



PUBLIC WORKS DEPARTMENT

January 30, 2014

Joel Klumpp, Team Leader
Technical Review & Oversight Team MC-159
Texas Commission on Environmental Quality
P.O. Box 13087
Austin, Texas 78711-3087

Re: City of Wichita Falls
PWS ID No. 2430001
Wichita County
TCEQ Full Scale Verification Site Visit Follow-up Requests

Dear Mr. Klumpp,

The City of Wichita Falls requested that the TCEQ send officials to witness the start of the City's Full Scale Verification test and to ensure that everything was in accordance with the TCEQ's guidance letters on the Direct Potable Reuse (DPR) project. The TCEQ agreed and sent a team for a 3 day site visit, beginning on January 27, 2014. During the site visit, the TCEQ team and City staff discussed numerous components of the TCEQ guidance documents for the DPR and the City was instructed to make formal written requests for follow-ups on several of those discussion items. This letter will serve as the City's formal written request to those items.

- January 22, 2014 Letter, Enclosure A, Page 3 of 21: The TCEQ has changed the City's trigger from Total Chlorine to Monochloramine.
The City of Wichita Falls has utilized a 1.5 ppm Total Chlorine residual as a trigger for over 20 years. The City has pointed out that with the differences between Monochloramine and Total Chlorine, requiring a 1.5 ppm Monochloramine trigger would effectively push the Total Chlorine trigger up to 2.0 ppm.
Therefore, the City of Wichita Falls respectfully requests that the TCEQ allow the City to continue to use 1.5 ppm as a Total Chlorine trigger, and if they desire a Monochloramine trigger, that it be set at 1.0 ppm.
As requested by the TCEQ, the City has attached documentation from several representative samples throughout its Distribution System for the TCEQ's consideration on this matter.
- It was noted by the City of Wichita Falls staff that the TCEQ documentation continually refers to the Full Scale Verification test as a 45-day test. However,

CITY OF WICHITA FALLS

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analysis of the Chlorine residual.

The City has since re-programmed and tested the SCADA system to automatically sound an audible alarm and shutdown the pumps at the River Road Wastewater Treatment Plant.

- The TCEQ noted that the two strap-on, Doppler Flow Meters (1 at River Road Wastewater Plant Discharge and 1 at the Cypress 2010 SWTP Discharge) would need to be validated by some means or method. After discussing the available options, the City said that it would get the units validated, somehow. The City received a backup, calibrated Doppler Flow Meter on 1/29/14. The City has used this backup meter to validate the accuracy of both on-line Doppler Flow Meters.
- The TCEQ requested that the shutdown trigger for violating the 50/50 blend ratio for Treated Effluent/Raw Lake Water be lowered from 15 minutes to 5 minutes. The City has made that change to the shutdown trigger.
- The TCEQ requested that the shutdown trigger for the on-line Nitrate monitor be lowered from the regulatory 10 ppm. Upon discussing what a recommended reduction should be, the TCEQ elected to go with the % error of the instrument. The City of Wichita Falls checked the O&M Manual for the on-line Nitrate monitor and found the % error to be 3%, so the City has lowered the shutdown trigger from 10 ppm to 9.5 ppm (5%).
- The TCEQ team found that the Temperature Correction Factor that the City of Wichita Falls was using was an older equation and requested that it be updated with a more current, approved calculation. The City of Wichita Falls has made that equation change.

And finally, there were a few items of clarification that the City would like to note for posterity.

- January 22, 2014 Letter, Enclosure A, Page 4 of 21: The TCEQ has altered the City's trigger for Human Pathogenic *Giardia* and *Cryptosporidium* and wants the City to trigger on the more generic *Giardia* and *Cryptosporidium*. The City included the designation of Human Pathogenic to its q-PCR trigger because PCR methods allow for the testing down to the species level, where the EPA Method 1623 only allows testing down to the genus level. This means that the EPA Method is not capable of determining whether or not the *Giardia* cysts or *Cryptosporidium* oocysts is infective of humans, just that it is *Giardia* or *Cryptosporidium*. However, using PCR, the City is only monitoring the 1 documented *Giardia* species (*G. lamblia*) and 1 documented *Cryptosporidium* species (*C. parvum*) that are capable of producing infection in humans. Testing for the remaining 4 morphologically distinct, non-human infective species of *Giardia* and the 12 non-human infective species of *Cryptosporidium*. The City discussed this with the TCEQ staff during the 9/13/13 meeting in Austin, Texas. At the time, the TCEQ staff agreed with the City that the PCR was only capable of species level analysis. During the meeting, the City informed the TCEQ

within the TCEQ correspondence, written clarification of the Full Scale Verification Protocol states that the Full Scale Verification test should be run for no less than a 30-day period (CIP period for the Microfiltration), after which a minimum of 10 additional days shall be run. This is consistent with the City of Wichita Falls notes from previous meetings with the TCEQ.

Therefore, rather than run for a length of time only stated in a Title, the City's interpretation of TCEQ guidance on the Full Scale Verification will allow us to test for 30 days, take a day to perform the CIP on the Microfiltration and Reverse Osmosis systems, and then run for an additional 10 days, thereby potentially making the Full Scale Verification test last for approximately 41 days. Using this methodology, with an initial start date of 1/27/14 for the Full Scale Verification test, the FSV will terminate on or near 3/8/14.

- The City of Wichita Falls would like to request the use of SM 20th Ed. 4500-NH₃ D. Ammonia-Selective Electrode Method for bench top ammonia testing in lieu of the TCEQ's recommendation in the September 5th letter, EPA 350.1 or SM 4500-NH₃ G. SM 4500-NH₃-D. is preferable due to the number of samples being testing since both test methods listed by the TCEQ require a manifold system and distillation.
- The City of Wichita Falls would like to request the use of the Hach APA 6000 Ammonia and Monochloramine Analyzer at the River Road Waste Water Treatment Plant for the detection of Monochloramine and the Wallace & Tiernan Micro 2000 analyzer for Total Chlorine. The TCEQ team requested that the City of Wichita Falls submit comparison data for the on-line analyzer to bench top testing. (Please see attachment Chlorine Titration vs. on-line).
- It was noted by TCEQ staff that the City was fully capable of running Dissolved Organic Carbon (DOC) with the current Total Organic Carbon (TOC) machine that it uses for regulatory reporting purposes. The TCEQ confirmed that the City was capable of the filtration process for DOC analysis.
Therefore, as per the TCEQ team, the City of Wichita Falls is requesting to perform the remaining DOC analysis for the Full Scale Verification test in-house, with DOC's going out every 7 days to confirm the City's in-house analysis.

Additionally, the TCEQ staff found a few items that they requested be changed or fixed as the DPR continued. The City has taken action to correct the following items, noted by the TCEQ:

- The TCEQ team found the 1961 Plant Weir Gate leaking a small amount of water at the Splitter Box.
The City of Wichita Falls staff has operated the Weir Gate and found the problem with the rubber seal. The rubber seal has been repaired and the leak stopped.
- The TCEQ team was concerned over a lack of "automatic" shutdown at the River Road Wastewater Treatment Plant. After discussing each trigger and determining that all but one of them were operator observation/analysis and thereby incapable of "automatic" shutdown, the TCEQ requested that the City pursue the one possible "automatic" shutdown. That automatic shutdown trigger would be tied to the on-line

of the human pathogenic species that would be utilized for their PCR analysis. Additionally, the City once again outlined the rationale for looking at human pathogenic species of Cryptosporidium and Giardia to the TCEQ team on 1/29/14, explaining the cost involved in analyzing for all species of Cryptosporidium and Giardia, thereby making it not feasible. The City believes that the TCEQ staff has agreed to this methodology on 2 separate occasions.

As requested by the TCEQ, the City has attached documentation on the PCR methodologies, to include Dr. Rebecca Guy's research paper, WHO Guidelines for Drinking Water Quality and correspondence with BioVir Laboratories.

