GEOGRAPHIC VARIATION AND ZOONOTIC POTENTIAL OF *CRYPTOSPORIDIUM* INFECTION IN DAIRY CATTLE

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Cryptosporidium parvum is a zoonotic protozoan parasite that produces life-threatening infection in people with compromised immune systems, and causes significant economic losses to the dairy industry due to morbidity in calves. The overall objective was to quantify and characterize the risk associated with zoonotic *C. parvum* infection in cattle, so that cost-effective intervention strategies may be implemented to mitigate the public health risk and to reduce economic losses. To that aim, four complementary studies were implemented, incorporating epidemiologic analytic approaches, geographic information system analyses and molecular techniques.

A series of cross-sectional studies were conducted using molecular genotyping methods to obtain species-specific estimates of the prevalence of *Cryptosporidium* in dairy cattle, and to investigate seasonal variations in prevalence. The empirical prevalence estimates were validated using a stochastic Bayesian approach. Subsequently, the crude and Bayesian risk estimates were used to investigate the spatio-temporal dynamics of *C. parvum* infection on dairy farms in an important watershed with various cluster detection methods. In addition, a case-control study was conducted applying uni-, and multivariable unconditional logistic regression analysis to determine the association between host, management, geographical, and meteorological factors and *Cryptosporidium* genotype. Finally, to investigate the global zoonotic risk of *Cryptosporidium*, our group collaborated with the University of Nairobi to identify *Cryptosporidium* genotypes from feces collected from urban and

peri-urban dairy cattle in Nairobi to determine their zoonotic potential.

Both empirical and stochastic methods revealed a summer peak in the prevalence of *C. parvum* in pre-weaned cattle. Empirical risk estimates highlighted both temporal and spatial clusters of *C. parvum* infection in a major watershed. Herd size, hay bedding and precipitation were significant risk factors associated specifically with the zoonotic genotype in calves. *Cryptosporidium ryanae*, a non-zoonotic genotype was found in pre-weaned calves in peri-urban Nairobi.

The findings of these studies will be useful to design control measures that reduce animal exposure and economic losses associated with *C. parvum* infection in cattle herds, and protect drinking water supplies by decreasing watershed contamination with this parasite.

BIOGRAPHICAL SKETCH

Barbara Szonyi received her Bachelor of Science degree in Animal Sciences, and her Doctor of Veterinary Medicine degree from Cornell University. During her veterinary training she participated in various disease surveillance and control project in Uganda and Ethiopia, which sparked her interest in epidemiology and global health research. Upon completing her D.V.M. degree, she immediately began pursuing a Ph.D. in epidemiology at Cornell's College of Veterinary Medicine. As a Ph.D. student, she collaborated with the University of Nairobi in *Cryptosporidium* risk assessment. In the final year of her Ph.D. studies, she was awarded a Fogarty Scholarship to investigate the molecular epidemiology of leptospirosis in Latin America.

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CHAPTER ONE

INTRODUCTION

Cryptosporidiosis is an emerging waterborne protozoan infection affecting humans and a wide range of animals worldwide (de Graaf *et al*., 1999; Xiao and Feng, 2008). To date the genus *Cryptosporidium* consists of 18 species and over 40 genotypes (Xiao and Fayer, 2008).

The majority of human cases of cryptosporidiosis is attributable to infection with *C. hominis* and *C. parvum*. Cryptosporidiosis in humans is associated with gastrointestinal infection which can be life threatening in immuno-compromised individuals. In HIV infected patients, *Cryptosporidium* may account for 50% of the cases of diarrhea (Morgan *et al*., 2000). In developing countries, *Cryptosporidium* is responsible for up to 19% of cases of diarrheal disease with significant effect on mortality (Gatei *et al*., 2006). The infection is transmitted by the fecal-oral route either by direct contact or through contamination of food and water (Smith *et al*., 2007).

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*., 2007, 2008; Fayer *et al*., 2005; Xiao *et al*., 2007). Recent studies showed that infection with these *Cryptosporidium* species in cattle is age-related. *Cryptosporidium parvum*, the only prevalent zoonotic species, is responsible for the majority of the infections in pre-weaned calves, whereas post-weaned and adult cattle are mostly infected with *C. bovis*, *C. andersoni* and *C. ryanae* (Fayer *et al*., 2006; Santin *et al*., 2004). Clinical appearance of *C. parvum* infection in calves can range from asymptomatic shedding of oocysts to severe diarrhea, dehydration and death (Fayer *et al*., 2009). Furthermore, damage to the intestinal epithelium can cause prolonged malnutrition and reduced growth rates in calves,

resulting in significant economic losses in dairy operations (Nydam and Mohammed, 2005). In contrast, the other three *Cryptosporidium* species in cattle are thought to be non-pathogenic and are considered unlikely to be zoonotic. Thus, only *C. parvum* is of primary concern from both the public health perspective and in terms of economic losses to the dairy industry due to morbidity in calves (Fayer *et al*., 2009).

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 *C. parvum* from *C. bovis* and *C. ryanae* (referred to as the *C. parvum*-like species) (Santin *et al*., 2004; Starkey *et al*., 2005). To effectively control the occurrence of *C. parvum* infection in cattle, molecular techniques need to be incorporated into epidemiological studies to obtain valid and reliable risk estimates of this parasite in the target population.

The New York City Watershed is currently the focus of a long-term project investigating the public health risk of waterborne cryptosporidiosis. Pathogens such as *Cryptosporidium* pose a significant threat to public health in the City's unfiltered water supply, because the oocysts are very resistant to chlorination, and they are regularly detected in reservoir effluents (Betancourt and Rose, 2004). Dairy calves are thought to be a primary source of zoonotic *Cryptosporidium parvum* contamination in watershed ecosystems (Xiao and Feng, 2008). In the NYC Watershed, the Catskill/Delaware drainage system is home to approximately 200 dairy farms. New York City implements extensive watershed management measures, with the goal to protect water quality while maintaining economic viability on these farms (NYC Department of Environmental Protection, 2009).

The general objectives of this work were to improve our understanding of the dynamics of *Cryptosporidium* infection in cattle, and to characterize the risk dairy

cattle pose to human health as a source of zoonotic *C. parvum* in two different ecosystems: in agricultural watersheds, and in an intensive urban smallholder system.

Although several cross-sectional studies have investigated the prevalence of *Cryptosporidium* infection in cattle, relatively few studies utilizes molecular techniques to distinguish *C. parvum* from the non-zoonotic *Cryptosporidium* species. The aim of the second chapter of this thesis was to ensure correct estimates of the risk associated with *Cryptosporidium* infection in dairy herds in the NYC Watershed, by obtaining species-specific prevalence estimates and validating the empirical estimates using a stochastic approach. Seasonal variation in the prevalence was also investigated.

Understanding the spatial and temporal pattern of *C. parvum* infection on dairy farms in watersheds would be useful in designing watershed management strategies to monitor and mitigate the risk of *C. parvum* contamination. However to date, the spatial and temporal variation in the risk of *C. parvum* infection in dairy herds in watersheds has not been investigated. In the third chapter we aimed to explore and map the temporal and spatial dynamics of the risk of *C. parvum* infection in dairy cattle in the NYC Watershed, and to identify potential high-risk clusters in space and time.

To effectively decrease the risk of infection with *C. parvum* in cattle, relevant risk factors for this parasite need to be identified. Due to the necessity of employing molecular typing to determine the zoonotic potential, relatively few studies investigated risk factors associated specifically with zoonotic *Cryptosporidium* infection in cattle. The fourth chapter of the thesis involved the identification of ecological and management related factors that increase the risk of infection with zoonotic *Cryptosporidium* in dairy calves using a case-control study design.

Cryptosporidium is one of the most common enteric parasites associated with

diarrhea in developing countries such as Kenya (Gatei *et al*., 2006). In spite of the high prevalence of human cryptosporidiosis in Kenya, little is known about the occurrence of different genotypes in Kenyan livestock (Kang'ethe *et al*., 2005). The lack of information in Kenya about the extent of zoonotic transmission of *Cryptosporidium* and the risk posed by livestock to human health prompted the fifth chapter of this thesis, which reports *Cryptosporidium* genotypes identified in the feces of urban dairy cattle in Nairobi.

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CHAPTER TWO

SEASONAL VARIATION IN THE PREVALENCE AND MOLECULAR EPIDEMIOLOGY OF CRYPTOSPORIDIUM INFECTION IN DAIRY CATTLE IN THE NEW YORK CITY WATERSHED[1]

ABSTRACT

A series of cross-sectional studies were conducted in the New York City Watershed to ensure a valid estimate of the risk associated with *Cryptosporidium* infection in dairy herds. The aims of the studies were to obtain species-specific estimates of the prevalence of *Cryptosporidium* in dairy cattle and to investigate seasonal variations in prevalence. The empirical estimates were validated using a Bayesian approach. Samples were collected on 32 study farms once in each of three different seasons using an age-stratified sampling design. The overall prevalence of *C. parvum*-like species and *C. andersoni* among the 1911 animals tested by the flotation method was 5% and 1%, respectively. Among pre-weaned calves, the prevalence of *C. parvum* -like species was twice as high in the summer (26%) compared to the winter (11%). Herd prevalence showed the same seasonal trend. Pre-weaned 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 of age. Sequencing of a portion of the 18s rRNA gene revealed that in the summer, 42% of the *C. parvum*-like oocysts shed by pre-weaned calves were zoonotic, compared to > 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 pre-weaned calves.

[1] This chapter has been prepared in the format for submission to the journal Parasitology Research

Determining whether seasonal variation in the prevalence and proportion of *Cryptosporidium* species shed by pre-weaned calves is due to management practices or ecological factors will have important implications for effective control of this parasite.

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*., 2007, 2008; Fayer *et al*., 2005; Xiao *et al*., 2007). A recent study showed that these *Cryptosporidium* species in cattle are age-related. *Cryptosporidium parvum*, the only prevalent zoonotic species, is responsible for about 85% of the infections in pre-weaned calves, whereas post-weaned 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*., 2007). 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 non-zoonotic *C. bovis* and *C. ryanae*. Thus, when traditional diagnostic methods are used, it is more accurate to commonly refer to C. *parvum*, *C. bovis* and *C. ryanae* as the *C. parvum*-like species (Santin *et al*., 2004; Starkey *et al*., 2005). However, for the purposes of risk assessment and risk mitigation, it is critical to differentiate between zoonotic and non-zoonotic species of this pathogen, and to obtain valid and reliable estimates of their occurrence in the target 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 Watershed is home to approximately 200 dairy farms, it provides over 80% of New York City's (NYC) 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 the empirical estimates using a stochastic approach.

MATERIALS AND METHODS

Target population and sample collection

A series of repeated cross-sectional studies were conducted targeting dairy herds in the Catskill/Delaware portion of the New York City 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 New York City 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). An age-stratified sampling design was applied, preferentially targeting pre-weaned 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 samples were collected from a total of 20 animals per visit. The sampled animals included all calves < 1 year of age up to a maximum of 12 animals; if more than 12 such animals were present, 9 samples were collected from pre-weaned calves (< 2 months old) and 3 samples from post-weaned calves (2-12 months of age). We also collected samples from 8 animals > 1 year of age including 4 heifers and 4 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 one week of collection using a standard quantitative centrifugation flotation technique (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 400X magnification with both bright-field and phase contrast illumination. A sample was considered positive by flotation when at least one oocyst with the correct morphological 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).

Statistical analysis

Empirical approach using maximum likelihood estimates

The animal prevalence was calculated 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 calculated as the proportion of herds with at least one positive animal on at least one of the 3 visits. The within-herd prevalence was estimated 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 New York City Watershed. The analysis was restricted to pre-weaned 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 pre-weaned calves is zoonotic (Fayer *et al*., 2007; Starkey *et al*., 2005; Xiao, 2009). Given these findings, restricting the

analysis to pre-weaned calves provided a more accurate estimate of the human health risk posed by dairy cattle regarding zoonotic *Cryptosporidium* within the watershed. A hierarchical model was fitted (Branscum *et al*., 2004), based on a single diagnostic test applied to multiple herds with binomial sampling (Appendix). Animal level prevalence was modeled as mixture distributions to allow for zero infection prevalence, as previous reports indicate that not all herds in the study area are infected (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, 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) 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 beta distributions of the average animal prevalence and the herd prevalence, respectively, with 95th percentiles of 0.3 and 0.6. Thus average animal prevalence was modeled as beta (12.82, 48.28) and herd prevalence was modeled as beta (9.51, 12.75). In a previous study, it was 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 sensitivity was modeled as beta (13, 5) and specificity as beta (97, 5). Models were run on the free software WinBugs version 1.4 (Spiegelhalter *et al*., 1996). The initial burn-in phase of 5000 iterations were discarded and the models were run for another 45,000 iterations to obtain estimates. Convergence was assessed by running multiple chains from various starting values (Branscum *et al*., 2004).

