Identifying host sources, human health risk and indicators of *Cryptosporidium* and *Giardia* in a Canadian watershed influenced by urban and rural activities

Michele I. Van Dyke, Corinne S. L. Ong, Natalie A. Prystajecky, Judith L. Isaac-Renton and Peter M. Huck

ABSTRACT

Cryptosporidium and *Giardia* were characterized in a watershed in southern Ontario, Canada, over a 2½ year period. River samples were collected every two weeks, primarily near a municipal drinking water treatment plant intake. *Cryptosporidium* and *Giardia* were frequently detected with an overall occurrence rate of 88 and 97%, respectively. *Giardia* concentrations were higher than *Cryptosporidium*, with median values of 80 cysts 100 L^{-1} and 12 oocysts 100 L^{-1}, respectively. Although pathogens rarely show a significant relationship with fecal or water quality indicators, this study determined that *Cryptosporidium*, but not *Giardia*, was significantly correlated with *Escherichia coli*, turbidity and river flow. There was no correlation between the two types of protozoa, and only *Giardia* showed a seasonal trend with higher concentrations at cold water temperatures. *Cryptosporidium* genotyping of all samples found that farm animals and wildlife were an important contributor of oocysts in the watershed, and that *Cryptosporidium* strains/genotypes of medium to high risk for human infection (*C. hominis*, *C. parvum* and *C. ubiquitum)* were detected in 16% of samples. This study was able to identify *Cryptosporidium* host sources and human health risk, and to identify differences between *Cryptosporidium* and *Giardia* occurrence in the watershed.

Key words | *Cryptosporidium*, *Cryptosporidium* genotype, drinking water, *E. coli*, *Giardia*, watershed

Michele I. Van Dyke (corresponding author)
Peter M. Huck
NSERC Chair in Water Treatment,
Department of Civil and Environmental Engineering, University of Waterloo,
Waterloo, Ontario,
Canada
E-mail: *mvandyke@uwaterloo.ca*

Corinne S. L. Ong
Natalie A. Prystajecky
Judith L. Isaac-Renton
British Columbia Centre for Disease Control Public Health Microbiology and Reference Laboratories,
Vancouver, British Columbia,
Canada

Corinne S. L. Ong*
Natalie A. Prystajecky
Judith L. Isaac-Renton
Department of Laboratory Medicine and Pathology,
University of British Columbia,
Vancouver, British Columbia,
Canada
*Present address:
School of Occupational & Public Health,
Ryerson University, Toronto, Ontario,
Canada

INTRODUCTION

The protozoan pathogens *Cryptosporidium* and *Giardia* are frequent causes of waterborne disease throughout the world. They can be transferred from human or animal sources through a fecal to oral route of exposure, and therefore waters containing either human sewage or animal waste (agricultural, domestic or wildlife) are important sources of infection. In North America illnesses caused by *Cryptosporidium* and *Giardia* have been associated with water consumption from recreational activities at both treated (i.e. pools, splash pads) and untreated (surface water) facilities (Yoder *et al.* 2010), and numerous drinking water outbreaks have also occurred. In Canada from 1974 to 2001, Schuster *et al.* (2005) investigated outbreak data from both private and public supply systems, and found that *Giardia* was responsible for 51 drinking water outbreaks and *Cryptosporidium* for 12 outbreaks. Worldwide, Karanis *et al.* (2007) found that *Cryptosporidium* and *Giardia* accounted for 50.8 and 40.6%, respectively, of 325 identified waterborne outbreaks caused by protozoa. It is recognized that the involvement of protozoa in waterborne disease is higher than that reported, since the causative agent is often not identified and those infected may not have sought medical attention.

The majority of drinking water outbreaks caused by *Cryptosporidium* or *Giardia* can be traced back to improper design or failure of the drinking water treatment system and

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are often associated with a weather event (Schuster 2005; Hrudey & Hrudey 2007). *Cryptosporidium* oocysts and *Giardia* cysts are resistant to disinfection by chlorine, and therefore effective removal (i.e. filtration) and inactivation (i.e. ultraviolet light) processes are required for drinking water. Information on protozoan removal through various water treatment strategies are well known, and implementation of effective technologies are critical in the production of drinking water with low public health risk (Betancourt & Rose 2004).

In recent years there has been a shift in drinking water guidelines and regulations towards a risk assessment-based determination of treatment requirements. These assessments require a knowledge of *Cryptosporidium* and *Giardia* concentrations in water sources (US EPA 2006; Health Canada 2010). However, enteric pathogen enumeration is costly and in Canada there is no specific requirement for pathogen testing of source waters. Surrogates are sometimes used to indicate the presence of pathogens, and water quality characteristics and risk inventories of source water are also used as a comparative measure of pathogen risk. To date, currently used surrogates (i.e. *Escherichia coli*) have not typically provided accurate information on *Cryptosporidium* and *Giardia* levels in water (Nieminski *et al.* 2010), and there remains a lack of information on protozoan pathogens in source water that allow for predictive evaluation. When appropriate and suitably applied methods and analyses are used, surveillance studies can be useful not only for providing source water data, but also to better assess potential indicators of pathogen occurrence, provide information for host and pollution source tracking, and determine the influence of environmental factors on *Cryptosporidium* and *Giardia* occurrence in the environment.

