting humans and livestock worldwide (de Graaf et al. 1999; Xiao and Feng 2008). Cattle are commonly infected by four *Cryptosporidium* species: *C. parvum*, *C. bovis*, and *C. ryanae* (formerly the deer-like genotype) in the intestine, and *C. andersoni* in the abomasum (Fayer et al. 2005, 2007, 2008; Xiao et al. 2007b). A recent study showed that these *Cryptosporidium* species in cattle are age-related. *C. parvum*, the only prevalent zoonotic species, is responsible for about 85% of the infections in preweaned calves, whereas postweaned and adult cattle are mostly infected with the host-specific *C. bovis*, *C. andersoni*, and *C. ryanae* (Fayer et al. 2006; Santin et al. 2004). These findings demonstrate that only neonatal calves are important sources of zoonotic cryptosporidiosis in humans, and it is this age group that is mostly affected by cryptosporidiosis in terms of prevalence of infection and associated morbidity and mortality (Xiao et al. 2007b). This information is critical for the design of cost-effective strategies to decrease the risk of this pathogen in dairy cattle populations.

One of the main challenges of quantifying the risk of *C. parvum* infections in cattle is that most studies use traditional diagnostic methods such as flotation that are capable of identifying *C. andersoni*, but molecular techniques are needed to distinguish the zoonotic *C. parvum* from the nonzoonotic *C. bovis* and *C. ryanae*. Thus, when traditional diagnostic methods such as microscopy are used, it is more accurate to commonly refer to C. *parvum*, *C.*

bovis, and *C. ryanae* as the "C. parvum-like" species (Fayer 2004; Santin et al. 2004; Silverlas et al. 2009; Starkey et al. 2005). However, for the purposes of risk assessment and risk mitigation, it is critical to differentiate between zoonotic and nonzoonotic species of this pathogen and to obtain valid and reliable estimates of their occurrence in cattle populations.

Various authors have reported contradictory findings regarding seasonality of the risk of *Cryptosporidium* infection in cattle populations. A number of studies conducted in New York State and other regions with similar climatic conditions indicated that winter is the greatest risk (Hamnes et al. 2006; Huetink et al. 2001; Mohammed et al. 1999), while others reported increased prevalence during the summer (Garber et al. 1994; Trotz-Williams et al. 2007) or no significant seasonal pattern of shedding of this protozoa (Starkey et al. 2005). Season itself may be a risk factor that is not amenable to intervention, but determining the presence of seasonal variation is of importance because the observed pattern might be associated with modifiable management practices. Hence, intervention strategies may be preferentially applied in high-risk months to effectively decrease the risk of infection.

While the Catskill/Delaware portion of the New York City (NYC) Watershed is home to approximately 200 dairy farms, it provides over 80% of NYC's drinking water that is largely unfiltered. Extensive efforts and resources are being invested to maintain the quality of the NYC drinking water and at the same time sustain the agricultural viability of the region. For these efforts to continue to be successful, it is essential to accurately quantify the risk that these dairy farms pose to water supplies as a source of zoonotic *Cryptosporidium*. Our objective was to ensure correct estimates of the risk associated with *Cryptosporidium* infection in dairy herds in the NYC Watershed. We adopted three specific aims: (1) to obtain species-specific estimates of the prevalence of *Cryptosporidium* in dairy cattle, (2) to investigate seasonal variations in prevalence, and (3) to validate our empirical estimates using a stochastic approach.

Materials and methods

Target population and sample collection

We conducted a series of repeated cross-sectional studies targeting dairy herds in the Catskill/Delaware portion of the NYC Watershed located in central New York State. The study population consisted of 32 farms that were drawn from herds enrolled in a voluntary program administered by the NYC Watershed Agricultural Council (Starkey et al. 2005).

Farms selected and enrolled in the study were visited once in each of three different seasons defined as winter (December–March), spring (April–June), and summer (July–September). We applied an age-stratified sampling design, preferentially targeting preweaned calves to improve the chances of detecting those animals shedding *C. parvum* oocysts (Starkey et al. 2006a; Wade et al. 2000). According to the protocol, we collected samples from a total of 20 animals per visit. The sampled animals included all calves younger than 1 year up to a maximum of 12 animals; if more than 12 such animals were present, 9 samples were collected from preweaned calves (<65 days of age) and 3 samples from postweaned calves (65 days–12 months of age). We also collected samples from eight animals older than 1 year including four heifers and four milking cows. The number of herds and animals to be sampled was based on an expected within-herd and animal prevalence of 30% and 3% (Levy and Lemeshow 1981).