Molecular typing of specimens

DNA was extracted 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 PCR protocol was used to amplify an 830bp 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). The primary reaction was carried out in 25 µl volume consisting of 1 µl of genomic DNA, 10.8 µl of reverse osmosis water, 2 µl of 10X PCR buffer (Fermentas, MD), 4.8 µl of $MgCl_2$ (25 mM), 0.4 µl of dNTP's (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 10X PCR buffer, 2.4 µl of $MgCl_2$ (25 mM), 0.4 µl of dNTP's (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). PCR products were visualized 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). Samples were sequenced in both directions, and the sequence chromatograms were aligned 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

A total of 1,911 fecal samples were collected on 32 dairy farms from 860 adult cattle and 1051 calves, including 507 pre-weaned and 544 post-weaned calves. The average number of pre-weaned calves present on the farms at sample collection was five, with continuous calving throughout the year. The animal-, and herd level prevalence of *Cryptosporidium* species as determined by flotation and stratified by age and season, were summarized in Table 2.1.

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%. Pre-weaned 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 post-weaned 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. The same seasonal trend in prevalence was detected at both the animal-, and the herd level: 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 pre-weaned calves sampled were shedding *C. parvum*-like oocysts. Conversely, the smallest farms that did not have more than 2-3 pre-weaned 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 1911 animals was 1%. Although the prevalence was low (close to 1%) in all age groups in all seasons, interestingly, the highest overall prevalence was found among pre-weaned 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 8 pre-weaned calves that were shedding *C. andersoni* at the time of sampling, 3 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 to only 20 oocysts per gram among pre-weaned calves. The proportion of farms with at least one animal shedding *C. andersoni* oocysts was in the range of 12-15% throughout the year. The cumulative herd prevalence of *C. andersoni* was 31%.

Bayesian estimates of the prevalence of *C. parvum*-like species in calves

The estimated within-herd prevalence distribution for each of the 32 study farms was displayed in Figure 2.1. The 95% credible interval was wide for the majority of the study farms, ranging from zero to 40-50%. Only 3 farms in the study (Farms 2, 8, and 32) had a 95% credible interval that did not include zero. These farms were among the largest herds in the study.

The means and 95% credible intervals for herd prevalence and prevalence distribution in the watershed are summarized in Table 2.2 The following posterior probabilities were also predicted: the probability that a randomly selected herd in the area is infection free ($P(\pi^* = 0 | \{yt\})$); 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 ($P(HP \leq 0.5 | \{yt\})$).

Consistent with the maximum likelihood estimates, the proportion of farms infected was highest in the summer (54%) and lowest in the winter (41%) (Figure 2.2).

Table 2.1 Maximum likelihood estimates of animal-, and herd-level prevalence of *Cryptosporidium* in cattle by season, as determined by microscopic examination

	Spring		Summer		Winter		Total	
Animal level	n[2]	p (%)[3]	n	p (%)	n	p (%)	n	p (%)
pre-weaned calves	150		182		175		507	
C. parvum-like spp[1]		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)
post-weaned 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)

[1] *C. parvum*-like spp: *C. parvum*, *C. bovis*, and *C. ryanae*
[2] n: number of animals (animal level) or herds (herd level) examined
[3] p (%): number and proportion (expressed as percentage) of animals or farms that tested positive by flotation

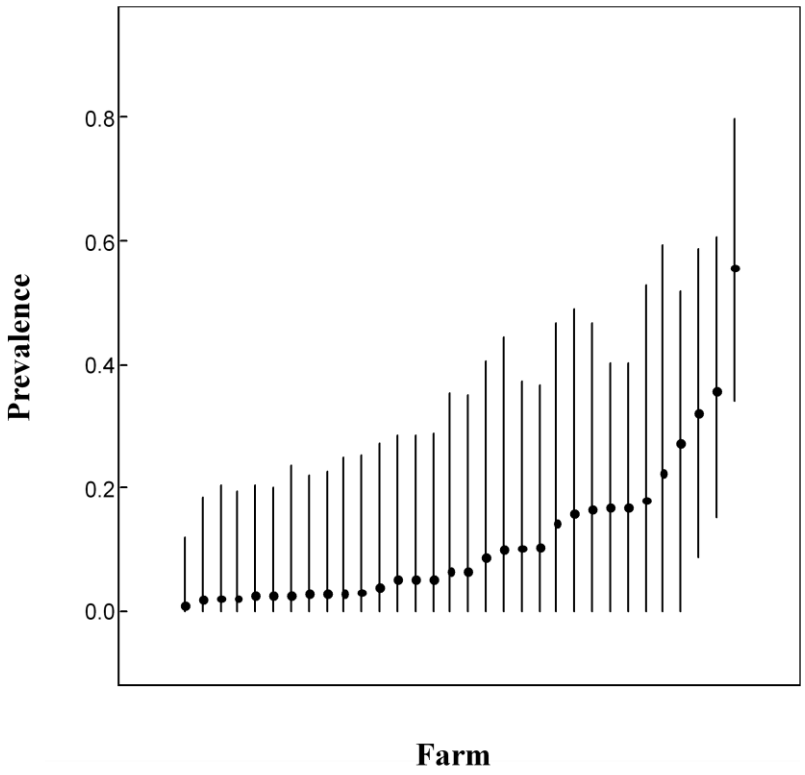

Figure 2.1 Bayesian estimate of within-herd prevalence for 32 dairy farms, ranked by their mean. Farm number is shown in brackets. For each study farm, the mean of the within- herd prevalence is indicated by a dot and a horizontal line represents the 95% credible interval

Table 2.2 Means, 95% credible intervals, and predictive probabilities for the prevalence of *C. parvum*-like species in pre-weaned calves based on Bayesian analysis

	Posterior distributions		**Posterior predictive probabilities**		
Dataset {yt}	Herd prevalence	Prevalence distribution	P ($\pi^* \leq 0.05 \mid \{y_t\}$)	P ($\pi^* = 0 \mid \{y_t\}$)	P (HP $\leq 0.5 \mid \{yt\}$)
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

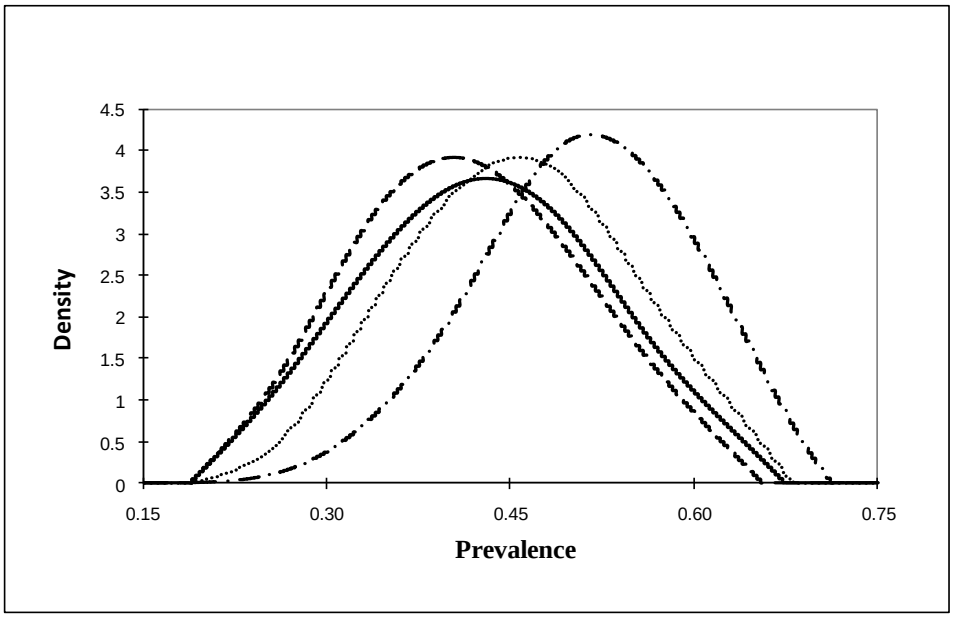

Figure 2. 2 Distribution of prior (solid) and posterior herd prevalence by season: spring (dots), summer (dashes and dots), and winter (dashes)

The prevalence distribution predicted that the average within-herd prevalence among all the herds in the target population ranged from 8% in winter to 16% in the summer, although there was 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 was infection free was > 50%; in contrast in the summer, it was predicted that over 50% of the farms had a prevalence > 5%. In the summer, there was only a 34% probability that herd prevalence was ≤ 50%; in the winter, this probability was 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 pre-weaned and 5 from post-weaned calves). All 5 sequences from post-weaned calves had 100% homology with *C. bovis* (Genbank accession AY120911). The number of different species of *C. parvum*-like organisms detected in pre-weaned calves in each season was summarized in Table 2.3. 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 non-zoonotic 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 pre-weaned calves (Figure 2.3).

Table 2.3 Number (n) of different *C. parvum*-like species identified by 18s rRNA gene sequencing in pre-weaned calves in each season.

Season	Species	n	Age (days)	Oocyst/gram of feces
Spring	*C. parvum*	12	20	115,686
	C. bovis	3	30	398
	C. ryanae	0		
Summer	*C. parvum*	15	17	105,733
	C. bovis	16	27	5613
	C. ryanae	5	43	182
Winter	*C. parvum*	17	14	154,255
	C. bovis	6	25	465
	C. ryanae	0		
Total	*C. parvum*	44	17	127,195
	C. bovis	25	27	3751
	C. ryanae	5	43	182

[1] The average age, and the average number of oocysts per gram of feces estimated by the quantitative concentration flotation method are also indicated

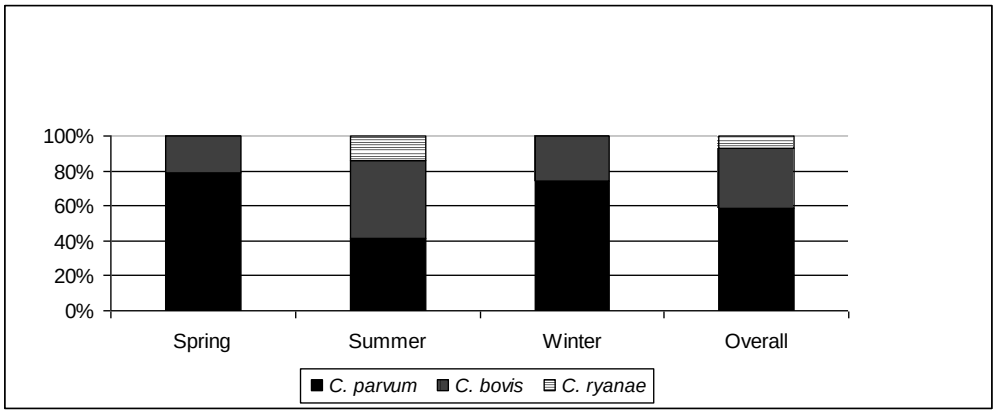

Figure 2.3 The proportion of *C. parvum*-like species shed by pre-weaned calves by season based on18s rRNA gene sequencing

In the winter and spring, at least 74% of the *C. parvum*-like samples from pre-weaned calves were zoonotic. However in the summer, only 42% of such samples were identified as zoonotic, while the rest belonged to the non-zoonotic *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. The overall prevalence of *C. parvum*-like species and *C. andersoni* among the 1911 animals tested by the flotation method was 5% and 1%, respectively. As expected, we detected the highest prevalence of *C. parvum*-like species among pre-weaned calves (18%).

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

In terms of the prevalence of *C. parvum*-like species, the present study was 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 it was confirmed that on average, approximately one-fifth of pre-weaned calves were infected with *C. parvum*-like species in the New York City Watershed.

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 40-50% 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 pre-weaned calves. According to the 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 of age in our study population. Molecular analysis of the specimens revealed that *C. parvum* did not occur in calves older than 2 months of age, while post-weaned cattle were only infected with the non-zoonotic *C. bovis* and *C. ryanae*. These findings were 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 over 6 months of age, *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). The results of the present study indicated that adult cattle in the New York City Watershed were either truly infection free, or that these animals were shedding *C. parvum*-like oocysts below the limit of detection. For the standard sugar flotation that have been used in our investigations, the threshold of detection was determined to be approximately 100 oocysts per gram of feces (Fayer *et al*., 2000; Xiao and Herd, 1993). 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 to smaller farms in the study area. This was consistent with findings in the neighboring watershed where the likelihood of shedding *C. parvum*-like oocysts increased with the number of pre-weaned 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 41-56%. 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 New York City 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 six 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 4 species of *Cryptosporidium* that affect cattle were detected in this study in pre-weaned calves. The youngest age we detected *C. parvum* was 7 days, which was in concordance with previous reports suggesting that calves acquired infection with this parasite within the first 1-2 days of life, and started shedding oocysts after a 5-6 day pre-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 was in agreement with other studies that found that calves acquired infection with these species early in life (Santin *et al*., 2004; Starkey *et al*., 2006b). The results of the present study also confirmed earlier suggestions that animals infected with *C. parvum* had significantly higher oocyst counts than animals infected with *C. bovis* and *C. ryanae* (Feng *et al*., 2007; Starkey *et al*., 2006b). We detected that pre-weaned 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 pre-weaned 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-level prevalences 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 the study design was that samples were collected on the same farms in three different seasons, which allowed the evaluation of the effect of season without statistical adjustment for farm effects (Mohammed *et al*., 1999). Seasonal variation in prevalence can be explained by at least 4 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 is 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 pre-weaned 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 pre-weaned calves were zoonotic, as opposed to at least 74% during the rest of the year.

These estimates were slightly lower than the result of another study in the United States, which indicated that *C. parvum* was responsible for 85% of the *Cryptosporidium* infections in pre-weaned 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 pre-weaned 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 vs. non-zoonotic 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.