In this paper, *Cryptosporidium* and *Giardia* are evaluated in a watershed used as a source of community drinking water and also for recreational activities. Protozoan concentrations are compared with several indicators of water quality and season to identify differences in terms of source, survival and transport influences. In addition, genotyping analysis of *Cryptosporidium* is conducted to identify human health risk and host sources. This study was conducted in the Grand River watershed located in southern Ontario, Canada, which is typical of other watersheds in North America and in developed countries worldwide. The area has an affluent economy, moderate humid continental climate with warm summers and cold winters, and the rivers are heavily influenced by urban wastewater, agriculture and wildlife. One community outbreak caused by *Cryptosporidium* occurred in the Region of Waterloo in 1993 resulting in approximately 200 cases (Region of Waterloo Public Health 2010). *Cryptosporidium* incidence rates in the Region of Waterloo (2005–2009) were 4.1 per 100,000 person-year, with 78% of these cases considered endemic and 22% travel related. *Giardia* resulted in 13.9 cases per 100,000 person-year, with 60% classified as endemic and 40% travel related (Region of Waterloo Public Health 2010). Human infections caused by these protozoa were higher in the summer months, possibly due to increased exposure to untreated recreational water (Region of Waterloo Public Health 2010). Therefore, results of this study will have direct application for treatment, regulatory and risk assessment studies of drinking and recreational water.

MATERIALS AND METHODS

Sample area

The study area was the Grand River watershed within the Regional Municipality of Waterloo. With a total area 6,965 km^2, it is the largest inland river system in southern Ontario (Cooke 2006). Three drinking water intakes along the Grand River are used as a source of community drinking water, and rivers and reservoirs in the watershed are also used for recreational activities. Approximately 76% of the watershed is agricultural, with poultry, swine and cattle as the major livestock (Dorner *et al.* 2004), and the remaining areas of the watershed are forested (17%) and urban (7%). As one of the fastest growing areas in Ontario, the Region of Waterloo has a current population of 525,000 living primarily within the cities of Waterloo, Kitchener and Cambridge, with about 80% of the population serviced by wastewater treatment plants (WWTP) and the remainder using septic systems (Cooke 2006).

Samples from the watershed were collected primarily at an urban location in the Grand River close to the intake for the Region of Waterloo drinking water treatment plant

(Figure 1, sample site 5); samples from this location ($n = 72$) were taken once every two weeks for 32 months. Within the Region of Waterloo, approximately 20% of drinking water is provided from the Grand River with the remaining 80% from groundwater sources. River water is treated through coagulation/flocculation/sedimentation, ozonation, multi-media filtration, ultraviolet irradiation and chlorine disinfection, followed by chloramination for distribution, and the plant maintains a 5-log inactivation of *Cryptosporidium*. Potential sources of pathogens upstream of this location include animals (livestock and wildlife) and treated sewage, including discharge from the Waterloo WWTP (Region of Waterloo), which has a capacity of 57,500 $m^3\ d^{-1}$ and at the time of this study used conventional secondary treatment including chlorine disinfection, although the plant has since upgraded to include ultraviolet disinfection.

Additional samples were taken from locations upstream of the drinking water intake at less frequent intervals. This included an urban area just after the Waterloo WWTP effluent enters the Grand River (Figure 1, site 4) that was sampled approximately every 3–4 months ($n = 14$). Other upstream locations included two major tributaries of the Grand River (Conestogo River and Canagagigue Creek; Figure 1, sites 2 and 3) and the Grand River north site (Figure 1, site 1); these locations were sampled at intervals ($n = 18$) to compare protozoan concentrations in the watershed during typical water quality conditions and during seasonal events including rain and snow melt. The Grand River north and the tributary sites are all heavily influenced by agricultural activities, including high livestock densities and a high level of tile drainage and agricultural land runoff into the rivers (Dorner *et al.* 2004, 2007). These upstream locations are also influenced by smaller WWTPs, each with capacities less than 8,000 $m^3\ d^{-1}$ and have secondary or tertiary treatment including either chlorine, ultraviolet or ozone disinfection. The mean water flow rate at each sample location using 2005–2008 data from the Water Survey of Canada (ec.gc.ca/rhc-wsc) was 1.5 $m^3\ s^{-1}$ at Canagagigue Creek, 10.6 $m^3\ s^{-1}$ at Conestogo River, 18.6 $m^3\ s^{-1}$ at the Grand River north site and 28.6 $m^3\ s^{-1}$ in the Grand River intake site.

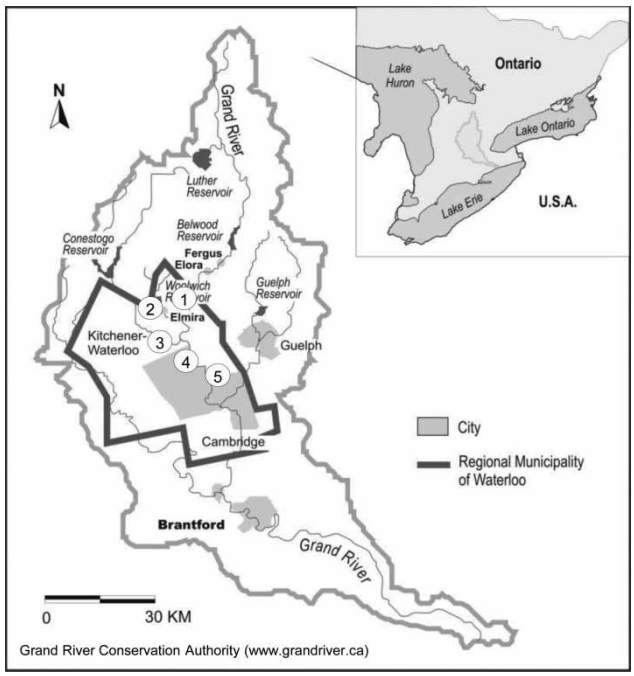

Figure 1 | Sample locations in the Grand River watershed, including the Grand River north (1) the Grand River downstream of a wastewater treatment plant discharge (4) and the Grand River upstream of a drinking water treatment plant intake (5). Samples were also collected at two tributaries just before they enter the Grand River, including the Canagagigue Creek (2) and the Conestogo River (3).