Sample processing and microscopic identification

Fecal samples were collected rectally from each animal into plastic cups that were immediately capped and labeled to identify their source based on the ear tag number. The samples were transported on ice to the Animal Health Diagnostic Center at Cornell University (Ithaca, NY), where they were processed within 1 week of collection using a standard quantitative centrifugation flotation technique (Georgi and Georgi 1990). For each sample, 1 g of feces was processed using sugar (sg 1.33) as the flotation medium. *Cryptosporidium* oocysts were confirmed at 400× magnification with both bright-field and phase-contrast illumination. We considered a sample positive by flotation when at least one oocyst with the correct morphologic characters was identified (*C. parvum-like* oocysts are 4–6 μm and spherical; *C. andersoni* is 7–9 μm and oval; both types contain a residuum and sporozoites, refract pink in sugar, and have a halo in phase; Wade et al. 2000). Due to the inability of microscopy to distinguish *C. parvum*, *C. bovis*, and *C. ryanae*, the collective term *C. parvum*-like is used to refer to these species when discussing findings based on microscopic examination alone.

Statistical analysis

Empirical approach using maximum likelihood estimates

We calculated the animal prevalence as the number of animals with positive test result on flotation divided by the total number of animals examined. The herd prevalence was defined as the number of herds with at least one positive animal at the time of visit divided by the total number of herds. Cumulative herd prevalence was calcu-

lated as the proportion of herds with at least one positive animal on at least one of the three visits. We estimated the within-herd prevalence as the proportion of positive animals in each herd.

Stochastic approach using Bayesian modeling

A Bayesian prevalence model was fitted to validate our empirical estimate of the prevalence of zoonotic *Cryptosporidium* in the NYC Watershed. We restricted the analysis to preweaned calves, because the risk of infection with zoonotic *Cryptosporidium* has been shown to be limited to this age group, and the majority of *Cryptosporidium* oocysts shed by preweaned calves is zoonotic (Fayer et al. 2007; Starkey et al. 2005; Xiao 2009). Given these findings, modeling the prevalence of *C. parvum-like* species in preweaned calves provides an accurate estimate of the human health risk posed by dairy cattle regarding zoonotic *Cryptosporidium* within the watershed. We fitted a hierarchical model (Branscum et al. 2004) based on a single diagnostic test (flotation) applied to multiple herds with binomial sampling (Appendix 1). We modeled within-herd prevalence as mixture distributions to allow for zero infection prevalence, as previous reports indicate that not all herds in the study area are infected with *Cryptosporidium* (Starkey et al. 2005; Wade et al. 2000). Using this model, we estimated the within-herd prevalence for each of the 32 herds, the distribution of prevalences for all herds in the region (prevalence distribution), the proportion of infected herds (herd prevalence), and predicted probabilities for a randomly selected herd in the region (including the predicted probability of zero prevalence).

The prior parameters for the model were based on a series of studies our group had conducted on dairy farms in New York State watersheds (Starkey et al. 2005; 2006a; Wade et al. 2000) using the most likely (modal) value and the estimated upper or lower 95th percentile for the parameter. We used the modal values of 0.2 and 0.42 for the β distributions of the average animal prevalence and the herd prevalence of *C. parvum*-like species in preweaned calves, respectively, with 95th percentiles of 0.3 and 0.6. Thus, average animal prevalence was modeled as β (12.82, 48.28) and herd prevalence was modeled as β (9.51, 12.75). In a previous study, we had determined in our laboratory that the sugar flotation has a sensitivity and specificity of 0.75 and 0.96, respectively (Starkey et al. 2007). Using these values, we modeled sensitivity as β (13, 5) and specificity as β (97, 5). Models were run on the free software WinBugs version 1.4 (Spiegelhalter et al. 1996). We discarded the initial burn-in phase of 5,000 iterations, and the models were run for another 45,000 iterations to obtain estimates. We assessed convergence by running multiple chains from various starting values (Branscum et al. 2004).

