Total and infectious *Cryptosporidium* oocyst and total *Giardia* cyst concentrations from distinct agricultural and urban contamination sources in Eastern Canada

Cindy Lalancette, Mylène Généreux, Jacinthe Mailly, Pierre Servais, Caroline Côté, Aubert Michaud, George D. Di Giovanni and Michèle Prévost

ABSTRACT

Cryptosporidium and *Giardia* (oo)cyst concentrations are frequently used for assessing drinking water safety. The widely used USEPA Method 1623 provides total counts of (oo)cysts, but may not be accurate for human health risk characterization, since it does not provide infectivity information. The total counts and infectious fraction of *Cryptosporidium* oocysts and the total counts of *Giardia* cysts were assessed in major fecal pollution sources. Fresh calf and cow feces, their manure, and the discharge point were sampled in a small rural sub-watershed ($n = 20, 21, 10, 10$). Median concentrations for total (oo)cysts were higher in calves (333 oocysts g^{-1}; 111 cysts g^{-1}) than in cows (52 oocysts g^{-1}; 7 cysts g^{-1}). Infectious oocysts were found in 17 (7%) of the samples and none were found in manure or at the discharge point. Urban sources were sampled in the influent and effluent ($n = 19, 18$) of two wastewater treatment plants. Peak concentrations were 533 oocysts L^{-1} and 9,010 cysts L^{-1} for influents and 89 oocysts L^{-1} and 472 cysts L^{-1} for effluents. Infectious oocyst fractions varied from below the detection limit to 7–22% in the effluent and influent respectively. These infectious fractions are significantly lower than those currently used for quantitative microbial risk assessment estimates.

Key words | contamination, *Cryptosporidium*, discharge, drinking water, *Giardia*, infectivity

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INTRODUCTION

Cryptosporidium spp. and *Giardia duodenalis* (syn. *G. lamblia*, *G. intestinalis*) are significant waterborne pathogens, accounting for 23.7 and 32% of protozoan drinking water outbreaks worldwide, respectively, during the periods 1954–2002 and 1983–2001 (Karanis *et al.* 2007). Increasingly rigorous regulations on its surveillance and removal have been implemented in numerous countries, such as the USA and England (United States Environmental Protection Agency (USEPA) 1998, 2005a, b; Lake *et al.* 2007).

Zoonotic *C. parvum* and *G. duodenalis* and anthropogenic *C. hominis* are commonly isolated from surface water (Percival *et al.* 2004). Major sources of these pathogens include livestock (Fayer *et al.* 1998; Starkey *et al.* 2005; Coklin

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et al. 2007), urban wastewater discharges (Ward et al. 2002; Quintero-Betancourt et al. 2003), and wildlife (Perz & Le Blancq 2001; Jiang et al. 2005; Bednarska et al. 2007).

As a consequence, additional specific *Cryptosporidium* monitoring is now required in source water in the USA and in high risk sources in the UK. These requirements are based on an economic analysis of the risk of infection, which considers the concentration and fraction of infectious oocysts (United States Environmental Protection Agency (USEPA) 2005b). The reference USEPA Method 1622/23 (United States Environmental Protection Agency (USEPA) 2005a) provides a total *Cryptosporidium* oocyst and *Giardia* cyst count, but does not differentiate between animal associated and human infectious species, the survival rate, or determine the infectivity of environmental oocysts and cysts (Di Giovanni et al. 1999; United States Environmental Protection Agency (USEPA) 2005a). Several published methods to estimate infectious oocysts, including cell culture with immunofluorescence assay (CC-IFA), cell culture with DNA PCR detection (CC-DNA), and cell culture with RNA RT-PCR detection (CC-RNA), were recently reviewed and systematically compared (Johnson et al. 2010). Recently, the dual direct detection with cell culture and immunofluorescence assay (3D-CC-IFA) was developed to provide a cost-effective and feasible approach for enumerating total and infectious oocysts for individual, unsplit water samples. Based on specificity of the anti-sporozoite antibody used in the 3D-CC-IFA assay, cell culture infectious oocysts detected using 3D-CC-IFA may be considered human infectious (Lalancette et al. 2010).

The main objective of this study was to estimate levels of total and infectious *Cryptosporidium* oocysts in two major contributing sources of rural (dairy cattle facilities) and urban (sewage) fecal contamination. Additional goals included: (a) applying the 3D-CC-IFA method to complex environmental samples, namely feces and sewage; and (b) comparing the percentage of positive samples and concentrations of total and infectious *Cryptosporidium* and total *Giardia* in dairy feces (adult cows and calves) and sewage (raw and treated). To our knowledge, this is the first report of direct measurements of total and infectious fractions of *Cryptosporidium* oocysts in individual animal fecal and sewage samples. These estimates can then be used to improve risk estimates using quantitative microbial risk assessment (QMRA).

MATERIALS AND METHODS

Description of sampling sites

The rural Ruisseau au Castor sub-watershed (11.2 km^2) is part of the Pike River watershed, and has an average monthly discharge flow of 2.57 $m^3 s^{-1}$. The highest discharge flows were observed in April and the lowest in August. The area drained by this stream is mainly agricultural (97%), and is predominantly dedicated to dairy ($n = 7$) and grain ($n = 5$) production. Sampling period extended from June to November 2009 and 6 dairy farms were randomly sampled. A total of 20 calf and 21 cow fresh feces samples were analysed and each sample represented a composite of four animals randomly chosen. Composite sampling was initially elected to maximize the potential to demonstrate infectious oocyst fractions. Stream samples (40 litres; $n = 10$) were randomly collected from June to November 2009, 50% were associated with dry weather conditions (<1.1 mm d^{-1}), typically related to the absence of significant contribution from surface runoff to stream discharge. From an operational perspective, sampling dates correspond to manure spreading operations on hay fields (June to September), small grain stubble (August and September) or following corn harvest (October and November).