Sample collection and water quality parameters

At each location, water was collected 2–3 m from the river's edge and 10–20 cm below the surface at a fast-flowing area. Water quality parameters were measured as described by Van Dyke *et al.* (2010). Samples for protozoan analysis were collected in two sterile 20 L polyethylene containers (I-Chem Cubitainer, VWR) and transported to the University of Waterloo for filtration according to US EPA Method 1623 (US EPA 2005). Briefly, a 25 L volume of river water was passed through Filta-Max filters (IDEXX) using a peristaltic pump (Solinst model 410, Solinst Canada Ltd), and the filters were shipped on ice for protozoan enumeration on the same day the samples were collected.

Protozoan enumeration

Protozoan enumeration was done at the BC Centre for Disease Control Environmental Microbiology Laboratory, Vancouver, British Columbia, Canada. Enumeration based on US EPA Method 1623 was done within 48 h of sampling.

Briefly, the protozoan (oo)cysts were eluted from the filters according to the manufacturer's instructions (IDEXX). The filtrate was then concentrated by centrifugation, and the pellet was purified by immunomagnetic separation using Dynabeads GC-Combo (Invitrogen). Following immunomagnetic separation, the purified product was applied to slide wells, stained with EasyStain (BTF Precise Microbiology Inc.) and enumerated by immunofluorescence microscopy, with confirmation using 4′,6-diamidino-2-phenylindole (DAPI) staining and differential interference contrast microscopy.

Cryptosporidium genotyping

For *Cryptosporidium* genotyping analysis, DNA was extracted directly from the enumerated slides based on the method described by Ruecker *et al.* (2005). Briefly, the coverslips and mounting medium were removed from microscopy positive slides. The material remaining on the slides was suspended in ATL lysis buffer (QIAamp DNA Micro Kit, Qiagen), carefully scraped using a pipette tip and transferred to a microcentrifuge tube. Slides were scraped using a total of four rinses with lysis buffer. The sample was then exposed to five freeze-thaw cycles (liquid nitrogen/65 °C) and purified using the QIAamp DNA Micro Kit with the addition of carrier RNA. A nested polymerase chain reaction (PCR) procedure targeting the 18S rRNA gene was performed as described by Xiao *et al.* (1999) with the following modifications: both PCR amplification steps used a 50 μL reaction volume including 2.75 μL of DNA template for the primary reaction followed by 1 μL of product for the secondary reaction, and Platinum Taq (Invitrogen) and 20 mg of bovine serum albumin were used in each reaction. PCR product from the secondary PCR step was sequenced using bidirectional sequence analysis using the ABI Prism 3130xl Genetic Analyzer (Applied Biosystems). The resulting assembled sequences were deposited to the GenBank National Center for Biotechnology Information database under accession numbers JQ313910 to JQ313989. The watershed sequences were then compared to those in the GenBank database using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) as well as using multiple sequence alignments and phylogenetic analyses (MegAlign, DNA STAR Inc.). Species or genotypes were assigned based on both the percentage similarity (≥99%) with GenBank sequences and branching within defined species or genotype clusters. Species or genotypes were not assigned for sequences with <99% homology.

Statistical analysis

Cryptosporidium and *Giardia* concentrations and those of the water quality parameters (*E. coli*, turbidity, water temperature, nitrate, ammonia and river flow) in each sample from the intake location were compared using the Spearman's rank correlation coefficient ($p < 0.01$; two-tailed). River flow data were not available at the intake location until May 2006. However we determined there was a strong correlation ($r_s = 0.94$; $n = 52$) between the flow data available at the Grand River intake and Grand River north locations, and therefore the Grand River north flow data were used as an indicator of fluctuating water levels in the Grand River for correlation analysis. Significant differences in *Cryptosporidium* or *Giardia* concentrations in river samples collected at low (<10 °C) or high (≥10 °C) water temperatures were compared using the Mann-Whitney *U* test ($p<0.05$; two-tailed). The Mann-Whitney *U* test was also used to compare protozoan concentrations at increasing *E. coli* ranges.

RESULTS

Protozoan concentrations in the watershed

Both *Cryptosporidium* and *Giardia* were frequently detected in the watershed (Table 1). The majority of samples ($n = 72$) were taken close to the intake for a drinking water treatment plant, and the occurrence of *Cryptosporidium* and *Giardia* at this location was 88 and 97%, respectively, with median concentrations of 12 oocysts and 86 cysts 100 L^{-1}, respectively. Although fewer samples ($n = 18$) were taken at the upstream locations in the watershed (Grand River north, Conestogo River and Canagagigue Creek), the occurrence and median concentration of *Cryptosporidium* at these sites were similar to those at the intake location, but the median *Giardia* concentration was lower (48 cysts 100 L^{-1}). The occurrence

Table 1 | Occurrence of *Cryptosporidium* and *Giardia* in the Grand River watershed. Sample locations are shown in Figure 1, and include the Grand River close to a drinking water treatment plant intake and a municipal WWTP effluent, or at upstream locations including the Grand River north ($n = 8$), Conestogo River ($n = 5$) and Canagagigue Creek ($n = 5$)

				(Oo)cysts 100 L^{-1}		
Protozoa	**Location**	***n***	**Occurrence (%)**	**Median**	**Mean**	**Range**
Cryptosporidium	Intake	72	88	12	18	<4–186
	WWTP	14	100	36	85	4–390
	Upstream	18	83	13	62	<4–900
	Overall	104	88	12	35	<4–900
Giardia	Intake	72	97	86	155	<4–1,384
	WWTP	14	100	398	531	16–1,864
	Upstream	18	94	48	343	<4–5,401
	Overall	104	97	80	238	<4–5,401

and median concentration of both *Cryptosporidium* and *Giardia* in samples taken in the river close to a municipal WWTP effluent were higher than those taken at other locations in the watershed. Both types of protozoa were detected in 100% of samples at the WWTP site, and median *Cryptosporidium* and *Giardia* concentrations were 36 oocysts 100 L^{-1} and 398 cysts 100 L^{-1}, respectively.