Molecular typing of specimens

We extracted DNA from all samples that tested positive for *C. parvum*-like oocysts with the flotation method, using QIAamp DNA Stool Mini Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). A two-step nested polymerase chain reaction (PCR) protocol was used to amplify a 830-bp fragment of the 18S rRNA gene using primers 5′-TTCTAGAGCTAATACATGCG-3 and 5′- CCCATTTCCTTCGAAACAGGA-3 for primary and 5′-GGAAGGGTTGTATTTATTAGATAAAG-3 and 5′ AAGGAGTAAGGAACAACCTCCA-3′ for the secondary PCR (Xiao et al. 1999). We carried out the primary reaction in 25-µl volume consisting of 1 µl of genomic DNA, 10.8 µl of reverse osmosis water, 2 µl of 10× PCR buffer (Fermentas, MD), 4.8 µl of $MgCl_2$ (25 mM), 0.4 µl of dNTPs (10 mM), 0.4 µl of each forward and reverse primer (10 µM), and 0.2 µl (5 U/µl) of *Taq* DNA polymerase. The secondary reaction consisted of 1 µl of the product from the primary reaction added to a mixture containing 13.2 µl of reverse osmosis water, 2 µl of 10× PCR buffer, 2.4 µl of $MgCl_2$ (25 mM), 0.4 µl of dNTPs (10 mM), 0.4 µl of each forward and reverse primer (10 µM), and 0.2 µl (5 U/µl) of *Taq* DNA polymerase. Both the primary and secondary reactions were run under the same conditions: initial denaturation (94°C for 3 min), followed by 35 cycles of amplification (94°C for 45 s, 55°C for 45 s, and 72°C for 1 min) and a final extension (72°C for 7 min). We visualized PCR products after electrophoresis on 1% agarose gel stained with ethidium bromide. After purification of PCR products using Exonuclease I/Shrimp Alkaline Phosphatase (Exo-SAP-IT; USB Corporation, Cleveland, OH), the products were sequenced using the internal primers described above in 9-µL reactions in an automated sequencer (3730 DNA Analyzer; Applied Biosystems, Foster City, CA). We sequenced the samples in both directions and aligned the sequence chromatograms from each strand using MEGA 4 software (Tamura et al. 2007). The DNA sequences were compared with GenBank DNA sequences to determine the species of *Cryptosporidium* in the sample using the Basic Local Alignment Search Tool (BLAST).

Results

Maximum likelihood estimates of prevalence

We collected a total of 1,911 fecal samples on 32 dairy farms from 860 adult cattle and 1,051 calves, including 507 preweaned and 544 postweaned calves. The average number of preweaned calves present on the farms at sample collection was five, with continuous calving throughout the year. The animal and herd levels of prevalence of

Cryptosporidium species as determined by flotation and stratified by age and season are summarized in Table 1.

Prevalence *of C. parvum*-like species based on microscopy

The prevalence of *C. parvum*-like species among the 1,911 animals tested with the flotation technique was 5%. Preweaned calves had the highest prevalence, with an overall average of 18% and a marked seasonal variation ranging from 11% in the winter to 26% in the summer. The prevalence in postweaned calves was low (0.5%–2%) throughout the year, and no adult cattle were infected with *C. parvum*-like species at any time during the study. The oldest animal shedding *C. parvum*-like oocysts was a 5-month-old calf. We detected the same seasonal trend in prevalence at both the animal and the herd levels: herd prevalence ranged from as low as 41% in the winter to 56% in the summer. The cumulative herd prevalence was 84%. The within-herd prevalence was greatly influenced by herd size (data not shown). We observed the highest within-herd prevalence among the largest herds in the study population, where up to 60% of the preweaned calves sampled were shedding *C. parvum*-like oocysts. Conversely, the smallest farms that did not have more than two to three preweaned calves on the premises at any given time often tested free of *Cryptosporidium* infection.