Empirical prevalence estimates obtained in this study were validated by fitting a Bayesian model to the data. Prior parameters for the model were based on previous data from studies that our group had conducted among the same target population and in the same laboratory. Therefore, these priors were considered to be an objective and unbiased representation of previous knowledge. Applying a Bayesian model allows to account 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 reflect these uncertainties. The 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 New York City Watershed.

APPENDIX

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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CHAPTER THREE

TEMPORAL AND SPATIAL DYNAMICS OF *CRYPTOSPORIDIUM PARVUM* INFECTION ON DAIRY FARMS IN THE NEW YORK CITY WATERSHED: A CLUSTER ANALYSIS BASED ON CRUDE AND BAYESIAN RISK ESTIMATES[1]

ABSTRACT

BACKGROUND

Cryptosporidium parvum is one of the most important biological contaminants in drinking water that produces life threatening infection in people with compromised immune systems. Dairy calves are thought to be the primary source of *C. parvum* contamination in watersheds. Understanding the spatial and temporal variation in the risk of *C. parvum* infection in dairy cattle is essential for designing cost-effective watershed management strategies to protect drinking water sources. Crude- and Bayesian seasonal risk estimates for *Cryptosporidium* in dairy calves were used to investigate the spatio-temporal dynamics of *C. parvum* infection on dairy farms in the New York City Watershed.

RESULTS

Both global (Global Moran's I) and specific (SaTScan) cluster analysis methods revealed a significant ($p<0.05$) elliptical spatial cluster in the winter with a relative risk of 5.8, but not in other seasons. There was a two-fold increase in the risk of *C. parvum* infection in all herds in the summer ($p=0.002$), compared to the rest of the year. Bayesian estimates did not show significant spatial autocorrelation in any season.

[1]This chapter has been prepared in the format for submission to the International Journal of Health Geographics

CONCLUSIONS

Although we were not able to identify seasonal clusters using Bayesian approach, crude estimates highlighted both temporal and spatial clusters of *C. parvum* infection in dairy herds in a major watershed. We recommend that further studies focus on the factors that may lead to the presence of *C. parvum* clusters within the watershed, so that monitoring and prevention practices such as stream monitoring, riparian buffers, fencing and manure management can be prioritized and improved, to protect drinking water supplies and public health.

BACKGROUND

Cryptosporidium is a protozoan parasite that is recognized as one of the most important biological contaminants in drinking water (Graczyk *et al*., 1997). Cryptosporidiosis is associated with gastrointestinal infection which can be life threatening in immuno-compromised individuals. The infection is transmitted by the fecal-oral route either by direct contact or through contamination of food and water (Smith *et al*., 2007). An experimental study of healthy adult volunteers revealed that the ingestion of as few as 30 *Cryptosporidium* oocysts can initiate infection (Graczyk *et al*., 1997). Water-borne transmission is facilitated by the long-lasting infectivity of the oocyst in the environment and its resistance to conventional water treatment technologies such as chlorination (Betancourt and Rose, 2004).

The New York City Watershed is currently the focus of a long-term project investigating the public health risk of waterborne cryptosporidiosis. Active surveillance in the city which began in 1994 has identified over 100 cases of cryptosporidiosis annually among NYC residents (NYC Department of Mental Health and Hygiene, 2009). A quantitative risk assessment model for cryptosporidiosis in

NYC predicted that the mean annual risk estimates for infection for all ages and persons with or without HIV/AIDS exceed the proposed acceptable annual risk level of 1 case of infection per 10,000 (Makri *et al*., 2004).

The New York City water supply system provides drinking water to almost half the population of New York State, which includes over 8 million people in the City and one million in Upstate counties, plus millions of commuters and tourists. The water is supplied from a network of 19 reservoirs and three controlled lakes that contain a total storage capacity of approximately 2 billion cubic meters. The total watershed area for the system is approximately 5,100 square kilometers extending over 200 kilometers north and west of NYC. The system is dependent on precipitation and subsequent runoff via streams and rivers to supply the reservoirs. The water is then moved via a series of gravity-fed aqueducts to the distributions system, where it is chlorinated before it reaches the consumers (NYC Department of Environmental Protection, 2009). Pathogens such as *Cryptosporidium* pose a significant threat to public health in the City's unfiltered water supply, because the oocysts are very resistant to chlorination, and they are regularly detected in reservoir effluents (Betancourt and Rose, 2004).

Dairy calves are thought to be a primary source of zoonotic *Cryptosporidium parvum* contamination in watershed ecosystems (Xiao and Feng, 2008). In the NYC Watershed, the Catskill/Delaware drainage system is home to approximately 200 dairy farms. To avoid building a huge filtration plant that could cost about $8 billion and the associated $300 million per year for operating costs, NYC implements extensive watershed management measures, including water quality monitoring and best management practices (BMP) on agricultural land, with the goal to protect water quality while maintaining economic viability on these farms (NYC Department of Environmental Protection, 2010). Watershed management requires a network design

that demands distinct spatial and temporal monitoring and protection efforts. However to date, the spatial and temporal variation in the risk of *C. parvum* infection in dairy herds in watersheds has not been investigated. Understanding the spatial and temporal pattern of *C. parvum* infection on dairy farms would be useful in designing or modifying watershed management strategies to monitor and mitigate the risk of *C. parvum* contamination in watersheds.

Bayesian approach has been used increasingly in geographical epidemiologic studies, because it stabilizes crude risk estimates by reducing variance heterogeneity. Thus, risk maps based on Bayesian rather than crude risk estimates are preferred because they are more accurate and visually appealing (Berke, 2005). In a Bayesian approach, a prior probability distribution for the values of a parameter (based on previous studies) is converted (under the influence of current observations) to a posterior distribution of that parameter. This posterior distribution is used to provide an estimate for the parameter (Rezaeian *et al*., 2007).

The objectives of the study were to 1) explore and map the temporal and spatial dynamics of the risk of *C. parvum* infection in dairy cattle in the NYC Watershed, and to 2) identify high-risk clusters in space and time. The study utilized both crude-, and Bayesian prevalence estimates to accurately describe the spatial epidemiology of this important zoonotic parasite among dairy herds in a large watershed ecosystem.

METHODS

Description of data and study area

The crude *C. parvum* prevalence estimates were based on a series of cross-sectional studies conducted in the Delaware portion of the NYC Watershed (Szonyi, 2010). The study farms were located within the Cannonsville drainage basin in the City's Delaware Water Supply System, which is the largest basin in the City's system,

encompassing an area of 1200 km^2 within Delaware County (NYC Department of Environmental Protection, 2009). Most of the dairy farms in the study area were family operated, small- scale farms occupying an average of 1-2 km^2 and maintaining a herd of approximately 100 mature dairy cows. There was a year-round calving pattern and most farmers spread calf manure on their fields regularly. The majority of the farmers maintained an open herd (*i.e*. regularly purchased cattle from other herds). Mature cattle were kept on pasture during the summer months and often had direct access to springs. Several farms used untreated spring water as water source for the barn. The study population was drawn from dairy herds enrolled in the Watershed Agricultural Program, which is a voluntary partnership between watershed farmers and the City, aimed at developing and implementing pollution prevention plans on farms to protect water quality. Thirty-two dairy farms were visited once in each of three different seasons defined as spring (April-June) summer (July-September) and winter (December-March). A total of 507 fecal samples were collected from pre-weaned calves (with or without apparent signs of illness) and screened for the presence of *Cryptosporidium* with a quantitative centrifugation flotation method and bright-field microscopy. A sample was considered positive by flotation when at least one oocyst with the correct morphological characters was identified (*C. parvum*-like oocysts are 4-6 µm and spherical; contain a residuum and sporozoites; refract pink in sugar and have a halo in phase) (Georgi, 1990).

Bayesian model

Prevalence data from the cross-sectional studies described above were used to fit a hierarchical Bayesian model using WinBugs version 1.4 software (Spiegelhalter, 1996). All prior estimates in the model were based on the results of epidemiologic studies that our group had conducted among dairy herds in New York State watersheds (Starkey *et al*., 2006a; Starkey *et al*., 2007) . Thus the sensitivity and

specificity of the flotation method was 0.75 and 0.96, respectively (Starkey *et al*., 2007). The Bayesian model was based on binomial sampling (Equation 1) (Branscum *et al*., 2004).

$$y_t / \pi_t, Se, Sp \approx Bin(n_t, \pi_t Se + (1-\pi_t)(1-Sp)) \qquad \text{(Eq 1)}$$

where y_t was the number of flotation-positive calves on farm *t*, n_t was the total number of calves tested on farm *t*, π_t was the prevalence of *Cryptosporidium* among pre-weaned calves on farm *t*, and Se and Sp were the sensitivity and specificity, respectively, of the flotation method. The term (π_t Se + (1- π_t)(1-Sp)) is the probability of a test positive result for a particular calf. Because of our concern regarding the potential over-dispersion in the estimate of π_t (due to the fact that animals are clustered by farm) we controlled for this dependency by conditioning the estimate on farm to achieve approximate conditional independence. In other words, we used a hierarchical modelling approach to be able to pool the information on the prevalence of *Cryptosporidium* from these herds without assuming that they belonged precisely to the same population. To achieve approximate conditional independence, we assigned Bayesian hyperpriors to π_t representing the mean *C. parvum* prevalence in pre-weaned calves in the population (μ), and the variability of this prevalence (ψ) due to aggregation by farms (Equation 2). Prior studies revealed that the average prevalence of *C. parvum* in a New York State watershed in pre-weaned cattle was 20% (Starkey *et al*., 2006a), therefore μ was modelled as beta (12.82, 48.28) with a most likely value of 20%. The same study revealed that the within-farm prevalence of *C. parvum* in this population ranged from 0-40%. Using this estimate, the variability among *C. parvum* prevalence in calves on different farms (ψ) was modeled as gamma (4.5, 0.5). Previous studies also revealed that not all herds in the study area were infected with *C. parvum* (Wade *et al*., 2000). To allow for the possibility of a *C. parvum*-free herd, π_t was modeled with a mixture distribution. A prior study in an adjacent watershed

estimated that 42% of the herds were infected with *C. parvum* (Starkey *et al*., 2006b). In the current study, the probability of a herd being infected (τ) was modeled as beta (9.51, 12.75) with a most likely value of 0.42. Thus, π_t was modeled as a mixture beta-distribution with the hyperpriors μ and ψ as described in equation 2.

$$\pi t / \mu, \psi \approx Beta(\mu\psi, \psi(1-\mu)) \quad \text{with the probability } \tau \text{ and } 1\text{-}\tau \text{ for } \pi_t = 0 \qquad \text{(Eq. 2)}$$

The number of animals and prevalence estimates used in the study are summarized in Table 3.1.

Potential clustering of zoonotic strains

The overall clustering tendency of the disease risk in the study region was assessed by a test of global spatial autocorrelation, which only investigates the presence but not the exact location of the cluster(s). Spatial autocorrelation arises when risk estimates from neighbouring farms are not independent, i.e., correlated. This correlation is measured using the Moran's I. High values for the I implies that disease rates for geographically closer farms are more highly correlated than those from farms that are geographically distant (Moran, 1950). The Moran's I statistic is defined as follows:

$$I = \frac{N}{\sum_i \sum_j w_{ij}} \frac{\sum_i \sum_j w_{ij}(X_i - \overline{X})(X_j - \overline{X})}{\sum_i (X_i - \overline{X})^2}$$

where N is the number of farms, $\overline{X}$ is the average prevalence on the farms, X_i and X_j are the prevalence on farm *i* and *j*, respectively, and w_{ij} is the spatial weight between farms *i* and *j*, determined by the distance between farms *i* and *j*. The Z Score associated with the index is based on the Randomization Null Hypothesis stating that "there is no spatial clustering". Thus Z-scores greater than 1.96 or smaller than −1.96 indicate significant spatial autocorrelation at the 5% level (Moran, 1950).

The spatial relationship among the farms was conceptualized with the inverse

distance model (the impact of one feature on another decreases with distance). The global spatial autocorrelation test was performed on both the crude and the Bayesian prevalence estimates by season, using the geographical information system (GIS) software ArcView 9.2 (ESRI, CA, USA).

The scan statistic implemented in the software SaTScan v8.0.1 (Kulldorff, 2009) was used to test for the presence of purely spatial, purely temporal, and space-time clusters, and to identify their location. The SaTScan statistic evaluates clusters in temporal, spatial and space-time setting by gradually scanning a window across time and/or space. Purely spatial analysis utilizes circular or oval scanning windows, while space-time analysis uses cylinders, with the base representing space and the height indicating time. For each window a likelihood ratio statistic is computed based on the number of observed and expected cases within and outside the window. The likelihood function assuming Poisson distributed cases is proportional to:

$$\left(\frac{c}{E[c]}\right)^{c}\left(\frac{N-c}{N-E[c]}\right)^{N-c} I(\,)$$

Where N is the total number of cases, c and $E[c]$ represent the observed and expected number of cases in a window, while N-c and $N - E[c]$ indicate the observed and expected number of cases outside the window. The indicator function $I(\,)$ is equal to 1 when the window has more cases than expected under the null hypothesis, and zero otherwise. The window with the highest likelihood ratio is the most likely cluster and is assigned a p value through 999 Monte Carlo simulations (Kulldorff, 1997). A Poisson model was fitted to the raw data for each season to examine the presence of purely spatial clusters. A Poisson model was also applied to the entire dataset to determine whether purely temporal (i.e. seasonal) or space-time clusters existed during the course of a year. At each farm location, cases were defined

as the number of calves that tested positive for *Cryptosporidium* by the flotation method, while the population size was the total number of calves that were tested. The maximum cluster size was set at the recommended value (50% of the total population at risk). Both circular and oval cluster shapes were evaluated.