The variability of protozoan concentrations throughout the watershed was assessed by collecting samples from different river locations on selected days. Samples were collected from three locations on the Grand River, including the intake, WWTP and north sites, and also at the Canagagigue Creek and Conestogo River tributaries (Figure 1). Results in Table 2 show that under dry to average weather conditions in the summer and fall, there was a trend towards higher protozoa concentrations at the WWTP location compared with the upstream or downstream locations in the Grand River and the tributary rivers. However, under heavy rain or snow melt conditions, protozoa concentrations were often similar or in some instances higher at the upstream locations. For instance on November 29, 2005 samples were collected during a severe storm event, and the Grand River north site had higher *Cryptosporidium* and *Giardia* concentrations than either the WWTP or intake locations.

A matrix spike was performed to determine the recovery of *Cryptosporidium* and *Giardia* for three samples, including two collected at the Grand River intake location and one collected at the WWTP location. The turbidity of the three samples ranged from 6.4 to 7.8, representative of average water quality conditions in the river. The recovery of *Cryptosporidium* ranged from 18 to 42% and the recovery of *Giardia* ranged from 19 to 31%. However, protozoan concentrations reported in this paper were not adjusted for recovery since these data were not determined for each sample.

Table 2 | Variability of *Cryptosporidium* and *Giardia* concentrations in the Grand River watershed in samples taken on the same day

		***Cryptosporidium* oocysts/*Giardia* cysts 100 L^{-1}**				
Sample date	**River flow[a] (m^3 s^{-1})**	**Grand River intake**	**Grand River WWTP**	**Grand River north**	**Canagagigue Creek**	**Conestogo River**
Nov. 29/05	51	69/69	150/750	900/5,401	NA[b]	NA
Mar. 26/07	64	16/52	32/16	12/52	4/52	32/28
Mar. 31/08	28	12/436	24/348	0/56	16/68	24/104
Jul. 12/06	6	20/68	390/173	24/4	NA	NA
Sept. 25/06	6	36/68	96/173	20/4	NA	NA
Jul. 16/07	5	7/21	40/1,200	3/41	3/7	19/27
Oct. 22/07	2	0/108	149/1,090	0/108	14/19	20/44

[a]River flow data from the Grand River north location.
[b]Data not available.

Seasonal differences in protozoan concentrations

Figure 2 shows *Cryptosporidium* and *Giardia* concentrations over time in samples collected from the Grand River intake site. Over the 2.5 year sampling period, there was a greater variability in *Giardia* concentrations (3 log units) compared with *Cryptosporidium* (2 log units). *Giardia* showed a seasonal trend with higher cyst concentrations during the winter months. Median protozoan concentrations were compared at cold (<10 °C) or warm (≥10 °C) river water temperatures, and Figure 3(a) shows the median and interquartile range values of oocyst and cyst concentrations at each temperature range. There was a significant difference between the median *Giardia* concentration at cold compared with warm water temperatures, but temperature did not affect the median *Cryptosporidium* concentration. In addition, the concentration of *Giardia* was significantly higher than *Cryptosporidium* at both cold and warm water temperatures. By looking at *Giardia* concentrations over the different seasons, we can see that the median cyst concentration was lowest in the summer and highest in the winter, with the greatest variability in the spring (Figure 3(b)).

Correlation with water quality parameters

Relationships between protozoa and various water quality parameters in the river samples were evaluated. As there was a larger protozoan data set available at the Grand River intake location, correlation analysis was done using samples from this site only. Water quality parameters (*E. coli*, turbidity, river flow, water temperature, nitrate and ammonia) measured in Grand River intake samples are shown in Table 3. Using the Spearman rank order method, *Cryptosporidium* concentration was found to be significantly correlated ($p < 0.01$) with *E. coli* ($r_s = 0.46$), turbidity ($r_s = 0.37$) and river flow ($r_s = 0.34$), but there was no relationship between *Giardia* and these water quality parameters (Table 4). Instead, *Giardia* levels were found to be positively correlated with ammonia concentration ($r_s = 0.52$), and negatively correlated with nitrate ($r_s = -0.31$). *Giardia* also had a significant negative correlation