Prevalence of *C. andersoni* based on microscopy

The overall prevalence of *C. andersoni* among the 1,911 animals was 1%. Although the prevalence was low (close to 1%) in all age groups in all seasons, interestingly, we found the highest overall prevalence among preweaned animals (1.5%), with a peak of 3.3% in the spring. The youngest animal infected with *C. andersoni* was 21 days of age. Among the eight preweaned calves that were shedding *C. andersoni* at the time of sampling, three animals were also shedding *C. parvum*-like oocysts simultaneously. However, adult cattle infected with *C. andersoni* shed an average of 98,286 oocysts per gram of feces, compared with only 20 oocysts per gram among preweaned calves. The proportion of farms with at least one animal shedding *C. andersoni* oocysts was in the range of 12% to 15% throughout the year. The cumulative herd prevalence of *C. andersoni* was 31%.

Bayesian estimates of the prevalence of zoonotic *Cryptosporidium* in preweaned calves

The estimated within-herd prevalence distribution for each of the 32 study farms is shown in Fig. 1. The 95% credible interval was wide for the majority of the study farms, ranging from zero to 40% to 50%. Only three farms in the study (Farms 2, 8, and 32) had a 95% credible interval that did not include zero. These three farms were among the largest herds in the study.

The estimated means and 95% credible intervals for herd prevalence and the prevalence distribution in the watershed are summarized in Table 2. We also predicted the following posterior probabilities: the probability that a randomly selected herd in the area is infection-free $[P(\pi^* = 0|\{y_t\})]$; the proportion of herds in the watershed that have a within-herd prevalence $< 5\%$ $[P(\pi^* \leq 0.05|\{y_t\})]$; and the probability that less than 50% of the herds in the area are infected $[P(\mathrm{HP} \leq 0.5|\{y_t\})]$.

Consistent with the maximum likelihood estimates, the estimated proportion of farms infected with zoonotic *Cryptosporidium* was highest in the summer (54%) and lowest in the winter (41%; Fig. 2). The prevalence

Table 1 Maximum likelihood estimates of animal and herd levels of prevalence of *Cryptosporidium* in cattle by season, as determined by microscopic examination

	Spring		Summer		Winter		Total	
	n	*p* (%)	*n*	*p* (%)	*n*	*p* (%)	*n*	*p* (%)
Animal level								
Preweaned calves	150		182		175		507	
C. parvum-like spp.		23 (15.3)		47 (25.8)		20 (11.5)		90 (17.7)
C. andersoni		5 (3.3)		1 (0.5)		2 (1.1)		8 (1.5)
Postweaned calves	192		170		182		544	
C. parvum-like spp.		4 (2.1)		1 (0.6)		1 (0.5)		6 (1.1)
C. andersoni		0		1 (0.6)		0		1 (0.2)
Adult cattle	292		275		293		860	
C. parvum-like spp.		0		0		0		0
C. andersoni		4 (1.4)		3 (1.1)		3 (1.0)		10 (1.2)
Herd level	32		32		32		32	
C. parvum-like spp.		14 (43.7)		18 (56.2)		13 (40.6)		27 (84.3)
C. andersoni		5 (15.6)		4 (12.5)		5 (15.6)		10 (31.2)

C. parvum-like species: *C. parvum*, *C. bovis*, and *C. ryanae*; *n*: number of animals (animal level) or herds (herd level) examined; *p* (%): number and proportion (expressed as percentage) of animals or farms that tested positive on microscopic examination

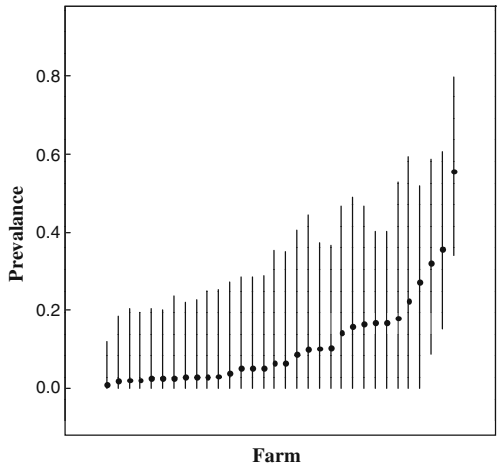

Fig. 1 Bayesian estimates of the within-herd prevalence of zoonotic *Cryptosporidium* among preweaned calves on 32 dairy farms, ranked by their mean. Farm number is shown in *brackets*. For each study farm, the mean of the within-herd prevalence is indicated by a *dot*, and a *horizontal line* represents the 95% credible interval

distribution predicted that the average within-herd prevalence among all the herds in the target population ranges from 8% in winter to 16% in the summer, although there is a wide variation indicated by the 95% credible intervals. In the winter and spring, the probability that a randomly selected herd in the target area is infection-free is >50%; in contrast, in the summer, it is predicted that over 50% of the farms have a prevalence >5%. In the summer, there is only a 34% probability that herd prevalence is ≤50%; in the winter, this probability is 82%.