- **Microfiltration Backwash Schedule:** The Siemens Microfiltration system was approved by the TCEQ for 3 minute backwash cycles every 25 minutes, based on Pilot data from 2000. However, the units have been in operation since 2008 and the City had noticed that the backwash cycle was leaving solids in/on the filters. Therefore, the City requested that Siemens evaluate the backwash system for effectiveness. Siemens was able to determine that a 5 minute backwash every 30 minutes was more effective than the previously programmed sequence of 3 minutes every 25 minutes. Siemens made this programming change in early 2013. The City of Wichita Falls spoke with Marlo Berg, via phone, on this issue and she stated that for the Full Scale Verification that the current backwash timing could be utilized and that a formal written request would need to be made when the Microfiltration system returned to normal operation with lake water. At this time, the City of Wichita Falls would like to request that it be allowed to change the backwash sequence/time to 5 minutes every 30 minutes, when it returns to normal operation with lake water.
- The City of Wichita Falls had requested a change in the Color Method from SM 2120 B (Visual Comparison Method) to SM 2120 C (Spectrophotometric Method). The City cited the subjectivity of 2120 B due to it being a human visual test (similar to the TCEQ disallowed Color Comparator for Chlorine). The City feels that the 2120 C method removes that subjectivity and will produce a more precise, repeatable result.
The TCEQ team agreed with the City's argument and is allowing SM 2120 C to be used during the Full Scale Verification test.
- It was noted during the site visit that there were no Methods specified in previous TCEQ correspondence for Total Culturable Virus, Hydrogen Sulfide and Perchlorate. The TCEQ team requested that the City make a "written statement" as to the Methods that would be employed for these analytes.
The City of Wichita Falls' contract laboratories will be using the following Methods:
 - Total Culturable Virus – EPA Method 1615
 - Hydrogen Sulfide – EPA 376.1 & SM 4500-S₂-F
 - Perchlorate – EPA 331.0
- The TCEQ staff has noted that the only "approved" edition of Standard Methods for us in drinking water is the 19th and 20th Editions. The City has been noting the most current Edition (22nd) in all of its Operations Manuals and Monitoring Plans. The City will make the appropriate changes to these documents to reflect the use of Standard Methods, 20th Edition (or 19th Edition where applicable).

- The TCEQ team had questions about the Inlet Pressure at location TCEQ 9J and the Outlet Pressure, Vacuum (psi) at location TCEQ 10J.

In the operation of the Microfiltration, the SCADA system receives the cell levels (ft) and the TMP for the cells. We are using the cell level to calculate the inlet or head pressure at the bottom of the membranes for location TCEQ 9J, and have already input this equation in the City's spreadsheets.

However, we do not get a vacuum pressure.

The Siemens PLC calculates the TMP from the feed and vacuum pressures every 30 seconds and supplies that TMP (PSI) to the SCADA, but we do not have the access to the values used in the Siemens logic.

Therefore, the City can only supply the Cell Level, Calculated Feed PSI based on that level, the Cell Flow and the Cell TMP, but no Outlet Pressure, Vacuum.

If you require any additional information on this matter, or if I can be of any further assistance, please do not hesitate to contact me at (940) 691-1153.

Sincerely,



Daniel K. Nix
Public Utilities Operations Manager
City of Wichita Falls

Cc: Russell Schreiber, P.E.; Director of Public Works
Mark Southard, Water Source/Purification Superintendent
Kerry Marony, P.E., Biggs & Matthews, Inc.
TCEQ Correspondence File

City of Wichita Falls Total Chlorine to MonoChloramine Comparison

Date	Address	Total Chlorine	MonoChloramine	Difference	Free Chlorine	Free Ammonia
1/30/2014	1300 7th	2.70	2.46	0.24	0.00	0.21
1/30/2014	1008 Southwest Pkwy	3.10	2.69	0.41	0.00	0.23
1/30/2014	1601 Southwest Pkwy	2.80	2.40	0.40	0.00	0.21
1/31/2014	4711 Southwest Pkwy	2.90	2.60	0.30	0.00	0.16
2/3/2014	811 MLK Blvd	2.60	2.16	0.44	0.00	0.25
2/3/2014	6200 Kemp	2.90	2.48	0.42	0.00	0.16
2/3/2014	4313 Seymour Hwy	2.70	2.46	0.24	0.00	0.28
2/3/2014	100 Central Freeway	2.90	2.56	0.34	0.00	0.25
2/3/2014	2314 Airport Dr	2.70	2.27	0.43	0.00	0.21
2/4/2014	2304 Kemp	2.27	1.80	0.47	0.00	0.25
2/4/2014	2300 Harrison	2.20	1.71	0.49	0.00	0.31
2/4/2014	4309 Old Jacksboro Hwy	1.60	1.20	0.40	0.00	0.59
2/4/2014	501 E. Midwestern Pkwy	2.00	1.83	0.17	0.00	0.30
2/4/2014	4516 Barnett	1.50	1.37	0.13	0.00	0.41
2/4/2014	2101 FM 369 N.	2.33	2.00	0.33	0.00	0.39
			Max	0.49		
			Min	0.13		
			Avg	0.35		

City of Wichita Falls Wastewater

Chlorine Analyzer Comparability Results

Date	Time	MONO			TOTAL		
		Titration	On-line	% +/-	Titration	On-line	% +/-
1/19/14	Midnight	3.10	2.91	-6.13%	3.85	3.76	-2.34%
1/19/14	8:00 AM	2.80	2.77	-1.07%	3.85	3.76	-2.34%
1/19/14	4:00 PM	2.80	2.98	6.43%	3.64	3.77	3.57%
1/19/14	8:00 PM	2.70	2.81	4.07%	3.60	3.76	4.44%
1/20/14	Midnight	2.70	2.63	-2.59%	3.80	3.75	-1.32%
1/20/14	8:00 AM	2.72	2.68	-1.47%	3.78	3.75	-0.79%
1/20/14	4:00 PM	3.20	3.28	2.50%	3.92	4.17	6.38%
1/21/14	Midnight	3.20	3.23	0.94%	4.10	4.15	1.22%
1/21/14	NOON	2.65	3.09	16.60%	3.54	4.18	18.08%
1/22/14	Midnight	2.70	2.94	8.89%	3.65	4.15	13.70%
1/22/14	8:00 AM	2.90	2.70	-6.90%	4.15	4.16	0.24%
1/22/14	4:00 PM	2.48	2.95	18.95%	3.30	4.16	26.06%
1/23/14	Midnight	3.10	3.07	-0.97%	3.95	4.18	5.82%
1/23/14	8:00 AM	3.10	3.10	0.00%	4.24	4.14	-2.36%
1/23/14	4:00 PM	3.31	3.20	-3.32%	4.20	4.14	-1.43%
1/24/14	Midnight	3.30	3.07	-6.97%	4.10	4.14	0.98%
1/24/14	8:00 AM	3.08	2.96	-3.90%	4.36	4.15	-4.82%
1/24/14	4:00 PM	3.15	3.19	1.27%	4.08	4.16	1.96%
1/25/14	Midnight	3.12	3.18	1.92%	4.09	4.15	1.47%
1/25/14	8:00 AM	3.01	3.11	3.32%	4.04	4.15	2.72%
1/25/14	4:00 PM	3.38	3.31	-2.07%	4.25	4.17	-1.88%
1/26/14	Midnight	3.30	3.20	-3.03%	4.25	4.16	-2.12%
1/26/14	8:00 AM	3.24	3.16	-2.47%	4.16	4.15	-0.24%
1/26/14	4:00 PM	3.40	3.33	-2.06%	4.15	4.19	0.96%
1/26/14	8:00 PM	2.80	3.15	12.50%	3.60	4.16	15.56%
		MIN		-6.97%	MIN		-4.82%
		MAX		18.95%	MAX		26.06%
		AVG		1.38%	AVG		3.34%

Completed Work Order Detail Report
Electronics

1/29/2014

Page 1 of 1

WO #	17569	Type	UnSch	Priority	5	Created	1/29/2014
Create Reason	Other	Created By	fhill	Printed		Scheduled	1/29/2014
Equipment	A-790 Titrator	Amperometric Titrator, W&T		Delinquent		Completed	1/29/2014
Location	05	Chlorinator/Hydro Room		Shutdown		Startup	
Task	WTP-CAL	WTP Instrument Calibration		Downtime Hours			

Instructions

Verify accuracy of amperometric titrator using a lab prepared known standard solution for total chlorine residual.