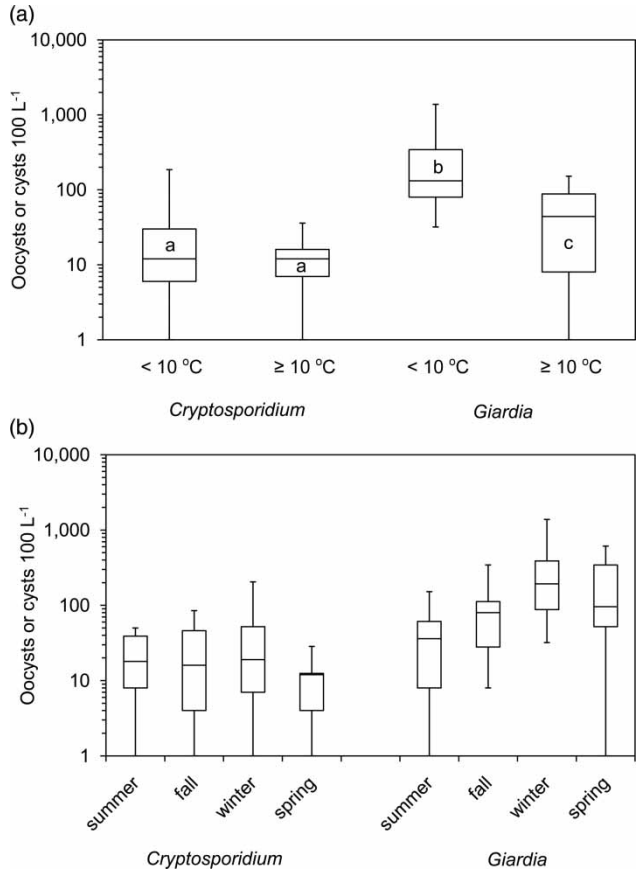

Figure 3 | The effect of temperature on protozoan concentrations in samples from the intake location. Each box represents the interquartile range, with the median shown as a line within the box, and minimum and maximum values shown by bars. Samples were grouped according to those with cold (<10 °C; $n = 51$) or warm (≥10 °C; $n = 53$) water temperatures (Figure 3(a)) or by season (Figure 3 (b)). Significant differences in protozoan concentrations between cold or warm temperatures are shown as different letters on each box (a,b,c).

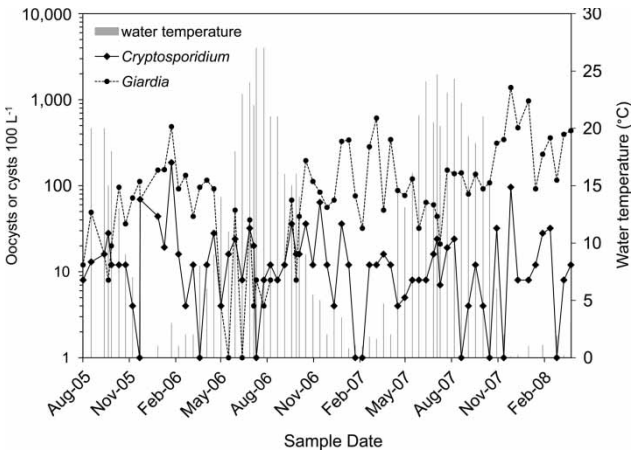

Figure 2 | Concentration of *Cryptosporidium* oocysts and *Giardia* cysts in samples collected at the intake location in the Grand River watershed from August 2005 to March 2008. Values below the detection limit of 4 (oo)cysts 100 L^{-1} are marked on the graph as 1 (oo)cyst 100 L^{-1}.

Table 3 | Water quality parameters in samples collected from the Grand River at the intake location

	Median	Mean	Range
E. coli (CFU 100 mL^{-1})	74	555	1–13,000
Turbidity (NTU)	8.2	13.3	1.9–183
Temperature (°C)	9	11	0–27
Nitrate (mg L^{-1})	1.1	1.4	0.1–6.4
Ammonia (mg L^{-1})	0.2	0.3	0–1.5

($r_s = -0.62$) with river water temperature, with higher cyst concentrations detected at colder water temperatures. It was determined that there was no significant correlation between *Cryptosporidium* and *Giardia* concentrations. The results in Table 4 also showed an association between certain water quality factors, including positive correlation values between turbidity and river flow, *E. coli* and nitrate concentration, and significant negative correlation values between temperature and both ammonia concentration and river flow.

Since *E. coli* is often used as an indicator of microbial water quality, this parameter was further explored by assessing median oocyst/cyst concentrations at log-fold increasing *E. coli* range values as shown in Figure 4 (1–10; 10–100; 100–1,000 and >1,000 CFU 100 mL^{-1}). None of the water samples had less than 1 CFU 100 mL^{-1} *E. coli*. Although there were fewer samples that fell into the low and high *E. coli* categories, it was determined that the median *Cryptosporidium* oocyst concentration was significantly higher in samples that had more than 100 CFU *E. coli* 100 mL^{-1} compared with samples that had less than 100 CFU 100 mL^{-1} (Figure 4(a)). However, there was no difference in the median concentration of *Giardia* cysts between the different *E. coli* ranges (Figure 4(b)).

Cryptosporidium genotypes

Each sample that was positive for *Cryptosporidium* oocysts was further analyzed to determine the genotypes that were present. This analysis was done by DNA sequencing of a PCR-amplified 18S rRNA gene fragment. Results in Table 5 show the species or genotypes identified in samples from the Grand River intake and WWTP sites or at the upstream locations (Grand River north, Conestogo River or Canagagigue Creek). An assignment of health risk for each identified species/genotype was based on the information available to date in the peer-reviewed literature regarding the host specificity and severity of disease in humans caused by these strains.