Two wastewater treatment plants (WWTP) using secondary biological treatment were chosen in the Greater Montreal Area (Canada). Both plants receive combined sewage with minor industrial flows, include primary settling, and do not disinfect or add coagulants. WWTP-S (47,000 $m^3 d^{-1}$) is equipped with aerated lagoons (AL) with an annual average hydraulic retention time (HRT) of 16 d. WWTP-V (20,000 $m^3 d^{-1}$) uses sequential bioreactors (SBR) with an annual average HRT of 7 h. Influents and effluents were grab sampled from February to November for WWTP-S (influent $n = 11$; effluent $n = 10$) and from April to November for WWTP-V (influent and effluent $n = 8$) in 2009.

Sample preparation prior to immunomagnetic separation (IMS) for *Cryptosporidium* and *Giardia* analysis

Fresh feces and manure

The animals were not monitored for signs of disease and were randomly sampled. Rectal grabbed samples from four animals in each group (calves <27 weeks and cows ≥27 weeks) were mixed in the randomly selected farms visited. Manure samples were also taken from both liquid management sources ($n = 9$) and solid management sources ($n = 1$). All samples were processed in the laboratory on the same day. A protocol was adapted from previous studies (Davies *et al.* 2003; Atwill *et al.* 2006b) using 0.5 ± 0.02 g of fresh feces or manure. When controls were performed, oocysts or cysts were mixed overnight at 4 °C with the 0.5 g samples. The feces were vortexed with 20 ml of Tris 50 mM 0.1% Tween 20 in 50 ml tubes until complete dispersion was observed. The samples were then shaken using a wrist action shaker (Barnstead Lab Line Multiwrist Shaker) at 900 rpm for 5 min and incubated at room temperature for 30 min. The homogenates were centrifuged at 2,000*g* for 10 min and the supernatants carefully discarded. The resulting pellets were homogenized with 6 ml of Milli-Q water, and the suspensions were then filtered (Millipore Steriflip® 100 µm, No. R7HN69592). Tubes and filters were rinsed with 2 ml of Milli-Q water, which was then combined with their filtrate. These 8 ml samples were pooled into a Leighton tube ready for purification via the IMS protocol described below.

Ruisseau au Castor surface water

Water grab samples (40 L) were collected at the outlet of the Ruisseau au Castor and processed the same day following USEPA Method 1623 (United States Environmental Protection Agency (USEPA) 2005a). For each sample ($n = 10$), water was filtered using one Pall Envirochek HV cartridge (No. DN 12009) until the pressure reached 25 psi or for a maximum of 40 L. The last 10 litres out of 40 (or for at a pressure of 15 psi) were used to add oocysts and cysts controls for every sample. The controls (ColorSeed, BTF) were added to a beaker of 2 L of sampled water, and agitated with a stirring bar. After several minutes of agitation, the water was pumped through the filter and the beaker rinsed four times with 2 L of surface water, with each rinse pumped through filter. The loaded filters were then stored overnight at 4 °C. Elution followed USEPA 1623 method plus a modification involving a 5% sodium hexametaphosphate rinse as initial step.

WWTPs influents and effluents

Wastewater samples were collected between 7:00 a.m. and 9:00 a.m., without consideration of hydraulic residence time. The 4 L samples of the influent and effluent were kept at 4 °C and analyzed within 24 h. Aliquots of 250 ml of each sample were repeatedly centrifuged at 2,000*g* for 10 min without the break using a Sorvall® Legent RT, until pellets of 0.5 ml per analysis were obtained. Controls for recovery (100 prestained gamma irradiated *C. parvum* and *G. duodenalis*; ColorSeed, BTF Pty. Ltd., North Ryde, Australia) and for infectivity (500 viable mouse propagated *C. parvum* Iowa isolate oocysts no older than 2 months; Waterborne, Inc., New Orleans, LA) were added when necessary before the last centrifugation round.

Immunomagnetic separation (IMS)

The IMS protocol was followed per manufacturer instructions (Dynabeads GC-Combo; Invitrogen, Carlsbad, CA) and USEPA Method 1623 (United States Environmental Protection Agency (USEPA) 2005a), but with subsequent *Cryptosporidium* oocysts and *Giardia* cysts isolation. Dynabeads anti-*Cryptosporidium* were first added to prepared samples, and Method 1623 followed up till the dissociation step, saving the supernatants from the first magnetic particle separation step. Oocysts were dissociated from IMS beads using acidified Hanks' balanced salt solution (catalog no. H9269; Sigma-Aldrich, St. Louis, MO) at pH 2.0 containing 1% porcine type II-S trypsin (catalog no. T7409; Sigma-Aldrich) for 1 h at 37 °C with vigorous vortex mixing for 10 s every 15 min as described for the 3D-CC-IFA protocol (Lalancette *et al.* 2010). Dynabeads anti-*Giardia* were then added to the supernatants saved from the *Cryptosporidium* IMS, and *Giardia* cysts recovered by following Method

1623 (United States Environmental Protection Agency (USEPA) 2005a), including 0.1 N HCl dissociation. Control experiments were conducted using combined high density spikes (2×10^5 *G. duodenalis* and 2×10^5 *C. parvum*, formaldehyde killed; Waterborne Inc.) in Milli-Q water, and demonstrated $\leq$5% of the oocysts and cysts were incorrectly separated (Dynabeads anti-*Cryptosporidium* 54 cysts: 1,036 oocysts and *Dynabeads* anti-*Giardia* 0 oocysts: 894 cysts counted in three fields at ×400 magnification).