Comments Poured a 200 ml sample of the prepared solution into the titrator cup. Added 1 ml of potassium iodide and 1 ml of pH4 acetate buffer. Placed on titrator and slowly added phenylarseline oxide from the pipette. I measured the total residual to be 1.78 mg/L. Superintendent Harold Burris repeated the procedure and measured 1.75 mg/L. Laboratory standard mean value was 1.79 mg/L; a difference of .56% on the first titration and 2.23% on the second titration. The acceptable range was 1.70 - 1.88 mg/L.

Meter And Gauge Readings

(None)

Work Order Part

(None)

Work Order Labor

Labor Class	Description	Labor Account No	Employee	Est. Hours	Actual Hours	Cost
ELEC 2	ELECTRONIC TECH II	(Unassigned)	FHILL	0	0.5	\$0.00
Labor Total						\$0.00

Work Order Total

\$0.00

Report Totals	Downtime Hours	0.00	Part Cost	\$0.00
			Labor Cost	\$0.00
			Vendor Cost	\$0.00
			Total Cost	\$0.00

Real-Time PCR for Quantification of *Giardia* and *Cryptosporidium* in Environmental Water Samples and Sewage

Rebecca A. Guy,^{1,*} Pierre Payment,² Ulrich J. Krull,¹ and Paul A. Horgen¹

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The protozoan pathogens *Giardia lamblia* and *Cryptosporidium parvum* are major causes of waterborne enteric disease throughout the world. Improved detection methods that are very sensitive and rapid are urgently needed. This is especially the case for analysis of environmental water samples in which the densities of *Giardia* and *Cryptosporidium* are very low. Primers and TaqMan probes based on the β -giardin gene of *G. lamblia* and the COWP gene of *C. parvum* were developed and used to detect DNA concentrations over a range of 7 orders of magnitude. It was possible to detect DNA to the equivalent of a single cyst of *G. lamblia* and one oocyst of *C. parvum*. A multiplex real-time PCR (qPCR) assay for simultaneous detection of *G. lamblia* and *C. parvum* resulted in comparable levels of detection. Comparison of DNA extraction methodologies to maximize DNA yield from cysts and oocysts determined that a combination of freeze-thaw, sonication, and purification using the DNeasy kit (Qiagen) provided a highly efficient method. Sampling of four environmental water bodies revealed variation in qPCR inhibitors in 2-liter concentrates. A methodology for dealing with qPCR inhibitors that involved the use of Chelex 100 and PVP 360 was developed. It was possible to detect and quantify *G. lamblia* in sewage using qPCR when applying the procedure for extraction of DNA from 1-liter sewage samples. Numbers obtained from the qPCR assay were comparable to those obtained with immunofluorescence microscopy. The qPCR analysis revealed both assemblage A and assemblage B genotypes of *G. lamblia* in the sewage. No *Cryptosporidium* was detected in these samples by either method.

Giardia and *Cryptosporidium* are protozoan parasites that cause widespread gastrointestinal illness. Ninety percent of reported outbreaks of these pathogenic protozoans occur through water, while 10% are related to food (29). Direct transmission by person-to-person contact also results in illness and is problematic for children in day care centers. *Giardia* is the most commonly diagnosed gastrointestinal protozoan in the world and is more frequently associated with outbreaks than *Cryptosporidium*. However, greater numbers of individuals are affected during outbreaks of *Cryptosporidium*. The Milwaukee outbreak in 1993 affected several thousand individuals served by a water treatment plant deficient for several days (15). The incidence of food-borne outbreaks due to protozoan pathogens is likely underestimated due to the difficulty of detecting low numbers of organisms, as enrichment techniques cannot be used (29). Detection of *Giardia* and *Cryptosporidium* on domestic fresh vegetables and fruits in Norway (27), a wealthy and modern country, has important implications for food safety in North America.

Waterborne outbreaks are associated with drinking water, wells, rivers, lakes, and recreational swimming pools. The reported frequencies of occurrences of contamination of surface water with *Giardia* and *Cryptosporidium* are from 60 to 96% in the United States (12, 22) and from 20 to 64% in Canada (11, 36). Their levels in surface waters are very low, ranging from 0.5 to 5,000 organisms in 100 liters of water (11, 21, 22, 23, 36). It has been proposed that action levels to prevent outbreaks

with these protozoa should occur when concentrations in 100 liters of water sampled are >5 *Giardia* cysts (37) and 10 to 30 oocysts of *Cryptosporidium* (6). Detection of such low numbers of organisms is difficult and requires very sensitive techniques.

Real-time PCR (qPCR) is a technique that provides great sensitivity in detection plus quantitative results (35). Detection of the amplified product using a fluorescent probe during PCR cycling eliminates post-PCR processing and produces results within 30 to 90 min, depending on the type of real-time instrumentation that is employed.

Both *Giardia* and *Cryptosporidium* have the potential for zoonotic transmission. Genetic variability exists within species that are infective to mammals (33, 38). Major genotypes have been identified in both *Giardia lamblia* and *Cryptosporidium parvum*. *G. lamblia* isolates in humans and other mammals fall into two major assemblages, A and B. Similarly, *C. parvum* isolates can be segregated into genotype 1 (recently named *Cryptosporidium hominis* 20) and genotype 2 (*C. parvum*). Molecular methods such as qPCR allow not only sensitive detection of pathogens but also the determination of genetic variability within isolates. A difference of 1 nucleotide is distinguishable using the appropriate probe chemistry, such as a molecular beacon (19), thereby providing invaluable information as to the sources of outbreaks and individual cases, permitting rapid action to be taken.

The objective of this study was to develop qPCR primers and TaqMan probes for detection of *Giardia* cysts and *Cryptosporidium* oocysts in environmental samples. We focused on methods of extraction of DNA from cysts and oocysts and the development of methods for overcoming PCR-inhibitory substances in water samples and sewage that would be detrimental to qPCR. The detection of *Cryptosporidium* using qPCR has

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TABLE 1. Primer-probe sets for detection of *Giardia* and *Cryptosporidium*

Primer-probe set	Type ^a	Sequence, 5'-3'	Nucleotide position ^a
β-Giardin P241	F	CATCCGCGAGGAGGTCAA	222-239
	R	GCAGCCATGGTGTCCGATCT	278-296
β-Giardin P434 (P1) ^b	P	FAM/AAATCCGCGGACAAACATGTACCTAACGA/BHQ-1	241-268
	F	CCTCAAGAGCCCTGAACGATCTC	411-432
	R	AGCTGGTCGTACATCTTCTTCTCT	462-485
	P	FAM/TTCTCCGTGGCAATGCCCGTCT/BHQ-1 ^d	434-455
β-Giardin P434 (H3) ^c	F	CCTCAAGAGCCCTGAACGACCTC	411-432
	R	AGCTGGTCATACATCTTCTTCTCTC	462-485
	P	Cy5/TTCTCCGTGGCGATGCCCTGTCT/BHQ-2 ^d	434-455
	F	CAAATTGATACCGTTTGTCTTCTG	583-607
COWP P702	R	GGCATGTCGATTCTAATTCAAGCT	711-733
	P	HEX/TGCCATACATTGTTGTCTTGACAAATTGAAT/BHQ-1 ^d	672-702

^a F, forward; R, reverse; P, TaqMan probe.^b P1, Portland 1 sequence of *G. lamblia* β-giardin gene (assemblage A) (GenBank no. M36728).^c H3, H3 sequence of *G. lamblia* β-giardin gene (assemblage B) (GenBank no. AY258616).^d Reverse complementary sequence of nucleotides.^e Relative nucleotide position in the coding sequence of the respective gene.

been reported (5, 8, 13, 14, 32); however, the primer-probe sequences are distinct from the ones described here. To date, there have been no reports of the use of qPCR for detection of *Giardia*. In addition, this is the first description of a multiplex qPCR assay for simultaneous detection of *Giardia* and *Cryptosporidium*.

MATERIALS AND METHODS

Parasites. Live *G. lamblia* cysts, produced by passage of the human strain H3 of *G. lamblia* through Mongolian gerbils, were obtained from Waterborne Inc. (New Orleans, La.). The cysts were in phosphate-buffered saline containing antibiotics and were stored at 4°C until they were used. The WB strain was a kind gift from Mike Belosevic, University of Alberta. The Robertis-Thompson strain of *Giardia muris*, produced by passage in *nutu* mice, was obtained from Waterborne Inc.