Sequences that matched those from high to medium risk species (*C. hominis*, *C. parvum* and *C. ubiquitum*) were detected in 13% of samples from the intake site, 50% of samples from the WWTP site and 0% of samples from the upstream locations. The fact that human infectious strains were not detected at the upstream sites may reflect the smaller data set available at this location. The most frequently identified species in the river samples was *C. andersoni*, which was isolated from 53% of samples and detected at all sampling locations in the watershed. *C. andersoni* has a relatively low health risk, but has been detected in humans (Leoni *et al.* 2006;

Table 4 | Correlations between *Cryptosporidium* and *Giardia* and water quality parameters in samples taken from the intake location

	Cryptosporidium	*Giardia*	*E. coli*	Turbidity	River flow[a]	Temperature	Nitrate	Ammonia
Cryptosporidium	1	0.12	**0.46**[b]	**0.37**[b]	**0.34**[b]	−0.07	0.09	−0.13
Giardia		1	−0.01	−0.17	0.14	**−0.62**[b]	**−0.31**[b]	**0.52**[b]
E. coli			1	**0.54**[b]	0.22	0.05	0.08	0.01
Turbidity				1	**0.52**[b]	0.04	**0.36**[b]	−0.14
River flow[a]					1	**−0.44**[b]	**0.38**[b]	0.16
Temperature						1	−0.06	**−0.63**[b]
Nitrate							1	−0.08
Ammonia								1

[a]River flow data from the Grand River north location.
[b]Significant correlation; $p < 0.01$ (two-tailed).

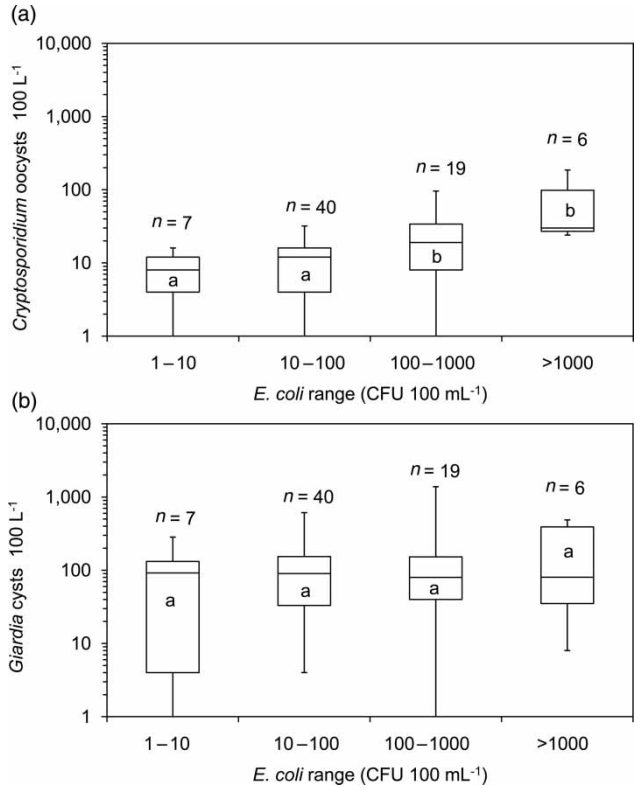

Figure 4 | *Cryptosporidium* (Figure 4(a)) and *Giardia* (Figure 4(b)) in samples from the intake location, categorized by range of *E. coli* concentration. Each box represents the interquartile range, with the median shown as a line within the box, and minimum and maximum values shown by bars. The number of samples (*n*) in each *E. coli* range are shown above each box. Significant differences in protozoan concentrations between each *E. coli* range are represented using different letters on each box (a,b).

Morse *et al.* 2007). Other species or genotypes detected in the watershed included *C. baileyi*, *C. bovis*, *C. muris*, muskrat genotypes I and II, skunk genotype and fox genotype. These strains are primarily associated with birds or animals (livestock, wildlife and rodents) and the human health risk is low or unknown (Xiao & Feng 2008). Five sequences did not show a close similarity with any identified species or genotypes.

Multiple species/genotypes were found in 22% of samples (Table 5). Of the 92 samples that were positive for *Cryptosporidium* by immunofluorescence microscopy, the species/genotype could not be determined in 27% of samples. The reason for this is likely due to the low concentration of oocysts in the sample and also the presence of PCR inhibitors that occur naturally in the river water. PCR inhibition of environmental samples is a common problem, in particular with surface water samples that contain a relatively high amount of particles and organic material.

Table 5 | *Cryptosporidium* species or genotypes identified in microscopy positive samples from the Grand River watershed. More than one species or genotype was identified in 22% of positive samples

		Number of positive samples			
Species/ genotype	**Health risk**	**Intake (*n* = 63)**	**WWTP (*n* = 14)**	**Upstream (*n* = 15)**	**Overall**
C. hominis	High	4	3	0	7
C. parvum	High	1	2	0	3
C. ubiquitum	Medium	3	2	0	5
C. andersoni	Low	36	4	9	49
C. baileyi	Low	3	0	2	5
C. muris	Low	0	1	0	1
C. bovis	Unknown	1	0	0	1
Muskrat genotype I	Unknown	2	2	1	5
Muskrat genotype II	Unknown	2	0	1	3
Skunk genotype	Unknown	0	1	0	1
Fox genotype	Unknown	1	0	0	1
Unassigned genotype	Unknown	4	0	1	5
No result		18	3	4	25

No relationship was found between the *Cryptosporidium* species/genotypes and either season or water quality parameters. To further examine potential links between *Cryptosporidium* species/genotypes and *E. coli*, samples were grouped into log-fold increasing *E. coli* ranges similar to that shown in Figure 4. Results (not shown) determined that the species type and diversity were more related to the sample number in each range and not *E. coli* concentration. A larger number of samples with low (1–10 CFU mL^{-1}) or high (>1,000 CFU mL^{-1}) *E. coli* concentrations would need to be evaluated to determine if *E. coli* concentration will influence the detection of high risk species.