Total and infectious *Cryptosporidium*

The 3D-CC-IFA protocol has been detailed elsewhere (Lalancette *et al.* 2010), but briefly described as follows. Oocysts were dissociated from IMS beads (and pre-treated for cell culture) as described in earlier section. Recovered oocysts were washed with infection medium and then seeded on HCT-8 cell monolayers for 72 h incubation. Immunodetection of cell culture foci of infection used a primary antibody (#A600UN, Waterborne, Inc.; 0.24 µL Ab: 150 µL PBS per chamber slide; 1 h incubation at RT) and an anti-rat secondary antibody (#F6258, Sigma-Aldrich; 0.68 µL Ab: 150 µL PBS per chamber slide; 1 h incubation at RT). After these steps had been taken, the oocysts were immunodetected with EasyStain GC combo (BTF) diluted 1/10 in BFT Fixing Buffer. This represents a minor modification of the published protocol. Microscopic counts were performed at a magnification of ×400 (×600 for samples with high background staining) using an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with FITC (U-N51006) and CY3 (U-N4107A) filters. Factors specifically affecting the recovery of infectious oocysts were previously evaluated by Lalancette *et al.* (2010), in particular the adhesion to cell monolayer. Recovery adjustments were made to both total and infectious oocysts using the recovery data of total oocysts.

Total *Giardia*

USEPA Method 1623 was followed (USEPA 2005b). IMS purified cysts were fixed with methanol on Dynal Spot-on slides (IDEXX No. 30201) and then stained with DAPI and EasyStain GC combo (BTF). Microscopic counts at ×400 and at ×1,000 magnification included FITC, DAPI, and differential interference contrast (DIC) observations.

RESULTS AND DISCUSSION

Recovery and infectivity controls

Table 1 presents an overview of the results of the recovery and infectivity assays completed in six different fecal matrices and wastewater. The average infectivity of seeded viable *C. parvum* Iowa oocysts varied from 21 to 33% in the 3D-CC-IFA assays. Average recoveries for ColorSeed™ seeded samples varied from 12 to 30% for *Cryptosporidium* (3D-CC-IFA) and from 9 to 36% for *Giardia* (USEPA Method 1623). Average recoveries of total counts were used to adjust measurements for raw sewage (RS), effluents, calf and cow feces and manure. Recovery estimations were repeated for each sampling event at the watershed discharge. Results show that matrices did not impact oocyst infectivity.

Table 1 | Recovery rates efficiencies of total and infectious *Cryptosporidium* using 3D-CC-IFA and reported as infectivity rates (foci counts per total seeded viable oocyst counts); and USEPA Method 1623 total *Cryptosporidium* and *Giardia* (oo)cyst ColorSeed™ recoveries

		Cryptosporidium **Infectivity rates of seeded viable *C. parvum* (%)**	**Seeded ColorSeed™ (%)**	***Giardia*** **Seeded ColorSeed™ (%)**
Rural	Calf fresh feces ($n = 3, 3, 3$)	30 (15)	12 (5)	9 (10)
	Cow fresh feces ($n = 3, 3, 3$)	33 (12)	19 (3)	30 (19)
	Liquid manure ($n = 1, 1, 1$)	25	30	36
	WS discharge n = (NA, 10, 10)	NA	24 (11)	12 (14)
Urban	WWTP Influents ($n = 3, 4, 5$)	27 (9)	18 (14)	13 (17)
	WWTP Effluents ($n = 2, 4, 5$)	21 (5)	26 (25)	36 (26)

WWTP: wastewater treatment plant, WS: watershed, NA: not avalaible.
Percentages of recoveries are expressed in average with their standard deviation in parenthesis.

Agricultural matrix total (oo)cysts and *Cryptosporidium* infectivity controls

The 3D-CC-IFA methods provided average recoveries for total *Cryptosporidium* oocysts of 12 ± 5% and 19 ± 3% for calf and cow feces, respectively (p-value = 0.1) (Table 1). The (oo)cyst isolation and purification modifications proposed by Davies *et al.* (2003) have been shown to improve separation results by up to 33% for calf feces and up to 37.5% for cow feces with a different detection protocol. Even using this improved separation technique; fecal matrices still caused significant interference with the 3D-CC-IFA microscopy. Chamber slides were screened, oocysts and foci carefully observed, but background interference from sample debris remained challenging. *Giardia* cyst recovery averaged 9 ± 10% for calf feces and 30 ± 19% for cow feces using 100 gamma irradiated cysts in 0.5 g of feces (p-value = 0.2). Higher average recovery rates of 76.4 to 97% were found for calf feces when spiking up to 10^{+6} cysts g^{-1} (Xiao & Herd 1993), which is consistent with increased recovery rates with higher spiked concentrations (Robertson *et al.* 2000). The fractions of spiked infectious *Cryptosporidium parvum* in calf and cow feces ranged respectively from 30 ± 15 to 33 ± 12%. Previous reports using the same procedure in Milli-Q water established an infectivity ratio of 19 ± 4% (Lalancette *et al.* 2010) without a storage at 4 °C during the procedure. Therefore, overnight incubation at 4 °C in fecal matrices did not appear to affect *C. parvum* infectivity.

Matrix spikes were directly performed for every sample of agricultural stream discharge analyzed using both 3D-CC-IFA and USEPA 1623 methods. For total *Cryptosporidium* oocysts using the 3D-CC-IFA method, average recoveries of 24 ± 11%, and USEPA Method 1623 12 ± 14% for *Giardia* were obtained. A large US study (n = 430) sampling surface water at 87 drinking water intakes (DWI) and using USEPA Method 1623 found average recoveries of 43% for *Cryptosporidium* and 53% for *Giardia* (Connell *et al.* 2000). Another review gathered data from 8 DWI and reported average recoveries varying from 12 to 50% for *Cryptosporidium* and from 6 to 47% for *Giardia* (Dechesne *et al.* 2006). These higher recovery rates were obtained in surface water (drinking water intakes) not in agricultural drainage catchments. We elected not to perform infectivity spikes, since this verification had previously been done on various surface waters including a heavily contaminated agricultural source (19 ± 15%, n = 3) (Lalancette *et al.* 2010).