Live *C. parvum* oocysts (IOWA strain) produced by passage in calves were obtained from Waterborne Inc. Oocysts in phosphate-buffered saline containing antibiotics were stored at 4°C and used within 3 months. The GCH1 isolate was obtained through the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergies and Infectious Diseases, NIH; *C. parvum* strain GCH1 oocysts were from Saul Tzipori.

DNA extraction. DNA was extracted from cysts using the DNeasy tissue kit (Qiagen, Hilden, Germany). A modification of the animal tissue protocol was employed, adding the following sequence of steps: (i) incubation in ATL (tissue lysis buffer; Qiagen) and proteinase K for 1 h at 55°C; (ii) three freeze-thaw cycles, each cycle consisting of 2 min in liquid nitrogen followed by boiling water; and (iii) three 20-s bursts of sonication (model W-220P cell disruptor; Ultrasonics Inc.). The manufacturer's protocol was followed for purification of DNA through the column, and the DNA was eluted from the columns in sterile double-distilled water (ddH₂O).

An alternative method to the use of the sonicator probe was the use of a cup horn (Sonics and Materials, Inc., Newton, Conn.). Samples in snap cap microcentrifuge tubes were placed in a floating rack and sonicated in a cup horn for 30 min at an output of 185 W and 87% amplitude. A slow flow of water was passed through the cup, maintaining the outflow temperature between 30 and 40°C.

DNA was quantified using the PicoGreen double-stranded-DNA quantitation reagent (Molecular Probes, Eugene, Ore.). Samples were diluted in Tris-EDTA to a volume of 25 µl and added to 25 µl of PicoGreen Tris-EDTA solution at a 1:200 dilution. Lambda DNA (GibcoBRL, Rockville, Md.) was used to generate a standard curve. Samples were run in triplicate wells, and fluorescence was determined using the 6-carboxyfluorescein (FAM) filter set on an Mx4000 multiplex quantitative PCR system (Stratagene, La Jolla, Calif.). Regression analysis to determine concentrations of unknowns was performed using Microsoft Excel.

Oligonucleotides. Primer-probe sets for detection of *Giardia* were designed against the coding region of the β-giardin gene (GenBank no. M36728) of the Portland 1 strain of *G. lamblia* (9). Two distinct primer-probe sets that produce

74-bp amplicons in qPCR were designed. The first primer set, P241, was based on region 222 to 296 of β-giardin, and the second set, P434, was based on region 411 to 485 (Table 1). Another primer-probe set, also in region 411 to 485 of β-giardin (Table 1), was designed based on the sequence of the H3 isolate of *G. lamblia* (GenBank no. AY258616).

The gene for the *Cryptosporidium* oocyst wall protein (COWP) (GenBank no. AF248743) (25) was selected as the gene for designing the primer-probe set for detection of *C. parvum*. A 151-bp region from 583 to 733 of the coding sequence of the precursor COWP gene was targeted (Table 1). In designing the sequences, 26 partial sequences coding for the oocyst wall protein, from different isolates and species of *Cryptosporidium*, were examined to identify regions of the gene specific to *C. parvum* and to the specific genotypes 1 and 2 of *C. parvum*. These sequences were entered into the ClustalW program (European Bioinformatics Institute), and a multiple alignment was performed to identify unique regions of the gene. The sequences and their GenBank accession numbers are as follows: *C. parvum* CBAH1 (AJ310765), *Cryptosporidium baley* (AF266276), *Cryptosporidium* sp. strain 715-dog (AF266274), *Cryptosporidium felis* (AF266263), *Cryptosporidium* sp. strain 815-bullsnake (AF266277), *Cryptosporidium meleagridis* (AF248742), *C. meleagridis* (AF266266), *Cryptosporidium wairi* (AF266271), *C. wairi* (U35027), *C. parvum* G2 (AF248743), *C. parvum* CPACH-1 (AJ310766), *Cryptosporidium* sp. strain 6-bovine (AF266273), *C. parvum* G2 (AF161577), *Cryptosporidium* sp. strain 411-mouse (AF266268), *Cryptosporidium* sp. strain 518-monkey (AF266272), *C. parvum* G1 (AF248741), *C. parvum* 181 (AF266265), *C. parvum* G1 (AF161578), *Cryptosporidium* sp. strain 351-ferret (AF266267), *Cryptosporidium* sp. strain 428-kangaroo (AF266269), *Cryptosporidium* sp. strain 490-pig (AF266270), *Cryptosporidium serpenti* (AF266275), *C. serpenti* (AF161580), *Cryptosporidium anderseni* (AF266262), *Cryptosporidium muris* (AF266264), and *C. muris* (AF161579).

All primers and TaqMan probes were designed using Primer Express versions 1.0 and 1.5 (Applied Biosystems) and were tested for specificity using an nBLAST homology search. The primers were synthesized by Sigma Genosys (Oakville, Ontario, Canada). Probes were synthesized by either Bioscience Technologies Inc. (Novato, Calif.) or Integrated DNA Technologies Inc. (Coralville, Iowa). The β-giardin probes P241 and P434 (Portland 1 sequence) were 5' labeled with FAM (emission wavelength [λ_{em}] = 518 nm), the COWP probe P702 was 5'-hexachlorofluorescein (HEX; λ_{em} = 553 nm) labeled, and the β-giardin probe P434 for assemblage B (H3 sequence) was labeled with 1-(ε-silicon-carboxypentyl)-1'-ethyl-3,3',3'-isotrimethylindodicarboxyamine-5 (Cy5; λ_{em} = 667 nm). All probes were 3' labeled with a nonfluorescent Black Hole Quencher (BHQ) dye: BHQ-1 for FAM and HEX and BHQ-2 for Cy5.

qPCR. PCR was carried out using the Brilliant Quantitative PCR core reagent kit buffers (Stratagene). Each 25-µl PCR used final concentrations of 4 mM magnesium chloride, 20 mM deoxynucleoside triphosphate, 300 or 600 nM each primer, 200 nM probe, a 1:50,000 dilution of the reference dye (carboxy-rhodamine [ROX]), and 2.5 U of SureStart Taq DNA polymerase. A volume of up to 5 µl of DNA was added. The PCRs were performed in an Mx4000 multiplex quantitative PCR system (Stratagene). The cycling conditions consisted of 10 min of incubation at 95°C followed by 40 cycles of alternating temperatures of 95°C for 15 s and 60°C for 1 min. Fluorescence data (three data

points) were collected at the end of each cycle, and each sample was run in triplicate wells. A no-template control was included in every assay, and no cycle threshold (Ct) values were consistently obtained after 40 cycles of PCR. Bovine serum albumin (BSA) (fraction V; Sigma, St. Louis, Mo.) was added to the PCR mix to a final concentration of 20 ng/ μ l for evaluations of environmental samples. The PCR volume was increased to 50 μ l for testing sewage extracts.

Sequencing. The amplicons obtained from the *Giardia* and *Cryptosporidium* multiplex qPCR assay using the β -giardin P241 primer-probe set and the COWP P702 primer-probe set were separated on a 2% agarose gel. The two bands (74 and 151 bp) were excised from the gel and purified using the Mini Elute gel extraction kit (Qiagen). The purified amplicons were sequenced at the Core Facility, York University (Toronto, Ontario, Canada) by use of the dideoxy method employing an ABI Prism 377 sequencer.

Environmental water sampling, filtration, and DNA extraction. Water samples were obtained from Etindale Pond and the Credit River, Mississauga, Ontario, Canada, and from Heart and Professor's Lakes, Brampton, Ontario, Canada, from May to September 2002. The samples were collected in 10-liter plastic carboys (Cole Palmer, Chicago, Ill.) and stored at 4°C until they were used (the same day). Samples (2 liters) were filtered through 3- μ m-pore-size, 47-mm-diameter cellulose nitrate filters (Sartorius, Goettingen, Germany) in a parabolic stainless steel funnel (Gelman, Ann Arbor, Mich.) using a vacuum of 250 to 375 mm of Hg generated by a Millipore vacuum/pressure pump (115 V; 60 Hz). Upon filtration of the sample, the funnel was rinsed with ddH₂O.