Molecular characterization of *C. parvum*-like specimens

We successfully amplified a segment of the 18s rRNA gene of 79 flotation-positive samples (74 from preweaned and 5 from postweaned calves). All five sequences from postweaned calves had 100% homology with *C. bovis* (GenBank accession AY120911). The number of different species of *C. parvum*-like organisms we detected in preweaned calves in each season is summarized in Table 3.

The majority of the *C. parvum*-like specimens (44) had 100% homology with *C. parvum* (AF093490), followed by 25 sequences that were identified as *C. bovis*, while only 5 specimens had 100% homology with *C. ryanae* (AY120910). Calves that were infected with *C. parvum* shed an average of 127,000 oocysts per gram of feces year-round, while those infected with the nonzoonotic species did not shed more than an average of 6,500 oocysts per gram at any time of the year. The average age of animals infected with *C. parvum* was the lowest (17 days), followed by *C. bovis* (27 days) and *C. ryanae* (43 days).

We observed a substantial seasonal shift in the proportion of zoonotic *Cryptosporidium* shed by preweaned calves (Fig. 3). In the winter and spring, at least

74% of the *C. parvum*-like samples from preweaned calves were zoonotic. However, in the summer, only 42% of such samples were identified as zoonotic, while the rest belonged to the nonzoonotic *C. bovis* and *C. ryanae* species.

Discussion

The goal of this study was to ensure correct estimates of the risk of infection with different *Cryptosporidium* species in cattle in an important New York State watershed. With the increased availability of molecular typing methods, our understanding of *Cryptosporidium* epidemiology is constantly evolving. Since the description of *C. andersoni* as distinct species from *C. muris* in 2000 (Lindsay et al. 2000), there has been an ongoing healthy and unavoidable discussion on the taxonomy of the genotypes and species of *Cryptosporidium* affecting cattle (Slapeta 2006; Xiao et al. 2007a). While recognizing the need for dialogue regarding *Cryptosporidium* taxonomy, the terminology used in this study reflects the currently adopted nomenclature.

The overall prevalence of *C. parvum*-like species and *C. andersoni* among the 1,911 animals tested by the flotation method was 5% and 1%, respectively. As expected, we detected the highest prevalence of *C. parvum*-like species among preweaned calves (18%).

Table 2 Estimated means, 95% credible intervals, and predictive probabilities for the prevalence of zoonotic *Cryptosporidium* in dairy herds in the NYC Watershed based on Bayesian analysis

	Posterior distributions		Posterior predictive probabilities		
Dataset $\{y_t\}$	Herd prevalence	Prevalence distribution	$P(\pi^* \leq 0.05 \mid \{y_t\})$	$P(\pi^* = 0 \mid \{y_t\})$	$P(\mathrm{HP} \leq 0.5 \mid \{y_t\})$
Spring	0.46 (0.28–0.64)	0.1 (0–0.47)	0.58	0.54	0.68
Summer	0.54 (0.36–0.7)	0.16 (0–0.59)	0.48	0.46	0.34
Winter	0.41 (0.23–0.60)	0.08 (0–0.5)	0.65	0.59	0.82
Total	0.47 (0.29–0.66)	0.1 (0–0.51)	0.58	0.53	0.62

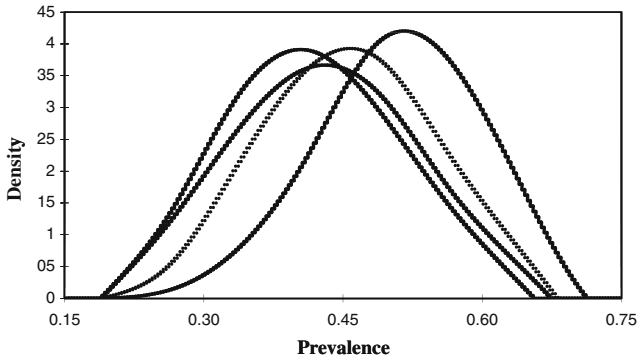

Fig. 2 Bayesian prior (*solid*) and estimated posterior distributions of herd prevalence of zoonotic *Cryptosporidium* by season: spring (*dots*), summer (*dashes and dots*), and winter (*dashes*)