Urban matrix total (oo)cysts and *Cryptosporidium* infectivity controls

Recoveries of total *Cryptosporidium* oocysts using the 3D-CC-IFA method in both WWTPs ranged from 18 ± 14% for influents to 26 ± 25% for secondary treated effluents (p-value = 0.6) (Table 1). Previous reports of recoveries of total *Cryptosporidium* (USEPA Method 1623) vary from 26% in influent (McCuin & Clancy 2006), to 25 and 53% in secondary effluents (McCuin & Clancy 2005, 2006), and about 32% in reclaimed effluents (Quintero-Betancourt *et al.* 2003). For *Giardia* cysts, the observed recovery efficiencies of 13 ± 17% for influent and 36 ± 26% for secondary treated effluents (p-value = 0.2) are consistent with prior reports of 27% for reclaimed effluents (Quintero-Betancourt *et al.* 2003). Using various *Giardia* cyst purification and isolation methods, (Robertson *et al.* 2000) reported recoveries from 30 to 85% in influent and 37 to 81% in wastewater effluents, with higher recoveries obtained when seeding high concentrations (5,000 cysts L^{-1}). Recoveries of total oo(cysts) are known to vary with the initially spiked concentrations in some datasets (Robertson *et al.* 2000; Messner & Wolpert 2002) and not in others (Petterson *et al.* 2007). While small datasets may provide acceptable estimates of the mean value and the 95th percentile, recovery datasets of $n > 20$ are suggested to reduce uncertainty for application of quantitative microbial risk assessment (QMRA) (Petterson *et al.* 2007).

Little information is available on the impact of the matrix on the recovery of infectious oocysts using cell culture based methods. Published recovery efficiencies for total oocysts in wastewater are available (Gennaccaro *et al.* 2003; Quintero-Betancourt *et al.* 2003; McCuin & Clancy 2005) but no recovery efficiencies for infectious oocysts are available in wastewater, only in river water (LeChevallier *et al.* 2003). Therefore, the influence of the wastewater matrix on the enumeration of total and infectious *Cryptosporidium* was assessed by seeding individual samples with viable *C. parvum* Iowa oocysts. Infectivity rates (foci counts

reported on oocysts counts) of 27 ± 9% were measured in influents and similar rates of 21 ± 5% obtained for secondary treated effluents (p-value = 0.5). Considering normal lot-to-lot variations in oocysts infectivity, infectivity rates were not significantly affected by the wastewater matrices.

(Oo)cysts from agricultural sources

Total *Cryptosporidium* and *Giardia* (oo)cysts

Figure 1 shows the oocyst concentrations found in various cattle fecal sources and at a watershed stream discharge point. The median concentrations measured for *Cryptosporidium* in calf and cow feces were 333 and 52 oocysts g^{-1} respectively, and for *Giardia* 111 and 7 cysts g^{-1} respectively. Maximum concentrations reached 44,067 and 808 oocysts g^{-1} for *Cryptosporidium*, and 1,939,333 and 145 cysts g^{-1} for *Giardia* in calf and cow feces, respectively. Higher percentages of positive composite samples were noted in calf feces (90%) than in cow feces (71%) for *Cryptosporidium*, and were marginally higher for *Giardia* (65% in calves and 57% in cows). Composite samples containing both cysts and oocysts were observed for 47% of calf and 60% of cow feces, respectively. Although co-infection by *Cryptosporidium* and *Giardia* for individual animals cannot be assessed using composite samples, it is common in calves (Thompson *et al.* 2003).

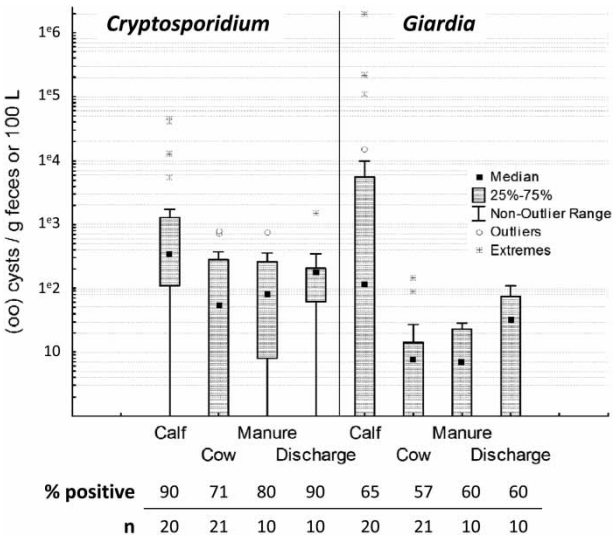

Figure 1 | Concentrations and % positive samples for *Cryptosporidium* (3D-CC-IFA) and *Giardia* (USEPA Method 1623) in rural fecal sources (April to November 2009) with data corrected for recovery (average recovery for each fecal matrix and daily recovery value for discharge samples). Calf, cow, and manure composite sample results expressed per g of feces and watershed discharge results per 100 L. Detection limits were 2 (oo)cysts g^{-1} for feces and manure, and ranged from 7 to 87 (oo)cysts 100 L^{-1}.