Mapping the risk of *C. parvum* in the watershed

Geospatial coordinates for each farm were collected with a Garmin eTrex Summit handheld global positioning system (GPS) device (Garmin International Inc, Olathe, Kansas, USA) and imported into the GIS software Manifold System 8.0 Ultimate Edition (Manifold, Carson City, NV, USA). The geographical coordinates were re-projected into the Universal Transverse Mercator coordinate system, Zone 18(N), North American Datum 1983, and overlaid with the shapefile of Delaware County, NY obtained from the New York State Geographic Information System Clearinghouse (www.nysgis.state.ny.us). Dot maps indicating the seasonal prevalence estimates on the study farms were created to examine the spatial dynamics of *C. parvum* in the watershed during the annual cycle, and to explore the differences between the crude-, and the Bayesian estimates.

RESULTS

Potential clustering of zoonotic strains

The Global Moran's I statistic was performed on both crude-, and Bayesian prevalence estimates to determine the presence of global autocorrelation in three different seasons. The results of this analysis were summarized in Table 3.2. The crude estimates revealed significant spatial autocorrelation in the winter with a Global Moran's I value of 0.18 ($p=0.03$), and no clustering in the spring and summer. In contrast, the Bayesian prevalence estimates did not show significant overall clustering tendency in any of the seasons examined.

Crude prevalence estimates were used to test for the presence of spatial,

temporal, and space-time clusters using the Poisson model with the scan statistic. The results of the SaTScan analyses were summarized in Table 3.3. The purely temporal analysis revealed a 2-fold increase in the risk of *C. parvum* infection in the summer affecting all the herds in the study area. A significant (p=0.003) oval-shaped cluster was identified in the winter, with a more than 5-fold increase in risk inside the cluster compared to the rest of the study area. In addition, significant space-time clusters were detected in the summer with both oval and circular scanning window settings. These space-time clusters included nearly 50% of the population at risk and overlapped geographically. Therefore, only the circular space-time cluster was shown in Figure 3.1.

Risk maps

Dot maps were created to explore the spatio-temporal dynamics of *C. parvum* infection in dairy herds in Delaware County, NY, based on crude-, and Bayesian prevalence estimates (Figures 3.1 and 3.2). The significant spatial and space-time clusters identified by the SatScan statistics were also indicated. Although cluster analyses based on Bayesian estimates did not show significant spatial clustering in any season, the map revealed a diffuse increase in the risk of *C. parvum* contamination in the summer.

Table 3.1 Characteristics of the data used in the study

Season	N samples[1]	N positive[2]	CP[3]	Range of CP[4]	BP[5]	Range of BP[6]
Spring	150	23	15	0-100	10	0-37
Summer	182	47	26	0-100	19	0-48
Winter	175	20	11	0-64	9	0-51

[1] Number of pre-weaned calves tested with the flotation method
[2] Number of pre-weaned calves positive for *C. parvum* with the flotation method
[3] Crude average prevalence of *C. parvum* among pre-weaned calves expressed as percent
[4] Range of crude within-herd prevalence of *C. parvum* expressed as percent
[5] BP Bayesian average prevalence of *C. parvum* among pre-weaned calves expressed as percent
[6] Range of Bayesian within-herd prevalence of *C. parvum* expressed as percent

Table 3.2 Results for the test of global spatial autocorrelation using Moran's I statistics based on crude-, and Bayesian risk estimates

Season	Estimate	Moran's I	Z-score	p-value
Spring				
	Crude	0.0087	0.41	0.67
	Bayesian	-0.0067	0.24	0.8
Summer				
	Crude	-0.17	-1.4	0.15
	Bayesian	-0.1	-0.64	0.51
Winter				
	Crude	0.18	2.07	0.03
	Bayesian	0.061	1.03	0.3

Table 3.3 The most likely temporal, spatial, and space-time clusters identified by the SaTScan statistics using the Poisson probability model, based on crude prevalence estimates

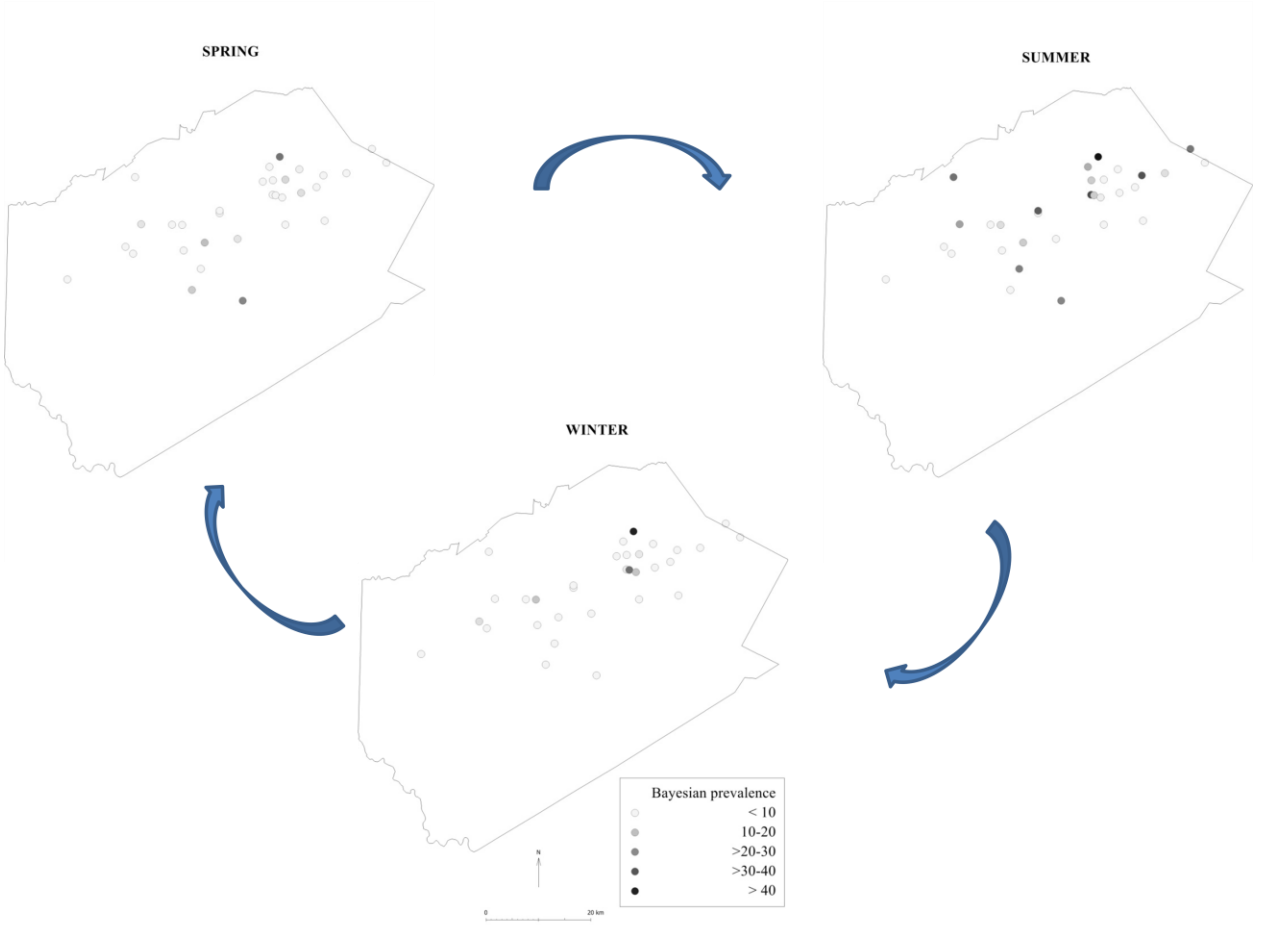
Analysis type	Observed[1]	Expected[2]	RR[3]	P-value	Population[4]	Shape
Purely Temporal	57	38	2	0.002	169	
Purely Spatial						
Spring	no significant cluster					
Summer	no significant cluster					
Winter	14	4	5.87	0.003	29	elliptical
Space-Time	37	20	2.3	0.031	83	circular
	40	17	3	0.004	78	elliptical

[1] Number of observed cases in cluster
[2] Number of expected cases in cluster
[3] Relative Risk
[4] Number of animals at risk in the cluster

Figure 3.1 Dot map of the spatio-temporal dynamics of *C. parvum* infection in dairy herds in Delaware County, NY, based on crude prevalence estimates. Significant circular clusters identified by the SaTScan statistics are also shown

SPRING
SUMMER
space-time cluster
WINTER
purely spatial cluster
Prevalence
< 10
10-20
>20-30
>30-40
> 40
N
0
20 km

Figure 3.2 Dot map of the spatio-temporal dynamics of *C. parvum* infection in dairy herds in Delaware County, NY, based on Bayesian risk estimates


SPRING
SUMMER
WINTER
Bayesian prevalence
< 10
10-20
>20-30
>30-40
> 40
N
0
20 km

DISCUSSION

This study was carried out to evaluate potential clustering of dairy herds that are infected with *C. parvum* in the NYC Watershed. This was the first study to evaluate the spatial and temporal variation in the risk of *C. parvum* infection in dairy cattle in an important watershed.

The decision to include only pre-weaned calves in this study was based on the results of a quantitative risk assessment (QRA) of *Cryptosporidium* in dairy cattle in the NYC Watershed which revealed, that despite representing only a small proportion of the population and producing a small fraction of manure, pre-weaned calves produced the vast majority of all zoonotic *C. parvum* oocysts shed within the dairy cattle population. Specifically, it was estimated that pre-weaned calves produced 99.5% of the total *C. parvum* oocyst burden with a calculated mean log oocyst shedding of 4.02×10^{10} daily. Thus it was estimated that pre-weaned calves produce nearly all the *C. parvum* oocysts that contaminate the watershed (Starkey *et al*., 2007).

One of the assumptions in the present models was that all *C. parvum*-like oocysts shed by pre-weaned calves were zoonotic. This assumption was made because molecular typing is required to determine the zoonotic potential of *C. parvum*-like oocysts, and this necessity would have further amplified the problem of small numbers. While over-estimating the zoonotic risk, we felt this assumption was reasonable, because recent studies that applied molecular typing revealed that the majority of *Cryptosporidium* infections in pre-weaned calves were indeed zoonotic (Santin *et al*., 2004; Starkey *et al*.; Szonyi *et al*, 2010).

Both global (Moran's I) and specific (SaTScan) cluster detection methods identified a significant spatial cluster of *C. parvum* infection in calves in the winter, with a relative risk (RR) of 5.8, based on crude risk estimates. No other purely spatial clusters were identified with either method. Thus, there was complete agreement

between the results of the two cluster detection methods. In addition, the scan statistics detected a significant space-time cluster in the summer with both circular (RR= 2.3) and elliptical (RR=3) window settings. Further investigation revealed the presence of a significant temporal cluster (RR=2) but the lack of a purely spatial cluster in the summer, which suggests that the space-time clusters identified in the summer were due to a temporal rather than a spatial increase in risk. The large sizes of the space-time clusters including nearly 50% of the population at risk (maximum allowed under the conditions specified) also supports the notion of a spatially diffuse increase in the risk of *C. parvum* infection in the summer.

It has been suggested that farms downstream of other farms may be contaminated with *Cryptosporidium* via runoff from farms upstream, although evidence for this epidemiologic link is lacking (Hansen and Ongerth, 1991; Ong *et al*., 1996; Sischo, 2000). The rationale for considering elliptical spatial clusters in this study was that farm-to-farm transmission via runoff would be expected to produce an elliptical rather than a circular cluster.

The term disease cluster is defined as an increase in the expected number of cases within a population bounded in space and time (Elliott and Wartenberg, 2004). Two different cluster detection methods were used in this study to ensure comparability and robustness of results. The scan statistics was selected for the investigation of temporal and space-time clusters, because recent studies identified SatScan as the most developed and robust space-time surveillance software package that takes multiple testing problems into account, and is considered the most powerful for detecting localized clusters (Robertson and Nelson, 2009).

The major limitation of the study was the low number of pre-weaned calves on the farms, resulting in unstable risk estimates as small populations have large variability in rates (Olsen *et al*., 1996) . For example, the small number of cases and

population at risk may have accounted for the high relative risk estimate (RR=5.8) for the winter spatial cluster. This limitation was corrected with the use of a Bayesian approach that has the ability to stabilize the raw estimates derived from a small number of individuals (Berke, 2004). The Bayesian approach also allowed the incorporation of prior knowledge about the risk of *C. parvum* infection in the target population, and accounted for the imperfections of the diagnostic test. However, the quality of this prior information might influence the quality of the estimate and hence could be a source of bias and limitations (Elliott and Wartenberg, 2004).