After filtration, the filter was removed, folded twice lengthwise with the upper surface facing out, and placed into a microfuge tube. DNA was extracted directly from the filter using the DNeasy kit. Following incubation in 180 μ l of ATL and 20 μ l of proteinase K for 1 h at 55°C, the filter was washed with 200 μ l of ATL and the wash was pooled with the initial cell lysate. The samples were then processed according to the manufacturer's instructions. DNA was eluted from the silica column using either one or two rounds of 50 μ l of ddH₂O.

PCR inhibitor removal. Following filtration of 2 liters of water through the 3- μ m-pore-size cellulose nitrate filter, the filter was treated with 20 ml of 0.5 M EDTA, pH 8.0, for 5 min and then washed with ddH₂O. After the filter was washed with 2 volumes of ATL, Chelex 100 (Bio-Rad, Hercules, Calif.) was added to the cell lysate to a final concentration of 20%. Polyvinylpyrrolidone (PVP 360) (ICN, Aurora, Ohio) at a concentration of 8% was also added to equal a final concentration of 2%. The 8% stock solution of PVP 360 was made in ATL lysis buffer (Qiagen), incubated at 55°C for 10 min, and pipetted gently to dissolve the PVP 360. The samples were incubated for 30 min at 55°C, subjected to freeze-thaw, and centrifuged at 12,000 $\times$ g for 10 min. The supernatant was processed on a DNeasy column following the procedure recommended by the manufacturer, and DNA was eluted from the column using 50 μ l of ddH₂O.

Collection and processing of sewage. *Giardia* and *Cryptosporidium* were concentrated from raw sewage obtained at the Autcuil and Fabreville wastewater treatment plants in Laval (Québec, Canada). One-liter samples of wastewater were processed by centrifugation for 30 min at 3,000 $\times$ g in 1-liter bottles in a Beckman J6 centrifuge equipped with a JS4.2 rotor. The pellets were resuspended in phosphate-buffered saline and pooled to a single volume (5.5 to 9.9 ml). Approximately 1 ml of the concentrate was used for detection of these pathogens using immunofluorescence (IF) microscopy. The remainder of the sample was sent immediately by courier to the Toronto laboratory for DNA extraction and qPCR detection of *Giardia* and *Cryptosporidium*.

DNA extraction from sewage. Following concentration of the sewage by centrifugation, each sample (5 to 9 ml) was split among microfuge tubes (1.5 ml was added to each of four to six tubes). The samples were centrifuged at 12,000 $\times$ g for 15 min, and the DNA was extracted from the pellets (~0.5 ml of packed pellets per entire sample). The tubes were tapped to dislodge the pellets, and 360 μ l of ATL was added and thoroughly mixed by vortexing. The samples were subjected to three cycles of freezing-thawing and cooled, and 40 μ l of proteinase K was added per tube. The tubes were placed in a cup horn sonicator for 30 min using an output of 185 W, following which the samples were incubated for 1 h at 55°C. The tubes were centrifuged for 1 min at 12,000 $\times$ g to remove debris, and the supernatant was treated with Chelex 100 and PVP 360 to final concentrations of 20 and 2%, respectively, and incubated for 30 min at 55°C. The tubes were centrifuged for 10 min at 12,000 $\times$ g, the pellets were discarded, and the supernatants from all tubes for each sample were applied equally to two DNeasy columns (Qiagen) to separate and collect the DNA. The eluted material (50 μ l [twice] of ddH₂O/column) was pooled to obtain a final volume of 200 μ l. The purification procedure required 3 to 4 h to perform but was necessary to ensure the removal of qPCR-inhibitory substances.

IF microscopy of sewage samples. Approximately 1 ml of concentrated sewage was purified by Percoll-sucrose flotation (density, 1.10 g/ml). The entire 1-ml

volume of the resuspended pellet was centrifuged on the gradient at 1,000 $\times$ g for 20 min. The upper layer and half of the lower layer were collected and centrifuged at 1,000 $\times$ g for 20 min and resuspended in 1 ml of water. Fifty microliters (per slide) was placed on microscope slides and dried. Cysts and oocysts were stained with anti-*G. lamblia* and anti-*C. parvum* monoclonal antibodies (Aqua-Glo G/C; Waterborne Inc.) and were counted by epifluorescence microscopy at $\times$ 400 using an Olympus B-50 research microscope. The whole surface of the slide was examined, and all characteristic structures that exhibited bright-green cyst or oocyst wall staining were counted.

RESULTS AND DISCUSSION

Simplex qPCR. The β -giardin P241 and COWP P702 primer-probe sets provided sensitive detection of DNAs extracted from *G. lamblia* cysts and *C. parvum* oocysts, respectively. DNA was detected across a range of dilutions of 7 orders of magnitude (Fig. 1A and B). Standard curves were generated using 10-fold serial dilutions of DNA purified from cysts and oocysts, with correlation coefficients ranging from 0.984 to 0.993 and slopes of -3.331 to -3.908 (Fig. 1). Both the β -giardin and COWP genes are expressed as single-copy genes within the nucleus of each trophozoite and sporozoite, respectively (10, 31). Cysts of *Giardia* contain two trophozoites that have undergone multiple steps of nuclear division, resulting in 16 copies of total genetic information within each cyst (1). Each *Cryptosporidium* oocyst contains four nucleated sporozoites. Therefore, there are 16 copies of the β -giardin gene per *Giardia* cyst and 4 copies of the COWP gene per oocyst. A theoretical estimate of the DNA content of one cyst is 195 fg, and that of one oocyst is 40 fg. Results from the qPCR assay demonstrated considerable sensitivity, as seen from detection of as little as 10 fg of DNA for *G. lamblia* and 40 fg of *C. parvum* DNA, corresponding to one copy of the β -giardin gene and four copies of the COWP gene.

Our selection of targets for developing primers and probes for qPCR assays was based on molecules that are unique to these organisms and whose biology is important. The β -giardin gene of *Giardia* codes for a structural protein that is a component of the adhesive disk of the parasite, important in binding of trophozoites to the intestinal epithelium of their host. PCR primers based on the β -giardin gene were first described by Mahbubani and colleagues (16) and have been used for detection of *Giardia* in environmental water samples (17).

The COWP gene was selected because it codes for a protein that is important in maintaining the integrity of the oocyst wall, allowing the parasite to withstand harsh environmental factors until it is ingested by a new host. Based on the multiple alignments of COWP sequences from different *C. parvum* isolates that are available in GenBank, we designed primer-probe set P702. This primer-probe has the potential to detect *Cryptosporidium* that is infective to humans, including *C. hominis*, *C. parvum*, *C. wrairi*, and *C. meleagridis*. The COWP gene has been used in endpoint and restriction fragment length polymorphism analyses for genotyping *Cryptosporidium* (40).

Multiplex qPCR. A multiplex qPCR assay using the β -giardin P241 (FAM-labeled) and COWP P702 (HEX-labeled) primers-probes detected *G. lamblia* and *C. parvum* (Fig. 1C) with sensitivities equivalent to that of a simplex assay (Fig. 1A and B). The amplicons generated by multiplex PCR were sequenced and proved to be identical to amplicons generated in the simplex PCR. It was observed that the addition of BSA or