DISCUSSION

This study assessed the occurrence of *Cryptosporidium* and *Giardia* in rivers in the Grand River watershed located in

southern Ontario, Canada. The watershed is in a mixed rural and urban area, and water quality is influenced by overland flow, groundwater contributions and wastewater discharges, with potential pathogen sources that include both animals (livestock and wildlife) and humans. In this study, both *Cryptosporidium* and *Giardia* were frequently detected in the rivers with overall occurrence rates of 88 and 97%, respectively, and median concentrations of 12 oocysts $100\,L^{-1}$ and 80 cysts $100\,L^{-1}$, respectively. These results are higher than previous studies in the Grand River watershed (LeChevallier *et al.* 2000; Dorner *et al.* 2007). The protozoan detection rates in our study were somewhat higher, but with similar concentration ranges, when compared with other drinking water sources and watersheds in North America that have similar urban and rural sources of pollution (Ong *et al.* 1996; Yang *et al.* 2008; Wilkes *et al.* 2009).

Of particular importance was the large variation in pathogen levels over the 2.5 year sampling period, with *Cryptosporidium* and *Giardia* concentrations that ranged by up to 2 and 3 log units, respectively. Information on the occurrence and concentration of these protozoan pathogens is valuable for a number of purposes, including a determination of the treatment requirements for municipal drinking water systems and also for assessing the human health risk through both drinking water and recreational water use. However, it is critical that the large variation in protozoan concentrations are taken into account during these assessments, since it is the peak values that will be the most important in terms of potential risk exposure.

The occurrence and concentrations of both *Cryptosporidium* and *Giardia* were found to be higher at a sample location close to the effluent of a municipal wastewater treatment plant. On days with dry to average weather conditions, protozoan concentrations tended to be higher at the WWTP site compared with upstream locations in the watershed, but on days with high surface runoff (rain or snowmelt) there was less difference between these locations, with sometimes higher upstream concentrations compared to the WWTP site. The northern portion of the watershed is heavily agricultural, and protozoan transport into the rivers will depend on proximity of the farms and animals to the river, manure spreading practices, tile drainage, and soil and transport properties. Therefore the relative input of protozoa from non-point sources may be related to environmental conditions that affect surface runoff into the rivers.

As part of our study, we assessed the relationship between *Cryptosporidium* and *Giardia* and various water quality parameters (*E. coli*, turbidity, river flow, ammonia, nitrate and water temperature). *E. coli* was used as an indicator for fecal contamination, while turbidity and river flow were indicators for precipitation and surface runoff. Ammonia and nitrate were measured as they can enter the watershed through agricultural or urban activities. There was no significant correlation between *Cryptosporidium* and *Giardia* concentrations, but different relationships were observed between the two types of protozoa and the water quality parameters. By analyzing samples taken close to a drinking water treatment plant intake, *Cryptosporidium* oocysts showed a significant correlation with *E. coli*, turbidity and river flow, but *Giardia* did not correlate with these parameters. Instead, *Giardia* concentrations were significantly correlated with temperature, nitrate and ammonia. These results suggest that *Cryptosporidium* and *Giardia* likely have different source, transport or survival characteristics in the watershed. Since turbidity was significantly correlated with *Cryptosporidium*, *E. coli* and river flow, this surface runoff parameter may be indicative of oocyst and *E. coli* transport into the rivers.

The relationship between *Giardia* and the nitrogen forms was likely related to water temperature and not a direct link between the parameters. Both *Giardia* and ammonia showed a moderate negative correlation with temperature. It is expected that ammonia conversion would be reduced at cold temperatures due to reduced metabolic activity in the river. Increased survival of *Giardia* at cold water temperatures was shown by comparing cyst concentrations at cold ($<10\,^{\circ}C$) versus warm ($\geq 10\,^{\circ}C$) river water temperatures, with higher cyst concentrations detected in the winter months. No relationship was found between water temperature and *Cryptosporidium* oocyst concentration. Improved *Giardia* survival at low temperatures has been shown previously (Bingham *et al.* 1979; deRegnier *et al.* 1989), and others have also detected higher concentrations of *Giardia* cysts in environmental water sources during the winter months (Isaac-Renton

et al. 1996; Ong et al. 1996) or at cold water temperatures (Atherholt 1998). These results suggest that *Giardia* cysts may be more sensitive than *Cryptosporidium* to temperature changes in the environment.

Although *E. coli* is often used as a microbial water quality indicator for untreated water, a significant correlation between *E. coli* and protozoan pathogens has rarely been shown (Nieminski et al. 2010). These relationships may be more common in environments in which both protozoa and *E. coli* are frequently detected. For example, Wilkes et al. (2009) found a significant ($p = 0.05$) correlation between *E. coli* and both *Cryptosporidium* and *Giardia* concentrations in a watershed in eastern Ontario, Canada, particularly in the fall and winter months. Atherholt et al. (1998) found a significant correlation between both *Cryptosporidium* and *Giardia* and the water quality indicators *E. coli*, turbidity and river flow, with *Cryptosporidium* showing higher correlation values. To further investigate the relationship between *E. coli* and protozoa in our study, oocyst/cyst levels were assessed at log-fold increasing *E. coli* range values. For *Cryptosporidium*, the median oocyst concentration generally increased with *E. coli* range, however the increase was small and only significant between *E. coli* range values of 10–100 and 100–1,000 CFU 100 mL^{-1}. Median *Giardia* concentrations did not show a significant difference between the *E. coli* ranges. Therefore, relying on *E. coli* levels as a basis for predicting protozoan concentration does not necessarily provide data that are useful in terms of human health risk. Of far more importance is a knowledge of the actual *Cryptosporidium* and *Giardia* concentrations of a particular source water, and more importantly the range and maximum expected concentrations, which can then be used for drinking water treatment design and assessment. Although the overall presence of *E. coli* will remain a useful tool for identifying general pathogen risk, the use of *E. coli* range values in predicting protozoan occurrence or concentrations must be viewed with caution.