With respect to the overall prevalence of *C. andersoni*, the current study is in concordance with a study our group had conducted on 109 farms in the NYC Watershed in 1998, where an overall prevalence of 1.1% was reported for this parasite (Wade et al. 2000). These results are also consistent with other reports from the United States (Fayer et al. 2000, 2007); however, investigators in India found a higher overall *C. andersoni* prevalence of 12.85% in postweaned and adult cattle (Paul et al. 2009).

In terms of the prevalence of *C. parvum*-like species, the present study is in agreement with a recent study conducted in an adjacent New York State watershed that reported an overall prevalence of 3.9% among the 453 animals examined, with a 20% prevalence among animals less than 61 days of age (Starkey et al. 2006a). Thus, we confirm that, on average, approximately one fifth of preweaned calves are infected with *C. parvum*-like species in the NYC Watershed.

Table 3 Number (*n*) of different *C. parvum*-like species identified by 18s rRNA gene sequencing in preweaned calves (<65 days of age) in each season

Season	Species	*n*	Age (days)	Oocyst/g of feces
Spring	*C. parvum*	12	20 (7)	115,686
	C. bovis	3	30 (26)	398
	C. ryanae	0		
Summer	*C. parvum*	15	17 (8)	105,733
	C. bovis	16	27 (10)	5,613
	C. ryanae	5	43 (22)	182
Winter	*C. parvum*	17	14 (7)	154,255
	C. bovis	6	25 (13)	465
	C. ryanae	0		
Total	*C. parvum*	44	17 (7)	127,195
	C. bovis	25	27 (10)	3,751
	C. ryanae	5	43 (22)	182

The average age and the age range as well as the average number and range of oocysts per gram of feces estimated by the quantitative concentration flotation method are also indicated.

Numerous studies conducted worldwide within the past decade reported wide variations in the prevalence of *C. parvum*-like species in dairy calves, ranging from 12% in Norway (Hamnes et al. 2006) to 40% to 50% in Australia and Zambia (Becher et al. 2004; Geurden et al. 2006) to 79% in India (Singh et al. 2006). Studies that applied repetitive sampling often reported higher period prevalences of >90% (Uga et al. 2000) and up to 100% (Castro-Hermida et al. 2002b; Xiao and Herd 1994) in preweaned calves. According to our study design, we only collected one fecal sample from each animal, which might have underestimated the actual prevalence in the study population, as intermittent oocyst excretion in cattle has been reported (McCluskey et al. 1995).

We did not detect *C. parvum*-like oocysts in cattle older than 5 months in our study population. Molecular analysis of the specimens revealed that *C. parvum* did not occur in calves older than 2 months, while postweaned cattle were only infected with the nonzoonotic *C. bovis* and *C. ryanae*. These findings are consistent with other reports, although some studies detected infection with *C. parvum*-like species in adult cattle (Fayer et al. 2006; Feng et al. 2007; Santin et al. 2004). In a study conducted in Maryland targeting cattle older than 6 months, *C. parvum*-like oocysts were detected in 20.7% of the 184 animals tested with the flotation method (Fayer et al. 2000), while another study in the eastern United States reported infection with *C. parvum* and *C. bovis* in 0.4% and 1.7% of the 541 cows examined, respectively (Fayer et al. 2007). Our results indicate that adult cattle in the NYC Watershed are either truly infection-free or that these animals are shedding *C. parvum*-like oocysts below the limit of detection. For the standard sugar flotation, we have been using in our investigations the threshold of detection, which is approximately 100 oocysts per gram of feces (Fayer et al. 2000; Xiao and Herd 1993; S.E. Wade, personal communication). Therefore, if adult cattle were consistently shedding *C. parvum*-like oocysts below the limit of detection, they would have been classified as negative for this parasite.