The degree of smoothing is a trade-off between high sensitivity (truly high risk areas correctly identified) and high specificity (areas without excessive risk correctly identified) such that sensitive but non-specific measure will generate many false positive findings, whereas a specific but not sensitive measure will miss areas with high risk (Elliott and Wartenberg, 2004). In this study contradictory results were encountered in the analysis of purely spatial clusters using Global Moran's I statistics. While crude estimates revealed significant spatial autocorrelation in the winter, Bayesian estimates indicated the lack of a spatial clustering in any season. Considering the limitations of using crude *vs.* Bayesian estimates in spatial analysis, the discrepancy in the results may be due either to the instability of the crude estimates leading to spuriously high values, or to the low sensitivity of the Bayesian model to detect areas with truly high risk.

Cryptosporidium is considered a non-point source pollutant in watersheds that is carried off the land surface during rain events (Atwill *et al*., 2006; Graczyk *et al*., 2000). Monitoring of stream sites in the study area revealed that event based (e.g. after a storm) *Cryptosporidium* concentrations were consistently higher than baseline results (up to 11.7 oocysts $50l^{-1}$), implicating runoff as contamination source (NYC Department of Environmental Protection, 2009). The close relationship between

activities in the drainage basin and the quality of its water resources forms the underlying premise for all watershed management programs. Best management practices that protect water supply on farms such as fencing, filter strips, stream crossings, animal trails and walkway, manure composting facility, and runoff management systems would ideally and ultimately be implemented on every farm in the watershed. However, until that goal is achieved, prioritization methodologies to address non-point source pollutants need to be developed, and the identification of "hot spots" is an integral part of this process.

The occurrence of spatial or temporal clusters may be due to rapid spread between locations in the case of a highly contagious disease, or the presence of common environmental risk factors (Stevens *et al*., 2009). The higher risk of *C. parvum* infection on dairy farms in the summer throughout the study area may be due to climatic or management factors that affect the entire area. This finding suggests that spreading manure in the summer (compared to other seasons) in any area of the watershed is associated with an increased risk of *C. parvum* contamination of the water supply. This finding is important because farms in the study area regularly spread untreated calf manure in the fields. The current recommendation is to avoid spreading manure in the spring and during frozen conditions, while summer is considered a lower risk period (NYC Department of Environmental Protection, 2009). If further studies confirm an increased risk of *Cryptosporidium* contamination in the summer, this knowledge will be useful to improve Nutrient Management Plans, which give recommendations about the most environmentally safe time and place to spread manure.

With the City's population expected to rise to 9.1 million by 2039 from 8.3 million in 2005, watershed management will continue to have an important part to play in protecting water quality (NYC Department of Environmental Protection,

2010). Over time, systematic and careful monitoring of disease-causing organisms and pollutants will determine the effectiveness of New York City's protection strategies and the continued success of its filtration avoidance plan. The identification of spatial or temporal "hot-spots" of *C. parvum* contamination within the watershed will have important implications for watershed monitoring and management, and need to be the focus of future investigations.

CONCLUSIONS

The identification of *C. parvum* clusters is a priority in designing cost-effective and targeted watershed management practices to ensure safety of the water supplies for public health. This study identified high risk clusters of *C. parvum* infection in dairy herds in both space and time in a large and important watershed, suggesting that further studies are needed to determine whether the presence of clusters are persistent and predictable. We recommend that future studies focus on the causes of these "hot spots" so that watershed monitoring and management strategies may be implemented and targeted to effectively decrease *C. parvum* contamination of the water supply.

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CHAPTER FOUR

A CASE-CONTROL STUDY OF FACTORS ASSOCIATED WITH THE RISK OF ZOONOTIC *CRYPTOSPORIDIUM* INFECTION IN DAIRY CALVES[1]

Objective – To identify risk factors associated with zoonotic *Cryptosporidium* infection in dairy calves

Design – Case-control study

Animals/Sample population – The target population consisted of dairy calves in the New York City Watershed. Cases consisted of calves infected with *C. parvum* and controls were either infected with *C. bovis* or were not infected with *Cryptosporidium*.

Procedures – Fecal samples were tested for the presence of *Cryptosporidium* spp. using the flotation concentration method. Samples were genotyped by sequencing of the 18s rRNA gene. The association between host, management, geographical, meteorological factors and *Cryptosporidium* genotype was assessed using univariable and multivariable logistic regression.

Results – A total of 108 cases and 283 controls were enrolled. The controls included 67 animals with *C. bovis* infection. Younger calves and calves housed in the cow barn were more likely to be infected with both genotypes. Herd size and hay bedding were associated with an increased risk of infection with *C. parvum,* while Jersey breed was a risk factor for *C. bovis* infection. There appeared to be a lower risk of infection with both species at higher latitudes, although this trend was not statistically significant. Compared to a flat surface, steeper slope was significantly associated with a decreased likelihood of infection with both genotypes, while precipitation influenced the risk of *C. parvum* infection only.

[1] This chapter has been prepared in the format for submission to the American Journal of Veterinary Research

Conclusions and Clinical Relevance – The unique aspects of the study were the examination of putative risk factors at the genotype level and the identification of factors that put calves at risk of infection with zoonotic strains of *Cryptosporidium*. The findings will be useful to design measures that reduce animal exposure, and decrease the public health risk and economic losses associated with *C. parvum* infection in cattle.

INTRODUCTION

Cryptosporidium parvum is a protozoan parasite that causes gastro-intestinal disease in humans and neonatal cattle. Clinical appearance of *C. parvum* infection in calves can range from asymptomatic shedding of oocysts to severe diarrhea, dehydration and death (Fayer *et al*., 2009). Furthermore, damage to the intestinal epithelium can cause prolonged malnutrition and reduced growth rates in calves, resulting in significant economic losses in dairy operations (Nydam and Mohammed, 2005).

Although cattle are commonly infected with four different species of *Cryptosporidium (C. parvum, C. bovis, C. ryanae* and *C. andersoni),* only *C. parvum* is of primary concern from both the public health perspective and in terms of economic losses to the dairy industry due to morbidity in calves (Fayer *et al*., 2009). Pre-weaned calves are thought to be the main source of *C. parvum* contamination in watersheds that threaten the quality of drinking water supplies. Pre-weaned calves may also be infected with *C. bovis,* which is considered host adapted, non-zoonotic, and non pathogenic in cattle (Fayer *et al*., 2005). One of the main challenges to identifying risk factors for *C. parvum* infections in cattle is that molecular techniques are required to distinguish *C. parvum* from the morphologically similar *C. bovis* and

C. ryanae (commonly referred to as the *C. parvum*-like species) (Fayer *et al*., 2009). It is necessary to decrease the risk of infection with *C. parvum* in cattle to protect water consumers and prevent economic losses in the dairy industry. To effectively control the occurrence of *C. parvum* infection in cattle, molecular techniques need to be incorporated into epidemiological studies to identify relevant risk factors associated with this parasite.

Cryptosporidium oocysts are tolerant to most disinfectants and can survive for months in the environment under favorable conditions (Fayer *et al*., 1997). Temperature, moisture, and UV radiation are among the most important factors affecting oocysts survival in the environment (Peng *et al*., 2008). While it has been shown that environmental conditions influence oocyst survival, their effect on the risk of infection has not been determined. It is also not know whether different environmental or ecological conditions facilitate infection with *C. parvum* compared to *C. bovis* in calves. It is important to identify ecological niches that favor oocyst survival and lead to increased risk of infection with the zoonotic genotype, as this information could be used to design preventive measures to decrease the risk of exposure.

The primary objective of this study was to identify risk factors associated with zoonotic *Cryptosporidium* infection in dairy calves. Specifically, we conducted case-control studies to examine the association between host, management, geographical, and meteorological factors and *C. parvum* infection in pre-weaned calves using *C. parvum* positive cases and two sets of controls: *Cryptosporidium* negative and *C. bovis* positive controls. In addition, risk factors for *C. bovis* infection were also investigated using *C. bovis* positive cases and *Cryptosporidium* negative controls.

MATERIALS AND METHODS

Study design

Unmatched retrospective case-control studies were carried out to identify potential risk factors for infection with *C. parvum* and *C. bovis* among dairy calves. The study population was recruited from animals that were enrolled in our ongoing studies in this target population on 44 dairy farms in the New York City Watershed (Szonyi *et al*, 2010; Wade *et al*., 2000). Briefly, fecal samples were collected from 1,072 pre-weaned calves (< 65 days of age) and screened for the presence of *Cryptosporidium* oocysts. There were 205 animals diagnosed as shedding *C. parvum*-like oocysts using a flotation technique. Subsequently, polymerase chain reaction (PCR) and sequencing of the 18s rRNA gene were performed on all flotation-positive specimens to determine the *Cryptosporidium* species in the sample. Sample size estimation[a] showed that with 2 controls per case for exposures of 25% in the control population and at 95% confidence, a study with 69 cases would have 80% power, while a study with 91 cases would have 90% power to detect odds ratios of 2.5 or more. The protocol for the studies was approved by the Institutional Animal Care and Use Committee at Cornell (Protocol # 00-4).

Sample collection and screening

Fecal samples were collected rectally from each animal into a plastic cup that was immediately capped and labeled to identify source based on the ear tag number. For each sample, 1 g of feces was processed using sugar (sg 1.33) as the flotation medium with a standard quantitative centrifugation flotation technique (Georgi, 1990). *Cryptospridium* oocysts were confirmed at 400X magnification with both bright-field and phase contrast illumination. A sample was considered positive when at least one oocyst with the correct morphological characters was identified at 400X magnification using bright-field and phase contrast illumination (*C. parvum*-like oocysts are 4-6 µm

and spherical; contain a residuum and sporozoites, refract pink in sugar and have a halo) (Wade *et al*., 2000).

Molecular typing of specimens

DNA was extracted from all samples that tested positive for *Cryptosporidium* oocysts and a select group of controls.[b] A two-step nested PCR protocol was used to amplify an 830bp 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). The primary reaction consisted of 25 µl including 1 µl of genomic DNA, 10.8 µl of reverse osmosis water, 2 µl of 10X PCR buffer,[c] 4.8 µl of $MgCl_2$ (25 mM), 0.4 µl of dNTP's (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 10X PCR buffer, 2.4 µl of $MgCl_2$ (25 mM), 0.4 µl of dNTP's (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). The PCR products were visualized after electrophoresis on 1% agarose gel stained with ethidium bromide. After purification of PCR products using exonuclease I-shrimp alkaline phosphatase,[d] the products were sequenced using the internal primers described above in 9-µL reactions in an automated sequencer.[e] After bi-directional sequencing, the sequence chromatograms were aligned using MEGA 4 software [f] (Tamura *et al*., 2007). We compared DNA sequences with GenBank DNA sequences to determine the species of *Cryptosporidium* in the sample using the Basic

Local Alignment Search Tool (BLAST).

Definition of cases and controls

A case was defined as a pre-weaned calf (<65 days of age) that tested positive by flotation for *Cryptosporidium*, and subsequent sequencing of the 18s rRNA gene revealed a 100% homology with *C. parvum* (GenBank accession number AF093490). Two set of controls were used in this study. The first set of controls, *Cryptosporidium* negative controls were selected randomly from pre-weaned calves (< 65 days of age) that tested negative by both the flotation concentration and by PCR targeting the 18s rRNA gene. Random selection was carried out using the animal's unique identification number and a random number generator using Microsoft Excel [g]. The second set of controls consisted of all pre-weaned calves (< 65 days of age) that tested positive by flotation for *Cryptosporidium*, and subsequent sequencing of the 18s rRNA gene revealed a 100% homology with *C. bovis* (GenBank accession number AY120911). Specimens that tested positive by flotation for *Cryptosporidium*, but either 1) subsequently failed to produce an 18s rRNA gene product, or 2) the amplicon did not have 100% homology with either *C. bovis* or *C. parvum*, were excluded from the study.

Data collection

A questionnaire was used to collect information on host factors and farm management practices. The data was collected by personal interview of the farm owner/manager. Geospatial coordinates for each farm were recorded at the calf housing facility with a handheld global positioning system (GPS) device.[h] Digital Elevation Models (DEM) for the study area were obtained from the New York State Geographic Information System Clearinghouse (www.nysgis.state.ny.us). The GPS data for farm locations and the DEM were imported into a geographical information system (GIS) software,[i] and re-projected into the Universal Transverse Mercator

(UTM) coordinate system (Zone 18N), North American Datum 1983. The DEM and farm coordinates were overlaid to obtain information on geomorphological features (*i.e.* elevation, slope and aspect) associated with each farm location. Elevation was converted from feet to meters (1ft = 0.305 m), and slope was expressed as percent gradient. The numerical slope aspect values were converted to categorical to reflect the direction in which the slope faces; *i.e.* slope aspect was equal to south (greater than or equal to 112° and less than 292° azimuth) or north (0-111°; 292-360° azimuth). To determine whether the geographic position of farms with respect to cardinal directions had effect on genotype, we included the easting and northing coordinates in the logistic regression analyses. The term "easting" refers to the eastward measured distance from the false east reading, which is uniquely defined in each UTM zone, while "northing" refers to the northward measured distance from the equator. Information on precipitation and temperature was obtained from the National Climatic Data Center, US Department of Commerce (www.ncdc.noaa.gov). For the analyses, all the rainfall measurements were converted from inches to millimeters (1inch=25.4 mm), and all the temperature measurements were converted from F to ˚C [˚C =(F-32)/9 X 5]. It has been shown that oocyst excretion starts 2-7 days post-infection, and lasts for up to two weeks (Fayer *et al*., 1997). The effect of precipitation and perhaps temperature is thought to exert an influence on the length of oocyst shedding intervals as well as the timing of shedding. Therefore, the average temperature and precipitation one month before the detection of oocyst shedding was considered, as this time frame appeared to be the most biologically plausible.