To our knowledge, studies describing the concentration (as opposed to prevalence) of oocysts in dairy cattle feces only include results for calves with concentrations reaching as high as 10^7 oocysts g^{-1} and no concentrations are available for adult dairy cows (Fayer *et al.* 1998; Starkey *et al.* 2005). Reported concentrations of oocysts in feces vary widely from not detected up to 10^7 oocysts g^{-1} using methods with diverse detection limits (Starkey *et al.* 2005). Ample information is available on the prevalence and speciation of oocysts in adult cow (Olson *et al.* 1997; Wade *et al.* 2000; Huetink *et al.* 2001; Santin *et al.* 2004; Maddox-Hyttel *et al.* 2006; Coklin *et al.* 2007; Mendonca *et al.* 2007) and a lower prevalence (up to 21%) in cows has been reported (Huetink *et al.* 2001). Concentration of oocysts in adult cows has been estimated and used for modeling based on concentrations measured in calves and feedlots (Dorner *et al.* 2004; Atwill *et al.* 2006b; Starkey *et al.* 2007). Starkey *et al.* in 2007 applied a wide gamma distribution (0.4856; 383,745) to describe the measured concentration of oocysts in calves (<2 months), and then used an inferred lognormal distribution (36.0; 28.2) for adult cows (>2 months).

Measuring the production of total and infectious oocysts in adult cows is critical in estimating the production at the watershed level and the associated risk in the surface water. Although adult cattle are mainly infected by species that are non pathogenic to humans (Fayer & Xiao 2007), the proportion of adult cattle in herds is high, the daily production of feces elevated and the resulting impact on oocyst loadings is significant. In the watershed studied, the average point estimates of daily oocyst loads from 742 adult cows (>6 months) represent 1.6×10^9 oocysts d^{-1}, as opposed to 1.2×10^9 oocysts d^{-1} for the 112 calves (<6 months). The production of total oocysts by adult cows is therefore predominant but may not be translated into human health risk.

In the watershed discharge, median concentrations reached 177 oocysts 100 L^{-1} and 32 cysts 100 L^{-1}, and peaked at 1,500 oocysts 100 L^{-1} and 100 cysts 100 L^{-1}. Higher percentages of positive samples were also observed for *Cryptosporidium* (90%) than for *Giardia* (60%) in this

discharge. In surface water with predominantly agricultural contamination, the prevalence of *Cryptosporidium* was estimated at 77%, with concentrations varying from not detected to 440 oocysts 100 L^{-1} (Ong *et al.* 1996; Isaac-Renton *et al.* 2005; Ruecker *et al.* 2007; Wilkes *et al.* 2009), while *Giardia* concentration varied from below detection limit (BDL) to 2,200 cysts 100 L^{-1}, with a prevalence of up to 44% (Ong *et al.* 1996; Isaac-Renton *et al.* 2005; Wilkes *et al.* 2009). Using a large database of 823 measurements in a rural watershed, marked increases in the prevalence of *Giardia* in the fall, 42% vs 9 to 12% in the spring and summer, and that of *Cryptosporidium* in the fall (72%) vs 17 to 19% in the spring and summer were reported by Wilkes *et al.* (2009). Isaac-Renton *et al.* (2005), reported peak contamination by *Cryptosporidium* occurred in an agricultural watershed in both spring and fall. Although cattle is the main oocyst input source in this watershed, other sources, such as wildlife (mice, rats, muskrat, deer, birds), humans, and companion animals (cats and dogs), can contribute (Ruecker *et al.* 2007).

Cryptosporidium infectivity and potentially viable *Giardia*

The *Cryptosporidium* infectivity ratio and the percentages of positives for infectious oocysts in the dairy farms studied are presented in Figure 2. Low averages of infectious ratios of 3.4% for calves and 3.0% for cows were observed and sporadically, up to 29% in calf feces (93 foci /330 oocysts, 1/7, and 1/6) and up to 45% in cow feces (5/11). Also, higher infectivity rates and positive samples were found in calves (17%) than cows (7%). Interestingly, for four different calf samples taken at three different farms, and one sample taken from the watershed discharge, excysted oocysts with sporozoites were observed on the cell monolayer, but their presence did not lead to infectious foci development. As only one life stage was observed, they were recorded as non-infectious oocysts. These oocysts were smaller ($n = 17$; 3.6×4.3 μm) than the control *C. parvum* Iowa oocysts ($n = 11$; 4.4×5.0 μm), and were similar in size to *C. ryanae* (Fayer *et al.* 2008). Typical foci were observed in control experiments performed with this calf fecal matrix seeded with *C. parvum* Iowa oocysts.

The low human infectious oocyst fraction found in the fresh feces most probably reflects the dominance of non-human pathogenic species, especially the dominance of *C. andersoni* in adult cows (Santin *et al.* 2004). However, these average ratios are misleading, since they reflect a majority of samples with no detected foci. In fact, very high infectious fractions detected by 3D-CC-IFA, up to 29% in calf feces and 45% in cow feces, reveal the sporadic presence of individual animals shedding high levels of human pathogenic species. These high fractions exceed the range typically reported for laboratory reference strains (Lalancette *et al.* 2010) and reveal the possible presence of strains highly infectious to humans within populations of dairy cattle. The 3D-CC-IFA results clearly support these high estimates, as they are based on a sufficient number of clear microscopic observations of both infectious foci and oocysts in composite samples. As expected, higher infectivity rates and positive samples were found in calves (17%) than cows (7%). *C. parvum* is the dominant species infecting calves (Santin *et al.* 2004; Fayer & Xiao 2007; Broglia *et al.* 2008) and is readily discriminated by 3D-CC-IFA (Lalancette *et al.* 2010). Several studies have shown that cows can harbor *C. parvum* infections, with a prevalence of 1% in post weaned calves (Santin *et al.* 2004) and in fecal material from a feed lot pen (Atwill *et al.* 2006a). Our results show that, when infected with an appropriate species, an adult cow can shed oocysts that are potentially very infectious to humans.