Larger farms had higher within-herd prevalence of *C. parvum*-like species compared with smaller farms in the study area. This is consistent with findings in the neighboring watershed where the likelihood of shedding

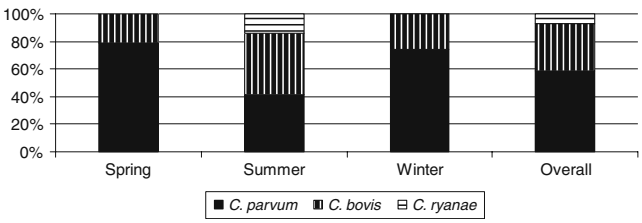

Fig. 3 The proportion of *C. parvum*-like species shed by preweaned calves (<65 days of age) by season based on18s rRNA gene sequencing

C. parvum-like oocysts increased with the number of preweaned calves in the herd (Starkey et al. 2006a). Several authors have described an association between the size of the farm and risk of *Cryptosporidium* infection, where the higher the number of susceptible calves, the greater the number of animals that become infected, which, in turn, results in increased environmental contamination (Garber et al. 1994)

Our study revealed that the point herd prevalence of *C. parvum*-like species ranged from 41% to 56%. However, the cumulative herd prevalence over the study period was 84%, with only 5 of the 32 herds testing negative at every visit. A herd that is not truly negative may be classified as negative if too few calves were tested to detect infection, or infected calves were shedding below the detection threshold at sampling, or the tested animals had recovered from earlier infection and did not shed at sampling time (Hamnes et al. 2006). Thus, a larger herd size is not only associated with an increased risk of infection as discussed above, but the probability of a herd testing positive also increases with the number of samples collected per farm (Garber et al. 1994). Therefore, it is possible that we could not accurately identify the infection status of the smaller herds in the study, due to the low number of calves present at any given time on these farms. This uncertainty is captured by the wide 95% credible intervals for within herd prevalence in the Bayesian analysis.

Due to the inherent difficulties in accurately assessing the infection status of small herds, it is questionable whether small farms that tested negative in this study are in fact truly free of *Cryptosporidium*. In a previous study conducted on dairy farms in the NYC Watershed, *Cryptosporidium* was found in the soil of 92% of the 37 farms examined, indicating high level of environmental contamination on these farms (Barwick et al. 2003). A comprehensive wildlife survey in the same watershed revealed that *Cryptosporidium* is ubiquitous in wildlife in this area; oocysts were detected throughout the year in 64% (25/39) of the mammalian species tested (Ziegler et al. 2007b), and 6 isolates recovered from rodents were identified as *C. parvum* (Ziegler et al. 2007a). These findings highlight the fact that wild mammal populations are persistently infected and serve as environmental sources of *Cryptosporidium* oocysts, since their feces may contaminate bedding material. We suspect that the high level of environmental contamination and the presence of rodent reservoirs provide continuous exposure of susceptible calves to *Cryptosporidium* oocysts. However, the average low number of calves on the small-scale farms in the NYC Watershed does not favor the propagation and detection of infection.

All four species of *Cryptosporidium* that affect cattle were detected in this study in preweaned calves. The youngest age of *C. parvum* we detected was 7 days, which is in concordance with previous reports that calves get infected with this parasite within the first 1 to 2 days of life and start shedding oocysts after a 5 to 6 days before patent period (Castro-Hermida et al. 2002b; Ongerth and Stibbs 1989; Quilez et al. 1996). The youngest calf shedding *C. bovis* and *C. ryanae* oocysts was 10 and 22 days of age, respectively. This finding is in agreement with other studies that found that calves acquire infection with these species early in life (Santin et al. 2004; Starkey et al. 2006b). Our results also confirm earlier suggestions that animals infected with *C. parvum* have significantly higher oocyst counts than animals that are infected with *C. bovis* and *C. ryanae* (Feng et al. 2007; Starkey et al. 2006b). We detected that preweaned calves, including a 21-day-old calf, were shedding *C. andersoni* oocysts at an average intensity of 20 oocysts per gram of feces. Although *C. andersoni* has been reported to mainly infect adult cattle (Enemark et al. 2002; Fayer et al. 2000; Huetink et al. 2001), several authors described this species in preweaned calves (Kvac and Vitovec 2003; Santin et al. 2004). It is possible that, similarly to the other *Cryptosporidium* species, calves also acquire *C. andersoni* infection early in life, but the intensity of shedding in young animals is low, which makes early detection difficult.