Methodology must also be considered when interpreting the protozoan enumeration data. In this study, protozoa were enumerated using US EPA Method 1623, which remains the most commonly used method for detecting *Cryptosporidium* and *Giardia* in water samples. Although protozoan recovery by matrix spike was not assessed for individual samples, data collected during typical water quality conditions showed recovery values that ranged from 18 to 42% for *Cryptosporidium* and from 19 to 31% for *Giardia*, which are typical and acceptable values according to the US EPA standard method. Based on the recovery data, it can be estimated that the actual concentrations of *Cryptosporidium* and *Giardia* in the watershed were likely three to five-fold higher than those shown in Table 1. Protozoan viability and infectivity are also important considerations in the interpretation of environmental data, and although our method did not specifically assess cell viability, protozoan (oo)cysts can remain viable in the environment for long periods of time (months) and it can be assumed that a proportion of the protozoa detected in this study were potentially infective to humans.

An additional consideration that affects the applicability of our results to drinking water applications includes human infectivity. The enumeration method used in this study targets all *Cryptosporidium* spp. and *Giardia* spp., however it is well known that within each genus there are a large number of species and sub-species that vary in their ability to infect humans. In recent years, PCR amplification with sequence analysis has allowed a comparative analysis and identification of microbial pathogens, and this information has advanced our understanding of the strains that can infect humans and animals, as well as those that predominate in causing human disease. *Cryptosporidium* genotyping analysis showed that a large number of *Cryptosporidium* species and genotypes were detected in the watershed samples. The most frequently detected species at each sample location was *C. andersoni*, which was detected in 53% of all samples. *C. andersoni* were commonly detected in beef and dairy cattle within the Region of Waterloo (Dixon et al. 2011) and this strain typically produces chronic and asymptomatic infections in these animals (Santín & Trout 2008). Since cattle are a common livestock animal on farms in the region and the major producer of agricultural manure (Dorner et al. 2004), the predominance of *C. andersoni* in the watershed is expected. Other studies have also shown that *C. andersoni* was the most frequently detected strain in surface waters (i.e. Ruecker et al. 2005, 2007; Yang et al. 2008; Nichols et al. 2010).

In addition to *C. andersoni*, a number of other *Cryptosporidium* species and genotypes were detected in the Grand

River watershed. *C. hominis* and *C. parvum* are the most common cause of human cryptosporidiosis, with *C. hominis* primarily associated with human infections while *C. parvum* can infect both humans and ruminants, primarily cattle (Nichols 2008). These two species combined were detected in 8% of samples from the Grand River intake location, and in 36% of samples at the Grand River WWTP site. *C. hominis* and *C. parvum* were not detected in samples taken at upstream sites in the watershed, but this may reflect the smaller number of samples collected at these locations. These results show that the proportion of human pathogenic strains may be higher at locations in the river directly influenced by human wastewater. It is interesting that *C. ubiquitum* (previously identified as the *Cryptosporidium* cervine genotype) also followed the same trend, and was detected in 5% of samples at the intake and 14% of samples at the WWTP site, but was not detected in the upstream samples. *C. ubiquitum* has been isolated from humans in Canada (Ong *et al.* 2002; Trotz-Williams *et al.* 2006; Wong & Ong 2006) and is ranked fourth (after *C. hominis*, *C. parvum* and *C. meleagridis*) in the number of reported cases in humans in industrialized nations (Fayer *et al.* 2010). It is therefore possible that *C. ubiquitum* may be found in humans in the region, although this species has a broad host range that includes domestic and wild ruminants, rodents, omnivores and primates (Fayer *et al.* 2010). This species has been isolated from other watersheds (Ruecker *et al.* 2007; Yang *et al.* 2008) and was dominant in samples from a watershed in New York, USA, that was primarily impacted by wildlife (Jiang *et al.* 2005).

Other *Cryptosporidium* species detected in the watershed are primarily associated with farm livestock and wildlife, including *C. baileyi* (poultry), *C. bovis* (cattle), *C. muris* (rodents), muskrat genotypes I and II, skunk genotype and fox genotype. Since both farming and wildlife affect water quality in the region, it is expected that *Cryptosporidium* species from related sources would be detected in the river samples. Since these strains have either seldom or never been detected in humans, their pathogenicity is most likely low or unknown. There is currently no data available on *Cryptosporidium* genotypes from humans in the Region of Waterloo, and therefore a direct comparison between watershed and human isolates could not be made. However, as additional data on sequence types and host specificity are made available, this rapidly expanding area may provide new insights on *Cryptosporidium* genotypes, sources and human infectivity. Overall results of the *Cryptosporidium* genotyping analysis show that 16% of samples taken from the watershed contained species that are known to be of high to medium risk to human health. The species identified in the watershed reflected potential sources of protozoa in the region, including farm animals, wildlife and humans.

CONCLUSIONS

Results of this study provide data that are important for regulatory and risk assessment studies of drinking water and recreational water exposure to protozoan pathogens. Using current detection and analysis techniques, accurate data on the occurrence and sources of protozoan pathogens were provided. Methods to analyze protozoan and water quality data were useful in identifying differences in the survival, transport or sources of *Cryptosporidium* and *Giardia* in the watershed. In addition, *Cryptosporidium* genotyping results were found to correspond with land use in the watershed area, and showed that farm animals, wildlife and humans all contributed oocysts to the rivers.

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