Statistical analyses

A systematic approach for data analysis was adopted. First, we examined the bivariable association between shedding *C. parvum* (zoonotic genotype) and each of the putative factors, in two sets of controls: 1, *Cryptosporidium*-negative controls and

2, *C. bovis*-infected animals. The significance of association between the putative risk factors and the likelihood of shedding *C. bovis* was also evaluated using *Cryptosporidium* negative controls. All analyses were conducted using standard statistical software. [j] Continuous variables were grouped into categories based on equal intervals. The functional form of the relationship between continuous variables and outcome were explored by plotting log odds ratios on a graph. Where a linear trend was supported by visual inspection, the variable was also considered as continuous. Screening of variables was performed using univariable logistic regression analysis to assess the effects of all variables on the outcome of infection. When the number of observations at a certain level of the independent variable and infection status was less than five, Fisher's exact test was used to evaluate the significance of association in the univariable analysis; such variables were omitted from the multivariable models. All other variables were considered for inclusion in the multivariable logistic regression models. Variables were retained in the model if the Wald-test p-value was < 0.05 and/or the variable significantly improved the model fit (likelihood ratio statistic < 0.05). Biologically plausible interaction terms were tested between final model variables at the first order level.

Because the sampling units, the animals, in this study were clustered in herds, it was assumed that this clustering would lead to a correlation in the likelihood of infection within the study population. This correlation between responses occurs because they are dependent on exogenous factors that are associated with these responses, *i.e.*, infection with the organism. Conditioning on an observed set of these factors by controlling for their effect in the analysis and including them as covariates in the logistic regression analysis will sometimes achieve approximate conditional independence. However, more often this correlation in the response arises from both observed and unobserved risk factors. It was assumed that the unobserved risk factors

were randomly distributed among farms and the overall significance of this assumption was evaluated by using a mixed-effect logistic regression model.

The final multivariable models were examined for goodness- of-fit using the Hosmer-Lemeshow statistics, (Hosmer and Lemeshow, 1989) and the stability of the models were assessed by examining the delta betas (Pregibon, 1981). The models were considered stable if removal of observations with the largest delta-beta values (*i.e*. those observations whose exclusions were predicted to have the largest influence on model fit) altered the odds ratio by < 25% and did not affect the significance of individual variables.

RESULTS

Descriptive

A total of 108 *C. parvum* cases and 67 *C. bovis* positive animals met the inclusion criteria and were enrolled in their respective category. In addition, 216 *Cryptosporidium* - negative controls were randomly selected. The 44 study farms were located within a distance of 66 kilometers in the east-west, and 31 kilometers in the north-south direction. The average elevation on the farms was 512 m (range 376-570 m) with a 6% slope (range 0-17.6 %). The average monthly precipitation ranged from 47 to 198 mm, while the mean monthly temperature ranged between -8.5 and 21.5 ˚C. Total herd size (including all ages) varied from 46 to 800 head of cattle.

C. parvum cases *vs*. *Cryptosporidium* negative controls

A graphical examination of the frequency distribution of the continuous variables (age, herd size, geographical location, and metrological features) and their relation to the log odds ratios of being a case indicated a non-linear trend. Thus, these variables were retained as ordered categorical.

In the univariable analysis, calves that were more than one month of age were at significantly decreased risk of *C. parvum* infection in comparison to younger calves

(Table 4.1). Herd size, outdoor housing, dirt flooring and being housed in a pen were associated with an increased risk, while keeping calves in a greenhouse was associated with a decreased risk of infection with *C. parvum*. Among geographical factors, steeper slope was significantly associated with a lower risk of *C. parvum* infection. The likelihood of infection was higher with a monthly precipitation of 100-150 mm compared to < 100 mm; however precipitation exceeding 150 mm was not associated with an elevated risk.

The final multivariable model for *C. parvum* infection using *Cryptosporidium* negative controls revealed that the risk of being a case was significantly increased with herd size > 200 compared to < 100, being housed in the cow barn, and with the use of hay bedding, while older age was associated with lower risk (Table 4.2). Compared to a flat surface, a slope of 5-10% was associated with a decreased risk; however a slope greater than 10% was not significant in the model. There appeared to be a lower risk at higher latitudes (northing), although this trend did not reach statistical significance at the 5% level. Precipitation of 100-150 mm compared to < 100 mm remained a significant risk factor in the multivariable model. No statistically significant interactions were identified. The model was robust to the exclusion of observations with the highest delta-beta values (extreme values), and provided adequate goodness-of-fit, as measured by the Hosmer-Lemeshow statistics (p=0.58).

Table 4.1 Univariable logistic regression analysis for associations between putative risk factors and *C. parvum* infection using *Cryptosporidium* negative controls in pre-weaned calves

	Cases (%)	Controls (%)	OR	95 % CI	Wald p-value
Host factors					
Age					< 0.001
< 1 month	100 (59.1)	70 (40.9)	1		
1-2 months	8 (5.2)	146 (94.8)	0.038	0.01-0.08	
Breed					
Holstein	101 (33.5)	201 (66.5)	1		
Jersey	7 (46.7)	8 (53.3)	1.74	0.61-4.93	0.29
Herd management					
Herd size					
< 100	12 (13.6)	76 (86.4)	1		
100 to 200	39 (24.2)	122 (75.8)	2	1.0-4.1	0.05
> 200	57 (76.0)	18 (24.0)	20.1	8.9-44.9	< 0.001
Greenhouse					
No	96 (35.8)	172 (64.2)	1		
Yes	12 (21.4)	44 (78.6)	0.48	0.24-0.96	0.04
Outdoors					
No	67 (26.2)	189 (73.8)	1		
Yes	41 (65.1)	22 (34.9)	5.25	2.91-9.46	< 0.001
In cow barn					
No	64 (41)	92 (59)	1		
Yes	42 (30.2)	97 (69.8)	0.62	0.38-1.0	0.06
Flooring					
Cement	50 (49.0)	52 (51.0)	1		
Dirt	58 (26.1)	164 (73.9)	2.71	1.66-4.49	< 0.001
Hay bedding					
No	36 (33.0)	73 (67.0)	1		
Yes	68 (33.8)	133 (66.2)	1.03	0.63-1.7	0.88
Dust bedding					
No	56 (33.1)	113 (66.9)	1		
Yes	48 (34.0)	93 (66.0)	1.04	0.64-1.67	0.86

Table 4.1 Continued

		Cases (%)	Controls (%)	OR	95 % CI	Wald p-value
Tied						
	No	57 (40.14)	85 (59.86)	1		
	Yes	51 (31.5)	111 (68.5)	0.68	0.42-1.0	0.11
Pen						
	No	55 (30)	128 (70)	1		
	Yes	53 (43.8)	68 (56.2)	1.81	1.12-2.92	0.015
Geographical features						
Easting (km)						
	< 500	29 (41.4)	41 (58.6)	1		
	500 to 520	52 (34.9)	97 (65.1)	0.75	0.42-1.35	0.351
	> 520	26 (25.0)	78 (75.0)	0.47	0.24-0.90	0.023
Northing (km)						
	< 4675	19 (43.2)	25 (56.8)	1		
	4675 to 4685	40 (34.5)	76 (65.5)	0.69	0.34-1.4	0.3
	> 4685	48 (29.4)	115 (70.6)	0.54	0.27-1.08	0.08
Elevation (m)						
	< 470	45 (58.4)	32 (41.6)	1		
	470 to 570	36 (20.8)	137 (79.2)	1.19	0.68-2.08	0.52
	> 570	27 (36.5)	47 (63.5)	0.79	0.44-1.41	0.43
Slope (%)						
	< 5	51 (42.9)	68 (57.1)	1		
	5 to 10	43 (29.5)	103 (70.5)	0.55	0.33-0.92	0.02
	> 10	14 (23.7)	45 (76.3)	0.41	0.21-0.83	0.01
Aspect						
	South	48 (33.8)	94 (66.2)	1		
	North	60 (33.0)	122 (67.0)	0.96	0.65-1.65	0.87
Meteorological factors						
Temperature (˚C)						
	< 0	37 (33.6)	73 (66.4)	1		
	0 to 11	22 (36.6)	38 (63.4)	1.14	0.59-2.2	0.69
	> 11	49 (31.8)	105 (68.2)	0.92	0.54-1.55	0.75
Precipitation (mm)						
	< 100	46 (28.8)	114 (71.3)	1		
	100 to 150	41 (47.7)	45 (52.3)	2.25	1.31-3.89	0.003
	> 150	21 (26.9)	57 (73.1)	0.91	0.49-1.67	0.76

Table 4.2 Final multivariable logistic regression model for associations between *C. parvum* infection and explanatory variables using *Cryptosporidium* negative controls in pre-weaned calves

	OR	95% CI	Wald p-value	LRS p-value
Age				< 0.001
< 1 month	1			
1-2 month	0.01	0.0025-0.051	<0.001	
Herd size				< 0.001
< 100	1			
100-200	2.87	0.81-10.1	0.099	
> 200	292	46-1836	< 0.001	
In cow barn				< 0.001
No	1			
Yes	14	2.5-78.81	0.003	
Hay				< 0.001
No	1			
Yes	7.05	2.4-20.1	< 0.001	
Northing (km)				0.002
< 4675	1			
4675 to 4685	0.22	0.03-1.3	0.096	
> 4685	0.24	0.05-1.12	0.071	
Slope (%)				0.003
< 5	1			
5 to 10	0.14	0.044-0.45	0.001	
> 10	0.57	0.1-3.0	0.51	
Precipitation (mm)				0.042
< 100	1			
100 to 150	3.35	1.2-9.5	0.02	
> 150	1	0.3-3.3	0.99	

C. parvum cases *vs*. *C. bovis* controls

After visual inspection, none of the continuous variables were deemed to have a linear relationship with the log odds of *C. parvum* infection using *C. bovis* controls. Thus all continuous variables were retained as ordered categorical.

Univariable analysis showed that compared to *C. bovis* controls, the likelihood of *C. parvum* infection increased significantly with herd size, outdoor housing, dirt flooring, and being in a pen, while older age, Jersey breed, being tied and housed in the cow barn were associated with a lower risk (Table 4.3). The risk appeared to be increasing at higher latitudes, but this trend was not consistent across the categories. The univariable effects of the meteorological factors were not significant.

The multivariable model for *C. parvum* infection using *C. bovis* infected controls revealed that the risk of being a case significantly increased with herd size and the use of hay bedding, while older age and Jersey breed were associated with lower risk (Table 4.4). The risk seemed to be increasing with precipitation, although this trend was not significant across the categories (*i.e.* only 100-150 mm was associated with a significantly higher risk compared to the reference category of < 100 mm). Similarly, there appeared to be an elevated risk associated with latitude (northing), but this trend was not consistently observed across all categories of this variable. There were no statistically significant interactions. The model was robust to the exclusion of observations with highest delta-beta values, and fitted the data adequately as measured by the Hosmer-Lemeshow goodness of fit statistics (p=0.44).

Table 4.3 Univariable logistic regression analysis for associations between putative risk factors and *C. parvum* infection using *C. bovis* controls in pre-weaned calves

		Cases (%)	Controls (%)	OR	95 % CI	Wald p-value
Host factors						
Age						
	< 1 month	100 (70.9)	41 (29.1)			
	1-2 months	8 (23.5)	26 (76.5)	0.12	0.05-0.30	< 0.001
Breed						
	Holstein	101 (69.2)	45 (30.8)			
	Jersey	7 (25.0)	21 (75.0)	0.14	0.05-0.37	< 0.001
Herd management						
Herd size						
	< 100	12 (33.3)	24 (66.7)	1		
	100 to 200	39 (61.9)	24 (38.1)	3.25	1.37-7.67	0.007
	> 200	57 (75.0)	19 (25.0)	6	2.52-14.26	< 0.001
Greenhouse						
	No	96 (61.5)	59 (38.1)	1		
	Yes	12 (60.0)	8 (40.0)	0.92	0.35-2.38	0.86
Outdoors[1]						
	No	67 (50.8)	65 (49.2)	1		
	Yes	41 (95.3)	2 (4.7)	19.8	4.62-85.53	< 0.001
In cow barn						
	No	65 (80.2)	16 (19.8)	1		
	Yes	43 (51.2)	41 (48.8)	0.25	0.12-0.51	< 0.001
Flooring[1]						
	Cement	58 (47.5)	64 (52.5)	1		
	Dirt	50 (94.3)	3 (5.7)	18.4	5.4-62.1	<0.001
Hay bedding						
	No	36 (54.5)	30 (45.5)	1		
	Yes	68 (65.4)	36 (34.6)	1.57	0.83-2.95	0.15
Dust bedding						
	No	56 (62.9)	33 (37.1)	1		
	Yes	48 (59.3)	33 (37.1)	0.85	0.46-1.58	0.62

Table 4.3 Continued

		Cases (%)	Controls (%)	OR	95 % CI	Wald p-value
Tied						
	No	57 (52.78)	16 (23.88)	1		
	Yes	51 (47.22)	51 (76.12)	0.28	0.14-0.55	<0.001
Pen						
	No	55 (50.0)	55 (50.0)	1		
	Yes	53 (81.54)	12 (18.46)	4.41	2.12-9.16	<0.001
Geographical features						
Easting (km)						
	< 500	29 (76.3)	9 (23.7)	1		
	500 to 520	52 (59)	36 (41)	0.44	0.18-1.05	0.068
	> 520	26 (54.2)	22 (45.8)	0.36	0.14-0.93	0.036
Northing (km)						
	< 4675	19 (50.0)	19 (50.0)	1		
	4675 to 4685	40 (78.4)	11 (21.6)	3.63	1.44-9.14	0.006
	> 4685	48 (56.5)	37 (43.5)	1.29	0.6-2.79	0.5
Elevation (m)						
	< 470	45 (68.2)	21 (31.8)	1		
	470 to 570	36 (51.4)	34 (48.6)	0.49	0.24-0.99	0.04
	> 570	27 (69.2)	12 (30.8)	1.05	0.44-2.46	0.91
Slope (%)						
	< 5	51 (65.4)	27 (34.6)	1		
	5 to 10	43 (58.9)	30 (41.1)	0.75	0.39-1.46	0.41
	> 10	14 (58.3)	10 (41.7)	0.74	0.29-1.88	0.53
Aspect						
	South	48 (55.2)	39 (44.8)	1		
	North	60 (68.2)	28 (31.8)	1.74	0.94-3.22	0.07
Meteorological factors						
Temperature (˚C)						
	< 0	37 (67.3)	18 (32.7)	1		
	0 to 11	22 (68.7)	10 (31.3)	1.07	0.41-2.72	0.887
	> 11	49 (55.7)	39 (44.3)	0.61	0.3-1.23	0.17
Precipitation (mm)						
	< 100	46 (56.1)	36 (43.9)	1		
	100 to 150	41 (68.3)	19 (31.7)	1.68	0.84-3.39	0.14
	> 150	21 (63.6)	12 (36.4)	1.36	0.59-3.14	0.45

[1]Fisher's exact test was used to assess the significance of the association.