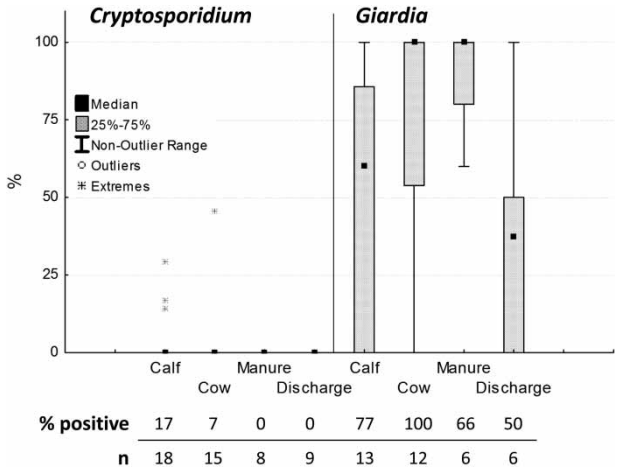

Figure 2 | Fractions (%) of human infectious *Cryptosporidium* (3D-CC-IFA) and potentially viable *Giardia* (DAPI+) in rural fecal pollution sources. Calf and cow fresh feces were composite samples. '*n*' = number of positive samples, and '% positive' is the ratio of infectious oocyst counts to total counts.

For *Giardia*, the estimates of potentially viable cysts were expected to be high, since viability was based on the presence of nucleic acid (positive DAPI staining), a less stringent criterion compared to the requirement of *Cryptosporidium* to form infectious foci on human cell lines. Figure 2 shows that *Giardia* cyst viability peaked at 100%, with the percentage of positives ranging from 77 to 100% for calves and cows. While the majority of cysts were potentially viable, the human health risk significance of these findings should be considered with caution. Indeed, livestock assemblage E is the dominant *G. duodenalis* genotype found in cattle, and the human pathogenic *G. duodenalis* genotype assemblage A only accounts for a small fraction (<0.5 to 17%) of cattle infections (O'Handley *et al.* 2000; Appelbee *et al.* 2003). Based on a review of several molecular epidemiology studies conducted on diverse hosts, Thompson *et al.* concluded that the public health risk from cattle-derived *Giardia* may be minimal, at least in North America and Australia, where genotyping has been undertaken and has confirmed the predominant presence of the livestock genotype (Thompson *et al.* 2003; Trout *et al.* 2004, 2007).

No infectious oocysts were found in the watershed discharge (Figure 2), although oocysts were almost always present in significant concentrations. The survival of parasites in soil following manure spreading is influenced by soil humidity, porosity, pH, and texture (Zarlenga & Trout 2004; Ramirez *et al.* 2009). The absence of infectious oocysts at our discharge point reflects both the expected dominance of non-human infectious species and environmental attenuation. The cumulative attenuation resulting from storage and environmental exposure in the field certainly reduced the already low infectious fraction observed in fresh feces. In similar watersheds, *Cryptosporidium* was frequently found in surface water, although human pathogenic species were rarely found, with *C. andersoni* being the most frequently isolated species (Xiao *et al.* 2001; Jiang *et al.* 2005; Ruecker *et al.* 2007; Yang *et al.* 2008). Wildlife, humans, and companion animals can also be sources of *C. parvum* (Xiao & Ryan 2004). However, in this watershed, these sources are likely to represent an insignificant volume of feces when compared to the loadings from the dairy production. Moreover, although *C. parvum* and *C. hominis* are the main species reported in human clinical isolates from urban and rural areas (Fayer & Xiao 2007), *C. hominis* is the dominant species found in specimens from urban residents (Llorente *et al.* 2007). The lower positive and median concentrations of *Giardia* cysts at the watershed discharge point, when compared to those found in fresh feces and manure, also suggest significant environmental decay.

(Oo)cysts from urban sources

Total *Cryptosporidium* and *Giardia* (oo)cysts

Cryptosporidium concentrations ranged from BDL to 533 oocysts L^{-1} in influent, and from BDL to 89 oocysts L^{-1} in WWTP effluents (Figure 3). Combined data from both WWTP revealed that 47% of influent and 56% of effluent samples were positive for *Cryptosporidium*. Our data are consistent with previously published concentrations in raw sewage influents (Robertson *et al.* 2000; Lemarchand & Lebaron 2003; Montemayor *et al.* 2005; McCuin & Clancy 2006) and WWTP effluents (States *et al.* 1997; Charles *et al.* 2003; Montemayor *et al.* 2005; Huffman *et al.* 2006; McCuin & Clancy 2006). As raw sewage is a more complex matrix and more difficult to purify than treated wastewater, higher prevalence in treated wastewater have been reported (Rose *et al.* 2004). However, for

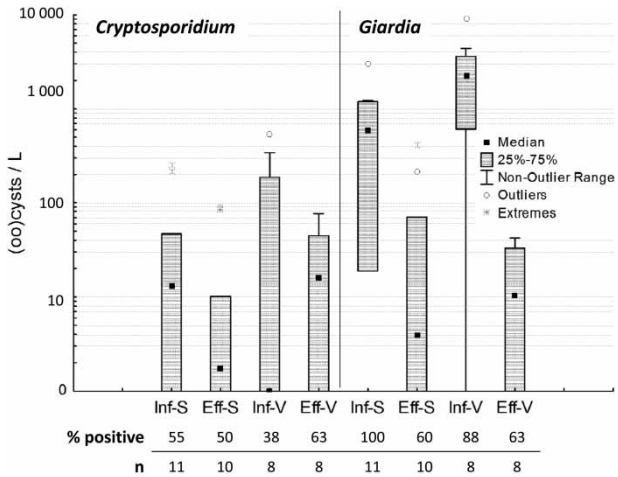

Figure 3 | Concentrations and % positive samples of *Cryptosporidium* (3D-CC-IFA) and *Giardia* (USEPA Method 1623) in urban fecal pollution sources (April to November 2009) (data corrected for average recoveries performed for each matrix). Detection limits for both parasites varied from 1.5 to 4 (oo)cysts L^{-1} for influents and from 0.33 to 2 (oo)cysts L^{-1} for effluents.