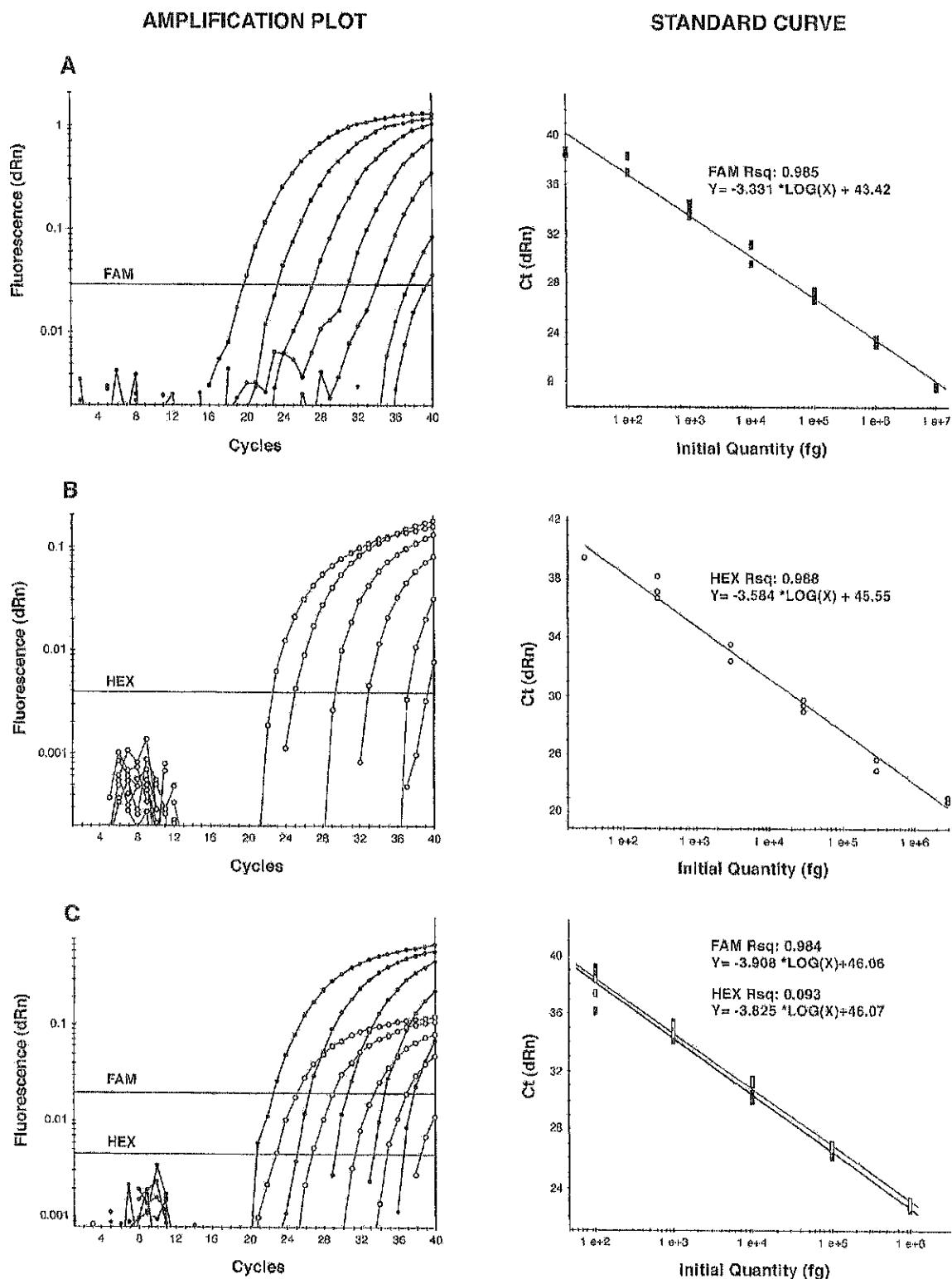


FIG. 1. Simplex and multiplex detection of *G. lamblia* (solid symbols) and *C. parvum* (open symbols) using qPCR. The standard curves (right) correspond to the amplification plots (logarithmic view) on the left. (A) Tenfold serial dilutions of *G. lamblia* cyst DNA ranging from 10 ng to 1 fg and detection using β -giardin P241. (B) Tenfold serial dilutions of *C. parvum* oocyst DNA ranging from 5.7 ng to 5.7 fg and detection using the COWP primer-probe set P702. (C) Tenfold serial dilutions ranging from 1 ng to 100 fg of DNA from *G. lamblia* cysts and *C. parvum* oocysts and simultaneous detection using the β -giardin primer-probe set P241 and the COWP P702 set. The β -giardin probe was FAM labeled, and the COWP probe was HEX labeled. Horizontal lines show thresholds. dRn, baseline-corrected normalized fluorescence; RSq, R^2 (indication of the fit of the standard curve to the standard data points plotted).

TABLE 2. Specificities of β -giardin and COWP probes for detection of *Giardia* and *Cryptosporidium* in qPCR

DNA source	Detection ^a		
	<i>G. lamblia</i>		<i>C. parvum</i>
	P241 ^b	P434 ^b	P702
<i>G. lamblia</i> H3	+	—	—
<i>G. lamblia</i> WB	+	+	—
<i>G. muris</i> (Roberts-Thompson)	+	—	—
<i>C. parvum</i> GCH1	—	—	+
<i>C. parvum</i> IOWA	—	—	+
<i>E. coli</i> (ATCC 8739)	—	—	—
<i>E. coli</i> O157:H7 (ATCC 35150)	—	—	—
<i>Ophiostoma novo-ulmi</i> (VA30)	—	—	—
<i>Microcystis aeruginosa</i> (UTCC 300)	—	—	—

^a +, detected; —, not detected.^b Portland 1 sequence (GenBank no. M36728).

BLOTTO to the PCR assay mixture reduced the efficiency of the amplification of the COWP gene in the multiplex qPCR from 100 to 70%. The efficiency of amplification of the β -giardin gene was not altered.

Primer-probe specificity in qPCR. The specificities of the primer-probe sets for *Giardia* and *C. parvum* were examined by performing qPCR assays with a panel of DNAs from related and unrelated organisms. All primer-probe sets were evaluated in qPCR with 500 pg of template DNA from the panel of organisms. The primers-probes were specific to the targets they were designed for and did not detect unrelated organisms (Table 2). The P241 primer-probe set detected *G. lamblia* and *G. muris*, the murine species of *Giardia*, but not *C. parvum*. The COWP P702 primer-probe set recognized the two *C. parvum* genotype 2 isolates GCH1 and IOWA but not *G. lamblia*. None of the primer-probe sets detected the waterborne bacteria *Escherichia coli*, *E. coli* O157:H7, and *Microcystis aeruginosa* or the fungus *Ophiostoma novo-ulmi*.

There is 3 to 5% variability in the sequence of the β -giardin gene among *G. lamblia* isolates (3; R. A. Guy, C. Xiao, and P. A. Horgen, unpublished data). Mismatches in the nucleotide sequences of the primers-probes were evident when the detections of different strains of *G. lamblia* with the probes were compared. The P241 primer-probe set detected the WB and H3 strains, corresponding to assemblages A and B of *G. lamblia*. However, the efficiency of amplification was lower when the mismatch was present compared to detection of a strain using the fully matched primer-probe set. *G. muris* was also detected using probe P241. Primer-probe set P434, designed against the sequence of the Portland 1 strain of *G. lamblia*, detected the WB isolate (GenBank no. AY258617; homologous to Portland 1 in the β -giardin gene region we examined) but did not detect the H3 strain of *G. lamblia* or *G. muris* (Table 2). In all cases, the matched primer-probe sets detected equivalent concentrations of the matched DNAs, demonstrating that all primer-probe sets were functional. The P434 primer-probe set was used to distinguish between assemblage A and assemblage B isolates of *G. lamblia*.

DNA extraction efficiency. Maximum sensitivity for detection of pathogens in environmental samples requires not only excellent performance from primer-probe sets but also the ability to extract the entire DNA from the cysts or oocysts in a

TABLE 3. Concentrations of DNA extracted from cysts and oocysts following modifications to the DNeasy DNA extraction method

DNA extraction modification ^a	Concn of DNA (ng/1.5 $\times$ 10 ⁵ cysts or oocysts) ^b	
	<i>G. lamblia</i>	<i>C. parvum</i>
	cysts	oocysts
1 h at 55°C + F/T	5.89 $\pm$ 1.76	6.21 $\pm$ 0.83
1 h at 55°C + F/T + sonication (probe)	24.28 $\pm$ 2.89	10.22 $\pm$ 1.52
1 h at 55°C + F/T + sonication (cup horn)	24.62 $\pm$ 3.42	13.59 $\pm$ 0.64
F/T + sonication (cup horn)	29.48 $\pm$ 2.52	16.60 $\pm$ 1.14

^a F/T, three cycles of freeze-thaw.^b Data are presented as the mean $\pm$ standard deviation of triplicate extractions per treatment group.

sample. Therefore, methods of DNA extraction from *Giardia* cysts and *Cryptosporidium* oocysts were evaluated and compared. The DNeasy system was selected as a preferred method because it is rapid and provides an inexpensive means of cleaning up the DNA from contaminants. A comparison was made of modifications of the DNeasy method, including freeze-thaw and sonication, to obtain the largest quantity and best quality of DNA for amplification and detection in qPCR. The PicoGreen method of dsDNA quantification (Molecular Probes) was used to minimize the numbers of cysts and oocysts that were required for these comparisons. It was determined that 1.5×10^5 cysts or oocysts was a sufficient number of cells for accurate quantification of extracted DNA.