Table 4.4 Final multivariable logistic regression model for associations between *C. parvum* infection and explanatory variables using *C. bovis* controls in pre-weaned calves

		OR	95% CI	Wald p-value	LRS p-value
Age					< 0.001
	1 month	1			
	1-2 month	0.05	0.01-0.15	< 0.001	
Breed					< 0.001
	Holstein	1			
	Jersey	0.023	0.004-0.132	< 0.001	
Herd size					< 0.001
	< 100	1			
	100-200	7.1	2.0-24.6	0.002	
	> 200	22.3	4.1-119	< 0.001	
Hay bedding					< 0.001
	No	1			
	Yes	5.66	1.21-26.3	0.027	
Northing (km)					0.001
	< 4675	1			
	4675 to 4685	0.56	0.1-3.23	0.52	
	> 4685	0.14	0.04-0.49	0.002	
Precipitation (mm)					0.018
	< 100	1			
	100 to 150	4.21	1.13-15.68	0.032	
	> 150	1.13	0.25-5.0	0.86	

C. bovis cases *vs. Cryptosporidium* negative controls

Based on visual inspection, none of the continuous variables had a linear relationship with the log odds of *C. bovis* infection. Thus all continuous variables were retained as ordered categorical.

Univariable analysis indicated that a herd size of > 200 cattle compared to < 100, Jersey breed, being housed in the cow barn, and being tied were significantly associated with an increased risk of *C. bovis* infection, while older age, dirt flooring, and being in a pen were associated with a decreased risk (Table 4.5). In addition, latitude (northing), elevation, and northern aspect were associated with a decreased likelihood of *C. bovis* infection.

The final multivariable model for *C. bovis* infection using *Cryptosporidium* negative controls revealed that the risk of being a case was significantly increased with Jersey breed and being housed in the cow barn, while older age was associated with lower risk (Table 4.6). There appeared to be an elevated risk associated with latitude (northing), but this trend was not consistently observed across all categories of this variable. Finally, the likelihood of infection was lower on steeper slopes. There were no statistically significant interactions. The model was robust to the exclusion of observations with highest delta-beta values, and fitted the data adequately as measured by the Hosmer-Lemeshow goodness of fit statistics (p=0.54).

Table 4.5 Univariable logistic regression analysis for associations between putative risk factors and *C. bovis* infection using *Cryptosporidium* negative controls in pre-weaned calves

		Cases (%)	Controls (%)	OR	95 % CI	Wald p-value
Host factors						
Age						
	< 1 month	54 (33.1)	109 (66.3)	1		
	1-2 months	13 (10.8)	107 (89.2)	0.24	0.12-0.46	<0.001
Breed						
	Holstein	45 (19.1)	201 (81.71)	1		
	Jersey	21 (72.4)	8 (27.6)	11.72	4.88-28.15	< 0.001
Herd management						
Herd size						
	< 100	24 (24.0)	76 (76.0)	1		
	100 to 200	24 (16.4)	122 (83.6)	0.62	0.33-1.17	0.14
	> 200	19 (51.4)	18 (48.6)	3.34	1.51-7.37	0.003
Greenhouse						
	No	59 (25.5)	172 (74.5)	1		
	Yes	8 (15.4)	44 (84.6)	0.53	0.23-1.19	0.12
Outdoors[1]						
	No	65 (25.6)	189 (74.4)	1		
	Yes	2 (8.3)	22 (91.7)	0.26	0.006-1.15	0.076
In cow barn						
	No	16 (14.8)	92 (85.2)	1		
	Yes	41 (29.7)	97 (70.3)	2.43	1.27-4.62	0.007
Flooring[1]						
	Cement	64 (28.1)	164 (71.9)	1		
	Dirt	3 (5.5)	52 (94.5)	0.14	0.04-0.49	0.002
Hay bedding						
	No	30 (29.1)	73 (70.9)	1		
	Yes	36 (21.3)	133 (78.7)	0.65	0.37-1.15	0.14
Dust bedding						
	No	33 (22.6)	113 (77.4)	1		
	Yes	33 (26.2)	93 (73.8)	1.21	0.69-2.11	0.49

Table 4.5 Continued

		Cases (%)	Controls (%)	OR	95 % CI	Wald p-value
Tied						
	No	16 (15.84)	85 (84.16)	1		
	Yes	51 (31.48)	111 (68.52)	2.43	1.3-4.57	0.005
Pen						
	No	55 (30.)	128 (69.95)	1		
	Yes	12 (15.0)	68 (85.0)	0.41	0.2-0.81	0.012
Geographical features						
Easting (km)						
	< 500	9 (18)	41 (82)	1		
	500 to 520	36 (27)	97 (73)	1.6	0.74-3.82	0.2
	> 520	22 (22)	78 (78)	1.28	0.54-3.0	0.56
Northing (km)						
	< 4675	19 (43.2)	25 (56.8)	1		
	4675 to 4685	11 (12.6)	76 (87.4)	0.16	0.06-0.39	< 0.001
	> 4685	37 (24.3)	115 (75.7)	0.4	0.2-0.8	0.01
Elevation (m)						
	< 470	21 (39.6)	32 (60.4)	1		
	470 to 570	34 (19.9)	137 (80.1)	0.37	0.19-0.73	0.004
	> 570	12 (20.3)	47 (79.7)	0.38	0.16-0.9	0.027
Slope (%)						
	< 5	27 (28.4)	68 (71.6)	1		
	5 to 10	30 (22.6)	103 (77.4)	0.73	0.4-1.34	0.31
	> 10	10 (18.2)	45 (81.8)	0.55	0.24-1.26	0.16
Aspect						
	South	39 (29.3)	94 (70.7)	1		
	North	28 (18.7)	122 (81.3)	0.55	0.09-0.31	0.03
Meteorological factors						
Temperature (˚C)						
	< 0	18 (19.8)	73 (80.2)	1		
	0 to 11	10 (20.8)	38 (97.1)	1.06	0.44-2.53	0.88
	> 11	39 (27.1)	105 (72.9)	1.5	0.79-2.83	0.2
Precipitation (mm)						
	< 100	36 (24.0)	114 (76.0)	1		
	100 to 150	19 (29.7)	45 (70.3)	1.33	0.69-2.57	0.38
	> 150	12 (17.4)	57 (82.6)	0.66	0.32-1.37	0.27

[1] Fisher's exact test was used to assess the significance of the association

Table 4.6 Final multivariable logistic regression model for associations between *C. bovis* infection and explanatory variables using *Cryptosporidium* negative controls in pre-weaned calves

		OR	95% CI	Wald p-value	LRS p-value
Age					< 0.001
	<1 month	1			
	1-2 month	0.23	0.1-0.5	< 0.001	
Breed					< 0.001
	Holstein	1			
	Jersey	3.69	1.02-13.31	0.046	
In cow barn					< 0.001
	No	1			
	Yes	7.69	2.21-26.71	0.001	
Northing (km)					< 0.001
	< 4675	1			
	4675 to 4685	0.14	0.03-0.62	0.009	
	> 4685	0.65	0.17-2.51	0.537	
Slope (%)					< 0.001
	< 5	1			
	5 to 10	0.23	0.09-0.56	0.001	
	> 10	0.12	0.04-0.39	<0.001	

DISCUSSION

Numerous recent studies have been conducted to identify risk factors for *Cryptosporidium* infection in cattle (Castro-Hermida *et al*., 2002; Maddox-Hyttel *et al*., 2006; Trotz-Williams *et al*., 2008). The results of these investigations greatly differ and are often contradictory, which has been attributed to differences in study design, management practices and climatic conditions (Silverlas *et al*., 2009). Arguably, the use of diagnostic techniques that do not distinguish the different species or genotypes of *Cryptosporidium* could also have contributed to inconsistent findings. To date, very few studies have incorporated DNA sequencing to distinguish risk factors for infection with zoonotic *vs.* non-zoonotic strains of *Cryptosporidium* in cattle (Duranti *et al*., 2009; Starkey *et al*., 2006). Although these studies had limited scope regarding the putative risk factors examined, their findings suggested that there was a difference in the subset of factors that predispose to infection by either genotype. The important and unique aspect of the current study was the examination of a range of host, management, and ecological factors at the genotype level, which allowed the identification of differences in risk factors associated with *C. parvum* and *C. bovis* infection. For the purpose of designing cost-effective strategies to mitigate the potential risk associated with different genotypes of *Cryptosporidium*, it is critical to compare and contrast the risk factors for zoonotic and non-zoonotic strains. We attempted to address this goal by comparing risk factors between animals shedding *C. parvum* and the ones shedding *C. bovis*.

The effects of age and breed

Calves < 1 month of age were at greater risk of infection with both *C. parvum* and *C. bovis*. Younger calves were also more likely to be infected with *C. parvum* compared to *C. bovis*. These findings are partially in agreement with previous studies

that demonstrated the relationship between age and *Cryptosporidium* infection in cattle. Specifically, the results concurred with previous reports that the majority of *C. parvum* infections occur in cattle < 1 month of age (Santin *et al*., 2004). However, our finding that younger age is also a risk factor for *C. bovis* infection seems to contradict other studies, that concluded that *C. bovis* infection was acquired later in life and was thus considered to be predominant in older calves (Santin *et al*., 2004). Jersey breed was a significant risk factor for *C. bovis* infection in both multivariate models that included *C. bovis* infection as outcome. However breed was not a significant risk factor for *C. parvum* infection. These results suggest that Jersey calves are more susceptible to *C. bovis* infection, but not less susceptible to *C. parvum* infection, compared to Holstein calves.

The effects of management

In this study we found that calves kept in the cow barn were at an increased risk of infection with both genotypes, which seems to support the idea of cow-to-calf transmission. In the past cows were not regarded as an important source of infection for calves, (Atwill *et al*., 1998) however recent studies indicated that cow-to-calf transmission might be an important route for acquiring *Cryptosporidium* infection in calves (Faubert and Litvinsky, 2000; Huetink *et al*., 2001). The use of hay bedding increased the likelihood of infection with *C. parvum* but not with *C. bovis* in the multivariable models. One study in Mexico found that hay bedding increased the odds of shedding *Cryptosporidium* oocysts in dairy calves, which was attributed to the humid and protective environment created by hay, favoring oocyst survival (Maldonado-Camargo *et al*., 1998). Finally, a herd size of > 200 head as compared to < 100 was associated with an increased risk of infection with *C. parvum*. In contrast, herd size did not remain a significant risk factor for *C. bovis* infection in the multivariable analyses. Previous studies have revealed a positive association between

the size of the farm and the risk of shedding *C. parvum*-like oocysts, attributed to environmental contamination by the higher density of animals (Garber *et al*., 1994). The results of the present study suggest that calves in larger herds are more likely to be infected with the zoonotic genotype.

The effects of geography and meteorology

Among the geomorphological factors in the multivariable analyses, a slope of 5-10% compared to a flat surface was significantly associated with a decreased likelihood of infection with both genotypes. One possible explanation for the effect of slope on infection is that steeper slopes may not be favorable for the accumulation of oocysts around calf holding facilities. In addition, the risk of infection with both *C. parvum* and *C. bovis* appeared to be lower at higher latitudes, although this trend was not statistically significant at the 5% level. Only precipitation had a significant effect in the multivariable models among the meteorological factors. Specifically, precipitation of 100-150 mm as compared to < 100 mm was found to be a significant risk factor for *C. parvum* infection using both sets of controls. Desiccation has been shown to be lethal for *Cryptosporidium* oocysts (Peng *et al*., 2008), thus precipitation may increase the risk of exposure by supporting oocyst survival. In wet periods, rain water could also transport feces from one calf to another, increasing the risk of calf-to-calf transmission.