concentrations corrected for recoveries, this trend is corrected. Extreme concentrations have been reported: up to 24,000 oocysts L^{-1} in raw sewage (45 times higher) and up to 44,500 oocysts L^{-1}, (500 times higher) in WWTP effluents (Robertson *et al.* 2000, 2006). When combining measurements from several sources, wide ranges of *Cryptosporidium* concentrations in raw sewage (1,000 to 10,000 oocysts L^{-1}) and in WWTP effluents (10 to 1,000 oocysts L^{-1}) may be expected (Medema *et al.* 2003). The prevalence of oocysts in this study varied from 38 to 55% of positive samples in raw sewage, and from 50 to 63% of positive samples in WWTP effluents, falling within the expected ranges of 30 to 100% previously documented (States *et al.* 1997; Charles *et al.* 2003; Quintero-Betancourt *et al.* 2003; Montemayor *et al.* 2005; Robertson *et al.* 2006). Modest removals of oocysts for this study WWTP (<2 log removal) are well supported by previous studies (Rose *et al.* 2004; Robertson *et al.* 2006; Crockett 2007).

Giardia concentrations varied from BDL to 9,010 cysts L^{-1} for raw sewage, and from BDL to 472 cysts L^{-1} in WWTP effluents (Figure 3), which fall within published ranges of concentrations for raw sewage (Payment 2003; Bertrand & Schwartzbrod 2007) and effluents (Payment 2003; Huffman *et al.* 2006). Samples positive for both parasites ranged from 61 to 95%. Significantly higher concentrations have been reported in some studies, up to 35,800 cysts L^{-1} (4 times the maximum observed in this study) in raw sewage, and up to 51,333 cysts L^{-1} (100 times the maximum observed in this study) in effluents (Robertson *et al.* 2000, 2006). A review of published concentrations reveals that *Giardia* concentrations in raw sewage range widely from 5,000 to 50,000 cysts L^{-1}, and from 50 to 500 cysts L^{-1} in WWTP effluents (Medema *et al.* 2003). Previous measurements at WWTP-S showed higher *Giardia* concentrations of 520 to 5,000 cysts L^{-1} in raw sewage, and from BDL to 720 cysts L^{-1} for plant effluents (Payment 2003). Prevalence ranged from 88 to 100% in raw sewages and from 60 to 63% in effluents, similar to the reported upper range of 100% for both types of water (Robertson *et al.* 2000, 2006; Payment 2003; Quintero-Betancourt *et al.* 2003). Modest removals of cysts for both WWTP (<2 log removal) are well supported by previous studies (Payment 2003; Rose *et al.* 2004; Robertson *et al.* 2006; Crockett 2007).

Cryptosporidium infectivity and potentially viable *Giardia*

As wastewater is considered to be the primary source of human infectious oocysts in urban watersheds (World Health Organization (WHO) 2009), sampling was primarily designed to measure the concentration and infectious oocyst fraction in raw sewage and treated wastewater. Indeed, genotyping has confirmed the presence of human pathogenic oocyst species in sewage (Xiao *et al.* 2001; Ward *et al.* 2002; Quintero-Betancourt *et al.* 2003; Di Giovanni *et al.* 2006). Average concentrations of infectious oocysts (not corrected for recovery) varied from BDL-13 foci L^{-1} in raw sewage, and from BDL-3 foci L^{-1} in treated secondary effluents. Figure 4 presents direct infectious oocyst fractions that range from BDL to 22% for combined raw sewage samples with oocysts present in 0 to 33% of samples. In combined effluents, infectious fractions varied from BDL to 7% (aside from one extreme data point), with oocyst positive samples varying from 20 to 40%. The extreme data point of 100% observed in WWTP-V was computed from a sample containing only 2 foci and 2 oocysts, which is close to detection limit.

Limited published information is available on the concentrations of infectious oocysts and the estimation of the infectious fraction in wastewater sources. Quantification of infectious oocysts using CC-FDM-MPN revealed

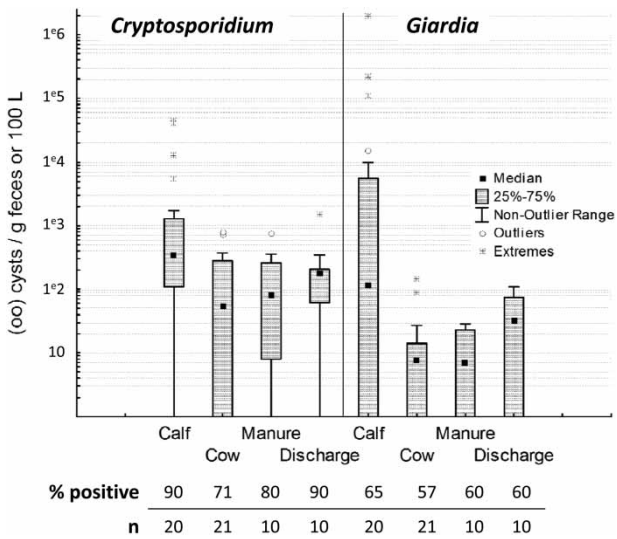

Figure 4 | Fractions (%) of infectious *Cryptosporidium* (3D-CC-IFA) and potentially viable *Giardia* (DAPI +) in urban fecal pollution sources. '*n*' = number of positive samples, and '% positive' is the ratio of infectious oocysts counts reported to total positive counts.

concentrations ranging from 0.17–0.27 foci L^{-1} in reclaimed water (Quintero-Betancourt *et al.* 2003), from BDL-1 foci L^{-1} in secondary treatment effluent (Gennaccaro *et al.* 2003), and from 0.37–50 foci L^{-1} raw sewage. In terms of infectious oocysts concentration in raw sewage and treated secondary effluent, our results are clearly comparable to those from the Rose *et al.* (2004) study and related papers. Interestingly, recent *Cryptosporidium* levels reported in source water used by drinking water plants suggest a decrease in the occurrence of this parasite (Regli & Cole 2010).