A good yield of DNA that was suitable for qPCR was obtained using the tissue protocol of the DNeasy kit, with 1 h of incubation in ATL lysis buffer (including proteinase K) followed by separation on the silica gel column. Importantly, the addition of a three-cycle freeze-thaw step and sonication (three 20-s bursts) increased the yield two- to fivefold (Table 3). DNA concentrations (determined by PicoGreen quantification) of the freeze-thaw- and sonication-treated cysts and oocysts approached maximal theoretical estimates of the concentration that was available from 1.5×10^5 cysts or oocysts. The percent efficiencies of extraction of DNA from *G. lamblia* cysts were 20.14% $\pm$ 6.01% without sonication and 83.02% $\pm$ 9.86% using the sonication probe. We adopted this modified DNeasy method for preparing DNA for our standard curves and for processing environmental samples.

We also evaluated the efficacy of DNA extraction using a closed sonication system, the cup horn sonicator (Sonics and Materials Inc.). Direct lysis of cysts and oocysts in the cup horn for 30 min was as effective in releasing DNA (108.18% $\pm$ 11.49% efficiency) as was preincubation of the cells in lysis buffer for 1 h at 55°C prior to sonication (84.18% $\pm$ 11.69% efficiency) (Table 3).

qPCR-inhibitory effects of concentrated environmental water samples. Complex matrices, such as surface water and sewage, contain numerous organic and inorganic substances with the potential to inhibit PCRs (30, 39). A study was conducted to determine the ability to use qPCR on DNA extracted from 2-liter water samples obtained from different sources. To detect the presence of inhibitors, the PCR mixtures containing the environmental water sample extracts were spiked with 500

TABLE 4. Effects of BSA on qPCR detection of spiked *Giardia* DNA in environmental water extracts

Water sample ^a	Ct ^b	
	No BSA	BSA
ddH ₂ O	21.11 ± 0.44	21.17 ± 0.19
Erindale Pond		
1	None	20.77 ± 0.21
2	None	20.67 ± 0.53
Credit River		
1	None	21.08 ± 0.29
2	None	21.46 ± 0.17

^a 1 µl from 100 µl of each sample extract was assayed.^b Mean ± standard deviation of triplicate wells.

pg of *G. lamblia* DNA, and DNA was detected using P241 in the qPCR assay. The Ct values from qPCR were compared to those obtained from the same concentration of DNA that was spiked into the PCR mixture containing ddH₂O. We observed that DNA extracts obtained from 2-liter samples of either pond water or river water were completely inhibitory to qPCR (Table 4). The addition of BSA (final concentration, 20 ng/µl) to the PCR mixture removed the inhibitory effect (Table 4).

Samples from two recreational lakes were evaluated for detection of *Giardia* and *Cryptosporidium* in a multiplex assay. The addition of BSA to the PCR mixture proved sufficient to eliminate inhibition when extracts of 2-liter concentrates from Heart Lake were tested. However, extracts from Professor's Lake were completely inhibitory even in the presence of BSA. Following application of the qPCR method, these samples were analyzed on an agarose gel to determine whether inhibition was due to a lack of amplicon or to interference with fluorescence detection, possibly due to quenching of the fluorophore by inhibitory substances. No amplicon was detected in wells containing the extracts from Professor's Lake; therefore, the inhibitory substance(s) was affecting the PCR and preventing the amplification of DNA. In contrast, the Heart Lake samples revealed two bands on the agarose gel of ~151 and 74 bp, corresponding to amplification of the COWP and β-giardin sequences.

Several chemical treatment methods were examined for removal of inhibitory substances from the Professor's Lake samples. Addition of Chelex 100 to a final concentration of 5 or 20% had no effect on reduction of inhibition. However, addition of 2% PVP 360 along with 20% Chelex 100 during the DNA extraction process effectively removed inhibitors. Amplification and detection of spiked DNAs of both *G. lamblia* and *C. parvum* was observed in the presence of extracts from Professor's Lake in the multiplex qPCR assay (Table 5). It was possible to detect the spiked DNA when 2.5 of the 50 µl of sample extract (1/20th of the total volume) was added to the qPCR. Five-microliter volumes of extract were partially inhibitory, increasing Ct values by 10 to 30%. The lower-molecular-weight material, PVP 40, was less effective at reducing inhibition than PVP 360. The use of 2.5 µl of the PVP 40-treated samples resulted in 15% inhibition, and 5 µl of sample was completely inhibitory (no Ct obtained in qPCR). Addition of Chelex 100 and PVP 360 during DNA extraction did not reduce the yield of DNA as determined by PicoGreen quantification (not shown).

TABLE 5. Effects of Chelex 100 and PVP 360 on multiplex qPCR detection of *Giardia* and *Cryptosporidium* DNAs spiked into lake water extracts

Water sample ^a	Ct ^b	
	β-Giardin P241	COWP P702
ddH ₂ O	24.88 ± 0.69	27.36 ± 0.40
Heart Lake		
Untreated	23.98 ± 0.09	27.09 ± 0.35
Treated ^c	24.34 ± 0.89	26.70 ± 0.89
Professor's Lake		
Untreated	None	None
Treated 1 ^c	24.89 ± 0.13	27.61 ± 0.19
Treated 2 ^c	25.15 ± 0.94	27.99 ± 0.60

^a 2.5 µl from 50 µl of each sample extract was assayed.^b Mean ± standard deviation of triplicate wells.^c Treated samples had 0.5 M EDTA, 20% Chelex 100, and 2% PVP 360 treatment during DNA extraction.

The chemical action of polyvinylpyrrolidone (PVPP; insoluble) and PVP (water soluble) in removal of inhibitors is based on the binding of phenolic groups. The formation of PVP-phenolic complexes removes molecules, such as humic acids, that contain phenolic groups (4). PVPP has been used to remove PCR inhibitors from *Cryptosporidium*-contaminated stool (18) and soil (4) samples. PVP 40 is a lower-molecular-weight form of PVP containing fewer reactive sites and possessing greater solubility in water than PVP 360. The lower effectiveness of PVP 40 than PVP 360 in blocking inhibition was likely due to the reduced capacity of PVP 40 to bind phenolic compounds in the samples.

Phenols in aquatic environments come from natural sources, such as biodegradation of humic substances, lignins, and tannins, and from man-made sources, such as derivatives of plastics and degradation of pesticides and herbicides (2). Concentrations of phenolics have been shown to vary between different bodies of water and also seasonally (34). In the present study, only one of the four water bodies required PVP 360 treatment to enable qPCR to be performed. Professor's Lake, a former sand and gravel quarry, is adjacent to a historic landfill site. It is possible that the landfill is a source of the phenol in the lake. An alternative source of the phenol-containing inhibitors may be herbicides applied to the extensive lawns that border the north shore of Professor's Lake.

qPCR versus IF microscopy for quantification of *Giardia* and *Cryptosporidium* in sewage. Sewage samples were processed and examined for the presence of *G. lamblia* and *Cryptosporidium*. The extraction of DNA from 1-liter sewage samples was done by Chelex 100 and PVP 360 treatment to remove inhibitors and sonication, using a cup horn to disrupt the cyst and oocyst walls. A great deal of fibrous material was associated with the sewage, and this had the potential to trap pathogens. Within several minutes of being subjected to sonication in the cup horn, clumps that were not disrupted by vortexing were visibly disrupted, possibly releasing trapped cysts or oocysts and exposing them to the lysis reagents and sonication.