Study design limitations

A large number of possible sources of bias and error in case-control studies have been discussed in the literature. Among the most common concerns are the identification of an appropriate control group (selection bias), and availability of accurate information on infection status and potential risk factors (information bias) (Kelsey, 1996). This study attempted to minimize selection bias by using a control group that was randomly drawn from the same source population as the cases.

Misclassification bias was kept to a minimum by the use of PCR which is one of the most highly sensitive and specific diagnostic tests for *Cryptosporidium* infection (Xiao, 2009). However, a potential source of bias was that conventional sequencing of the 18s rRNA gene is unable to detect mixed *Cryptosporidium* infections (Santin and Zarlenga, 2009). Thus if any of the cases were infected with both genotypes, only the dominant genotype would have been identified by this method. Since age has been shown to affect the likelihood of *Cryptosporidium* infection in cattle, and may also be associated with other hypothesized risk factors, we controlled for the confounding effect of age by considering all independent variables in the multivariable analysis, even if they were not significant at the univariable level.

In conclusion, this study identified and compared several host, management, and ecological risk factors associated with *C. parvum* and *C. bovis* infection in cattle. The findings of this study will be useful in designing measures that reduce animal exposure, and effectively decrease the public health risk and economic losses associated with *C. parvum* infection in cattle herds.

a. StatCalc, EpiInfo version 6 software, Center for Disease Control, Atlanta, GA

b. QIAamp DNA Stool Mini Kit, Qiagen, Valencia, CA

c. Fermentas, MD

d. Exo-SAP-IT; USB Corporation, Cleveland, OH

e. 3730 DNA Analyzer; Applied Biosystems, Foster City, CA

f. MEGA version 4.1, Tempe, AZ

g. Microsoft Excel 2000, Microsoft, Redmont, WA

h. Garmin eTrex Summit, Garmin International Inc, Olathe, KS

i. Manifold System 8.0 Ultimate Edition, Manifold Net Ltd, Carson City, NV

j. Intercooled STATA version 11 for Mac, Stat Corp LP, College Station, TX

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CHAPTER FIVE

FIRST REPORT OF *CRYPTOSPORIDIUM* DEER-LIKE GENOTYPE IN KENYAN CATTLE[1]

ABSTRACT

The objective of the study was to identify *Cryptosporidium* genotypes from feces collected from urban and peri-urban dairy cattle in Nairobi, Kenya, in order to determine their zoonotic potential. A total of 34 samples that were diagnosed positive by the modified Zielh-Neelsen technique were preserved in potassium dichromate (2.5%) and the DNA was extracted using the QAIamp DNA stool kit. Two *Cryptosporidium* isolates examined at the 18S rRNA locus were identified as the deer-like genotype by DNA sequencing. As public health officials are facing the difficult decision of whether to allow urban livestock production because of its economic benefits and a livelihood asset to the urban communities, or to ban it for its public health risks, the finding of non-zoonotic genotypes in a smallholder dairy system has significant public health as well as economic implications that merit further investigation.

INTRODUCTION

Cryptosporidium is a globally important intracellular pathogen of humans and animals. Recent studies in the United States and several other countries in Europe suggest that cattle are infected with at least four *Cryptosporidium* species or genotypes: *C. parvum*, *C. bovis*, *C. andersoni* and *Cryptosporidium* deer-like genotype (Feng *et al*., 2007; Langkjaer, 2007; Thompson *et al*., 2007; Plutzer *et al*., 2007).

[1] This chapter has been prepared in the format for submission to the journal Veterinary Parasitology

These *Cryptosporidium* species in cattle show a host age related susceptibility: *C. parvum* predominates in pre-weaned calves, *C. bovis* and the *Cryptosporidium* deer-like genotype in post-weaned calves and *C. andersoni* in older calves and adult cattle (Santin *et al*., 2004; Fayer *et al*., 2006), although *C. bovis* and the *Cryptosporidium* deer-like genotype have been reported in all age groups (Feng *et al*., 2007). These findings indicate that *C. parvum* is the major species responsible for diarrhea in calves, and is the only zoonotic species in cattle (Thompson *et al*., 2007). *Cryptosporidium parvum* infects the small intestine of young calves causing enteritis and diarrheal disease, whereas *C. andersoni* infects the abomasums of juvenile and mature cattle and is not associated with overt clinical signs, although reduced milk production has been reported (Santin *et al*., 2004., Olson *et al*., 2004). *Cryptosporidium* deer-like genotype and *C. bovis* have not been associated with signs of disease (Fayer *et al*., 2006). Because of the difficulty in differentiation between species of *Cryptosporidium* based on the morphological characteristics of the oocyst, molecular techniques are used to determine the genotype and asses the risk of human infection (Fayer *et al*., 2006).

Urban dairy production is an essential source of income and nutrition for an increasing number of households in African cities, but its intensive nature provides an environment that is conducive to emerging zoonotic pathogens such as *Cryptosporidium* (Kang'ethe *et al*., 2005). Therefore, urban dairying as a source of human infection is a great concern. Previous studies of human cryptosporidiosis in Kenya showed a prevalence of 2-5% in children (Gatei *et al*., 2006; Simwa *et al*., 1989) while in HIV infected patients, a prevalence of 17% was reported (Mwachari *et al*., 1998). However, the prevalence of zoonotic vs. non-zoonotic genotypes of *Cryptosporidium* in cattle raised under urban smallholder system has not been

investigated, and the extent of zoonotic transmission in such production systems is unknown (Kang'ethe *et al*.; 2005). The objective of this study was to identify *Cryptosporidium* genotypes collected from feces of urban and peri-urban dairy cattle in Nairobi in order to determine their zoonotic potential.

MATERIALS AND METHODS

Sources of samples

Fecal samples were obtained from cattle in Dagoretti Division of Nairobi from randomly selected urban and peri-urban households. All 143 calves aged 7-30 days were sampled in the selected households between August and December of 2006. Feces were collected directly from the rectum of animals into plastic gloves and transported to the laboratory the same day in an icebox for diagnosis of *Cryptosporidium* spp. All samples were collected from cattle of mixed local breeds and processed within one week of collection. The samples were stained by the Modified Ziehl-Neelsen method (Casemore *et al*., 1985) and examined under bright field light microscope. Positive samples were preserved with 2.5% potassium dichromate at 4 °C until further processing.

DNA extraction

After an initial step of washing four times in distilled water to remove the preservative, DNA was extracted from 34 samples identified positive by the Modified Ziehl-Neelsen method using QIAamp DNA Stool Mini Kit according to the manufacturer's instructions (Qiagen, Valencia, CA).

Molecular detection of *Cryptosporidium* genotypes

A two-step nested PCR protocol was used to amplify an 830bp fragment of the 18S rRNA gene (Xiao *et al*., 1999). PCR products were visualized after electrophoresis on 1% agarose gel stained with ethidium bromide. Samples were sequenced in both directions and sequence chromatograms from each strand were

aligned and inspected using MEGA version 4.0, 2007 (Tamura *et al*., 2007). The basic local alignment search tool (BLAST) was used to compare DNA sequences with GenBank DNA sequences and to determine the species of *Cryptosporidium* in the sample.

RESULTS

We were able to extract DNA from 34 samples that were identified positive by the modified Ziehl-Neelsen staining method. PCR positive results for the 18SrRNA gene of *Cryptosporidium* were obtained from samples from two animals. Both animals were 14-day-old bull calves from separate households. Genetic sequence analysis indicated that both of these isolates had 100% homology with the deer-like genotype listed in GenBank (accession number AY587166).

DISCUSSION

The aim of this study was to genetically characterize isolates of *Cryptosporidium* from urban dairy cattle in Nairobi in order to determine the risk of human infection.

Two samples that amplified with PCR were identified as the non-zoonotic deer-like genotype. It is unclear why only two out of the 34 samples amplified successfully after numerous attempts, but several factors might have played a role. The Modified Ziehl-Neelsen technique is nonspecific, staining a range of other gastro-intestinal parasites (Sunnotel *et al*, 1999). Therefore, misclassification by the initial screening process cannot be ruled out. Pre-PCR processing, such as concentration and purification might have led to parasite loss. It is also possible that the small volume of fecal material used in the extraction missed the oocyst if the parasite load in the feces was low. Furthermore, genetic denaturing might have occurred during long transport times between laboratories. Another possible explanation for the failure of PCR is that ruminant feces contain high levels of PCR inhibitors that could have interfered with

the reaction (Thornton *et al*., 2004).

This is the first report of the deer-genotype in cattle from Africa. The SSU rRNA sequences in our study were identical to those previously reported from the United States and China (Santin *et al*., 2004; Feng *et al*., 2007). A series of studies indicated that *C. bovis* as well as the deer-like genotype were prevalent and widespread in dairy cattle on the East Coast of the Unites States (Santin *et al*., 2004; Fayer *et al*., 2006; Fayer *et al*., 2007) . In a recent study, both genotypes were found in a small number of cattle in China and India (Feng *et al*., 2007). Several countries in Europe, such as Denmark, Hungary and Northern Ireland recently reported that *C. bovis* and the deer-like genotype were prevalent in their cattle populations (Langhjaer *et al*., 2007; Plutzer *et al*., 2007; Thompson *et al*., 2007). In Africa, *C. bovis* was shown to be widespread in calves in Zambia (Geurden *et al*., 2006). Both *C. bovis* and the deer-like genotype were detected in pre- and post-weaned calves as well as milking cows (Feng *et al*., 2007). The deer-like genotype was found in small number of calves (5%) at 3 weeks of age in the United States (Santin *et al*., 2004) and in one pre-weaned calf in China (Feng *et al*., 2007). Our finding of the deer-like genotype in pre-weaned calves is in agreement with previous studies and suggests that calves may acquire infection early in life.

In developing countries, *Cryptosporidium* is responsible for up to 19% of cases of diarrheal disease with significant effect on mortality. In HIV infected patients, *Cryptosporidium* may account for up to 50% of the cases of diarrhea (Gatei *et al*., 2000). *Cryptosporidium* is one of the most common enteric parasites associated with diarrhea in Kenya (Gatei *et al*., 2006). In spite of the high prevalence of human cryptosporidiosis in Kenya, little is known about the prevalence of different genotypes in Kenyan livestock, and the risk posed by livestock to human health (Kang'ethe *et al*., 2005). This is the first time that *Cryptosporidium* isolates from Kenyan cattle have

been analyzed genetically.

The finding of non-zoonotic genotypes in urban and peri-urban dairy cattle has significant public health as well as economic implications that underscore the need for molecular characterization of bovine cryptosporidiosis. As public health officials are facing the difficult decision of whether to allow urban livestock production for its economic benefit, or to ban it for its public health risks, epidemiologic studies using molecular techniques to distinguish between the genotypes are essential tools in formulating sound management policies to improve human health and livelihoods.

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CHAPTER SIX

CONCLUSION

The aim of this body of work was to improve our understanding of the dynamic of *Cryptosporidium* infection in dairy cattle. Specifically, the work focused on quantifying and characterizing the public health risk posed by dairy cattle as a source of zoonotic *Cryptosporidium* in an important watershed and in an urban dairy production system, such that cost-effective mitigation strategies could be recommended. This aim was accomplished in four complementary studies that employed DNA sequencing methods in combination with epidemiologic analytical approaches.

The first study utilized molecular techniques to obtain species-specific estimates of the prevalence of *Cryptosporidium* infection in dairy herds in the New York City Watershed. The study incorporated findings of previous investigations that our group had conducted in the target population through the use of a stochastic Bayesian approach, which allowed improving the estimates of the risk associated with zoonotic *Cryptosporidium* infection in this population. The study confirmed previous observations that only pre-weaned calves were the source of zoonotic *Cryptosporidium* in dairy herds. The study also increased our knowledge of the effect of season on the risk of infection.

The second study built upon the risk estimates obtained in the first study to investigate the spatio-temporal dynamics of *C. parvum* infection in dairy calves and to identify potential high-risk clusters in the New York City Watershed. The study employed two different cluster detection techniques and identified high-risk clusters in both space and time in this important watershed. This finding has implications for watershed management, and will aid in the improvement of monitoring and prevention activities to protect drinking water supplies.

Knowledge gained in previous studies in the target population were integrated in the third study to determine risk factors for infection with zoonotic *vs* non-zoonotic *Cryptosporidium* in dairy calves using a case-control study design. The study revealed that younger age and being housed together with adult cattle were associated with an increased risk of infection with both *C. parvum* and *C. bovis*, while larger herd size and hay bedding increased the likelihood of infection only with the zoonotic genotype. The findings of this study may be useful to design measures that reduce animal exposure and decreased the public health risk and economic losses associated with *C. parvum* infection in cattle.

In the final chapter we identified *Cryptosporidium* species using DNA sequencing to determine the zonotic risk associated with urban dairy production in the East African city of Nairobi. This study added to our knowledge regarding the global distribution of *C. ryanae* (formerly the deer-like genotype). The finding emphasized that dairy cattle worldwide may be infected with non-zoonotic *Cryptosporidium* genotypes, and underscored the need to incorporate molecular techniques in *Cryptosporidium* risk assessment in dairy cattle worldwide.