In the present study, both the animal and the herd levels of prevalence of *C. parvum*-like species peaked in the summer and dropped to their lowest levels in the winter. Both our empirical estimates and Bayesian modeling confirmed this seasonal trend. One advantage of our study design was that we collected samples on the same farms in three different seasons, which allowed us to evaluate the effect of season without statistical adjustment for farm effects (Mohammed et al. 1999). Seasonal variation in prevalence can be explained by at least four different scenarios: seasonal calving, which results in a higher number of susceptible animals in the calving season; crowding of animals indoors, which leads to increased animal-to-animal transmission; better survival of oocysts in the environment due to favorable climatic conditions; and seasonal differences in management practices that affect the risk of infection (Atwill et al. 1999; Castro-Hermida et al. 2002a; Garber et al. 1994; Hamnes et al. 2006). In the NYC Watershed, there is a year-round calving pattern, and crowding indoors are not characteristic of the summer months when cattle spend most of their time outside. Thus, the most likely explanation for the observed seasonal variation in prevalence in this watershed is either seasonal differences in management or favorable conditions for oocyst survival.

Concurrently, with an increase in the prevalence of *C. parvum*-like species among preweaned calves, there was seasonal variation in the proportion of zoonotic *Cryptosporidium* shed by these animals. Sequencing of a portion of the 18s rRNA gene revealed that in the summer, only 42% of the *C. parvum*-like species shed by preweaned calves

were zoonotic, as opposed to at least 74% during the rest of the year. These estimates are slightly lower than the result of another study in the United States, which indicated that *C. parvum* is responsible for 85% of the *Cryptosporidium* infections in preweaned calves (Santin et al. 2004).

Combining the results suggests that seasonal variation in the prevalence of *C. parvum*-like species in this population was due to an increased risk of infection with the non-zoonotic species in preweaned calves in the summer. It is plausible that the oocysts of different *C. parvum*-like species favor different environmental conditions. Alternatively, different management practices might also favor the propagation of zoonotic versus nonzoonotic species. Determining whether seasonal variation in the prevalence and proportion of *Cryptosporidium* species shed by calves is due to management practices or ecological factors will have important implications for effective intervention.

We validated our empirical prevalence estimates by fitting a Bayesian model to our data. Prior parameters for the model were based on previous data from studies our group had conducted among the same target population and in the same laboratory. Therefore, we considered these Bayesian priors to be an objective and unbiased representation of previous knowledge. Applying a Bayesian model allows accounting for imperfections in the diagnostic test and for random variations in the input parameters, giving a range of possible values for the target population that reflects these uncertainties. Our prevalence estimates based on maximum likelihood and Bayesian methods were in close agreement, lending credence to the conclusion that we obtained a valid and reliable estimate of the risk of infection with zoonotic *Cryptosporidium* in dairy herds in the NYC Watershed.

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Ethical standards The authors declare that the experiments comply with the current laws of the country in which they were performed.

Conflict of interest The authors declare that they have no conflict of interest.

Appendix 1

Syntax for the Bayesian model

```
Model;
{
Se ~ dbeta(13, 5) ## Se=sensitivity of the test
Sp ~ dbeta(97, 5) ## Sp=specificity of the test
tau ~ dbeta(9.51, 12.75) ## tau=proportion of infected herds
mu ~ dbeta(12.82, 48.28) ## mu=average prevalence
psi ~ dgamma(4.5, 0.5) ## psi=variability among herd prevalences
alpha <- mu*psi ## first parameter of prevalence distribution
beta <- psi*(1-mu) ## second parameter of prevalence distribution
for(i in 1:k)
{
inf[i] ~ dbern(tau) ## for each herd, it is estimated whether it is infected or not using a Bernoulli distribution with mean tau
pi.star[i] ~ dbeta(alpha,beta) ## estimated prevalence of each herd
pi[i] <- pi.star[i] * inf[i] ## estimated prevalence of each herd allowing for zero prevalence by multiplication with inf[i]
prob.tpos[i] <- pi[i]*Se + (1-pi[i])*(1-Sp) ## probability of a positive test result is the probability of a true positive + probability of a false positive
y[i] ~ dbin(prob.tpos[i], n[i]) ## the number of positive test results in an infected herd follows a binomial distribution
}
```

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