Our results differ from previous studies in terms of the improved estimates of infectious oocyst fractions, since the 3D-CC-IFA provides more direct and reliable results (Lalancette *et al.* 2010) compared to previously used MPN estimates of split heterogeneous environmental samples, especially those with low oocyst concentrations. The estimates of BDL-33% from this study suggest a lower fraction of infectious oocysts in raw sewage and treated secondary effluent compared to other studies. Rough estimates of 14% in raw sewage and 25% in treated disinfected reuse water were previously reported (Gennaccaro *et al.* 2003) and similar forecasts range between 0.3 and 40% in reclaimed effluent. Estimations of infectious oocyst fractions in water are challenging because of other factors such as: (1) the use of concentrations corrected for recovery; (2) the variability between samples when using a methodology with split sampling; (3) the low concentrations and paucity of paired positive results, which cause an even greater challenge for analysis of surface water or treated drinking water (LeChevallier *et al.* 2003).

Moreover, above methodological considerations, the species composition may have a significant impact as CC-IFA based methods detect foci from oocysts of common human pathogenic species, such as *C. parvum*, *C. hominis*, and *C. meleagridis*, but not animal associated species such as *C. andersoni* and *C. muris* (Ward *et al.* 2002; Johnson *et al.* 2010; Lalancette *et al.* 2010).

The determination of the infectious oocyst fraction has a major impact on the results of risk analysis directed to the determination of drinking water treatment. An average value of 37% of infectious oocysts in surface water has been proposed based on a study using composite data from cell culture PCR (CC-PCR) and conventional microscopy (LeChevallier *et al.* 2003). This approximation served as a reference to define the underlying distribution of infectivity in the model used by the US EPA to review the LT2 legislation (United States Environmental Protection Agency (USEPA) 2005b) leading to the use of probability distribution mode of 40% for surface water samples. It must be noted that the infectious oocyst fraction inferred by LeChevallier was obtained using a prevalence ratio of independent samples for infectious and total oocysts, and the infectivity method used (CC-PCR) has recently been shown to produce false positives for 17% of mock infection cell monolayers inoculated with 3 oocysts (Johnson *et al.* 2010). The rough estimates produced by Gennaccaro *et al.* (2003) and Quintero-Betancourt *et al.* (2003), the improved estimates from this study and the growing body of molecular genotyping confirming the presence of non-human infectious species in source water (Ruecker *et al.* 2007; Yang *et al.* 2008) suggest that the use of the EPA distribution should be questioned and lower values considered.

In this study, *Giardia* cysts detected by USEPA Method 1623 were considered potentially viable when DAPI stained nuclei were observed (DAPI positive) and internal structure were observed as determined by Method 1623. However, as for *Cryptosporidium*, the antibodies used for *Giardia* staining in Method 1623 cannot discriminate genotypes, which mean human pathogenic and non-pathogenic *Giardia* genotypes may be enumerated (Bertrand & Schwartzbrod 2007). In this study, we observed high cyst viability (up to 100%) in both raw sewage and secondary effluents (Figure 2), with cysts present in 40 to 86% of samples. However, DAPI staining should be viewed only as a marker of viability and not considered an indicator of infectivity. Large decreases in viability were observed for both plants regardless of retention time, with reductions of 84% in AL with 16 days versus 100% for sequential bioreactors with 7 h of retention time. Although the viability procedure used does not confirm the presence of human infectious species, *G. duodenalis* cysts from assemblages A (also rarely found in cats and dogs) and B (also rarely found in dogs and rats) are commonly found in wastewater (Thompson 2004; Bertrand & Schwartzbrod 2007).

CONCLUSION

In this study, the fractions of human infectious *Cryptosporidium* oocysts were assessed directly for environmental

water and fecal matrices influencing drinking water treatment plant intakes, using the 3D-CC-IFA method. The sampling strategy aimed to better qualify human health risks posed by distinct sources of fecal pollution. The 3D-CC-IFA infectivity measurements provide more accurate data for QMRA than simple estimates based on total microscopic oocyst counts and assumptions of human infectious fractions. In rural sources, *Cryptosporidium* spp. and *Giardia* spp. were commonly found in calves and cows. Importantly, the *Cryptosporidium* oocysts were not often found to be infectious using 3D-CC-IFA, indicating a lower human health risk for these sources. Calves had sporadically higher concentrations of both parasites than cows, and the proportion of infectious oocyst samples was higher in calves than in cows. Manure generally contained parasites, but no infectious oocysts were detected. Discharge from the studied rural watershed contained both parasites in significant concentrations (BDL to 1,518 oocysts 100 L^{-1} and BDL to 108 cysts 100 L^{-1}), but no infectious oocysts were found. *Cryptosporidium* spp. was the most prevalent in rural pollution sources, while *Giardia* was most common in urban pollution sources. In urban sources, *Cryptosporidium* spp. and *Giardia* spp. were commonly isolated from raw sewage (<550 oocysts and <10,000 cysts L^{-1}) and at lower concentrations (<90 oocysts and <500 cysts L^{-1}) in the effluents of the two WWTPs. Human infectious *Cryptosporidium* were observed in 20% of the samples, and the percentages of infectivity did not rise above 22% for raw sewage and 7% for WWTP effluent (with one exception). In both rural and urban pollution sources, the presence of human infectious oocysts was limited, and when present, they were most of the time well below the 40% reference value commonly used QMRA estimate and would lead to lower risk estimates. Data collected in this study are currently being used to refine QMRA models for the studied watersheds. A similar approach as taken in this study may prove useful for refining risk assessments for other watersheds.

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