The efficiency of extraction from sewage was estimated by spiking half of one sample with 10⁵ cysts of *G. lamblia* and 10⁵ *C. parvum* oocysts and comparing the extraction of an equivalent number of cells in the absence of sewage. We found that

TABLE 6. Multiplex qPCR detection of 1 ng of spiked DNA to determine the presence of inhibitors in 1-liter sewage sample extracts

Sample	Vol added to qPCR assay (μ l)	Ct ^a	
		β -Giardin P241	COWP P702
ddH ₂ O	10	23.30 $\pm$ 0.63	23.88 $\pm$ 0.25
Extraction control 1 ^a	10	23.23 $\pm$ 0.23	23.12 $\pm$ 0.29
	1	23.31 $\pm$ 0.49	23.63 $\pm$ 0.55
Extraction control 2 ^a	10	23.95 $\pm$ 0.54	23.30 $\pm$ 0.27
	1	23.31 $\pm$ 0.73	23.51 $\pm$ 0.41
Auteuil A1	10	None	None
	1	24.20 $\pm$ 0.31	24.12 $\pm$ 0.26
Auteuil A2	10	26.37 $\pm$ 0.61	25.00 $\pm$ 0.55
	1	23.51 $\pm$ 0.70	23.52 $\pm$ 0.46

^a Extraction controls, all DNA extraction methods minus sewage.^b Mean $\pm$ standard deviation of triplicate wells.

the efficiency of extraction in the presence of sewage was not reduced.

A total of six sewage extracts were examined for the presence of inhibitors of qPCR by spiking the qPCR mixture containing the sewage extracts with 1 ng each of DNAs from *G. lamblia* and *C. parvum*. Inhibition was observed when 10 μ l of the 200- μ l sewage extract was tested in qPCR but not for 1 μ l of sample (Table 6). Therefore, to quantify cysts and oocysts in these samples, 2- μ l volumes of the sample extracts were used in qPCR except for sample Auteuil 1 (which inhibited PCR at 2 μ l and partially at 1 μ l), where 0.5 μ l of the 200- μ l sample was used. The multiplex assay detected *Giardia* in all six sewage samples but no *Cryptosporidium* (data not shown).

Quantitative analysis of *G. lamblia* cysts in the sewage samples was done using the P434 primer-probe set to avoid the problem of potential mismatches in the sequence of the DNA of *Giardia* in sewage when using the P241 primer-probe set. The primers and probe for one set of P434 were based on the Portland 1 sequence (assemblage A) of *G. lamblia*, and the other set was the same region of the gene but using the H3 sequence (assemblage B). We used these primer-probe sets in separate assays and summed the numbers obtained from each to obtain the total number of *G. lamblia* cysts present in each sample. *Giardia* was detected in all six samples from the Auteuil and Fabreville (Québec, Canada) treatment facilities, with cyst concentrations ranging from 2,653 to 13,408/liter of sewage (Table 7). No Ct values were obtained in the negative control samples, consisting of the extraction procedure in the absence of sewage, suggesting that Ct values obtained in the sewage samples were specific to *G. lamblia*. The values obtained from qPCR were similar to those obtained by enumerating cysts using IF microscopy (Table 7).

Both assemblage A and B genotypes were detected in the samples, with the majority of cysts (58 to 91%) being of the assemblage B genotype (Table 7). Recently, Caccio and colleagues (3) reported genotyping of *G. lamblia* from clinical stool samples using PCR and restriction fragment length poly-

TABLE 7. Comparison of qPCR and IF microscopy for detection of *G. lamblia* and *Cryptosporidium* in 1-liter sewage samples

Sample ^a	No. of <i>G. lamblia</i> cysts			No. of <i>C. parvum</i> oocysts	
	qPCR		IF microscopy	qPCR	IF microscopy
	Assemblage A	Assemblage B			
NC	0	0	0	0	0
A1	496	5,146	5,642	2,380	0
A2	2,476	8,340	10,816	9,880	0
A3	5,672	7,736	13,408	7,980	0
F1	838	1,815	2,653	9,900	0
F2	2,196	3,663	5,859	6,660	0
F3	545	3,331	3,876	4,290	0

^a NC, negative control in qPCR; A, Auteuil Treatment Facility; F, Fabreville Treatment Facility.

morphism of the β -giardin gene. Both genotypes were detected in the stool samples, with a predominance of the assemblage A genotype. We have also detected assemblage A and B genotypes in stool; however, the majority of samples were genotype B (Guy et al., unpublished).

Interestingly, no *Cryptosporidium* was detected in any of the samples (Table 7), as determined by qPCR using both the multiplex assay and a simplex assay with the COWP P702 primer-probe and also using IF microscopy. The lack of detection of *Cryptosporidium* was not due to inhibition of either COWP amplification or fluorescence of the HEX fluorophore, because *C. parvum* DNA spiked into qPCR wells containing extracts from the sewage samples was detectable (Table 6). Seasonal variation in the abundance of *Giardia* and *Cryptosporidium* in sewage has been reported, with higher levels of *Giardia* and low to no detection of *Cryptosporidium* in the fall months (7), the time of year we sampled. *Giardia* cysts are consistently seen in sewage influent. For instance, Wallis and colleagues (36) detected *Giardia* in 73% of raw sewage in a cross-Canada survey. Detection of *Cryptosporidium* was sporadic: only 6.1% of samples contained *Cryptosporidium* (36), and low numbers of oocysts (<500) were observed (36). High levels of *Giardia* and low-level sporadic detection of *Cryptosporidium* has also been reported by others (7, 24, 26, 28).

The results obtained in the present study from spiking experiments revealed that there were additional qPCR-inhibitory substances in the DNA extracts from the sewage samples, as it was not possible to use larger volumes of the 200- μ l sewage extract (no greater than 2 μ l, and 0.5 μ l in the case of the sample Auteuil 1) in the qPCR. Therefore, the quantitative evaluation limit for the qPCR method using sewage samples was 100 oocysts. Lack of detection of *Cryptosporidium* in the sewage suggests that oocysts were either absent or present at very low levels.

In summary, new methods for qPCR were described, and their application to pathogen detection in environmental samples, including water and sewage, was demonstrated. With the appropriate adaptation of sample extraction, qPCR is applicable to a variety of detection systems. These approaches may provide a sensitive method for detection of low numbers of pathogens, and their application would contribute to understanding the distribution and abundance of *Giardia* and *Cryp-*

*to*sporidium in the environment and the risks they pose to human health.

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REFERENCES

- Bernander, R., J. E. D. Palm, and S. G. Svärd. 2001. Genome ploidy in different stages of the *Giardia lamblia* life cycle. *Cell. Microbiol.* 3:55-62.
- Bruzzoniti, M. C., C. Sarzanini, and E. Mentasti. 2000. Preconcentration of contaminants in water analysis. *J. Chromatogr. A* 902:289-309.
- Caccio, S. M., M. De Giacomo, and E. Pozio. 2002. Sequence analysis of the β -glucuronidase gene and development of a polymerase chain reaction-restriction fragment length polymorphism assay to genotype *Giardia duodenalis* cysts from human faecal samples. *Int. J. Parasitol.* 32:1023-1030.
- Cullen, D. W., and P. R. Hiesch. 1998. Simple and rapid method for direct extraction of microbial DNA from soil for PCR. *Soil Biol. Biochem.* 30:983-993.
- Fontaine, M., and E. Guillot. 2002. Development of a TaqMan quantitative PCR assay specific for *Cryptosporidium parvum*. *FEMS Microbiol. Lett.* 214: 13-17.
- Hess, C. N., and J. B. Rose. 1995. Developing an action level for *Cryptosporidium*. *J. Am. Water Works Assoc.* 87:81-84.
- Heltman, T. L., L. M. Frederick, J. R. Viste, N. J. Guselle, U. M. Morgan, R. C. A. Thompson, and M. E. Olson. 2002. Prevalence of *Giardia* and *Cryptosporidium* and characterization of *Cryptosporidium* spp. isolated from wildlife, human, and agricultural sources in the North Saskatchewan River Basin in Alberta, Canada. *Can. J. Microbiol.* 48:530-541.
- Higgins, J. A., R. Payer, J. M. Trout, L. Xiao, A. A. Lal, S. Kerby, and M. C. Jenkins. 2001. Real-time PCR for the detection of *Cryptosporidium parvum*. *J. Microbiol. Methods* 47:323-327.
- Holberton, D., D. A. Baker, and J. Marshall. 1988. Segmented alpha-helical coiled-coil structure of the protein giardin from the *Giardia* cytoskeleton. *J. Mol. Biol.* 204:789-795.
- Holberton, D. V., and J. Marshall. 1995. Analysis of consensus sequence patterns in *Giardia* cytoskeleton gene promoters. *Nucleic Acids Res.* 23: 2945-2953.
- Isaac-Renton, J. L., W. Moorehead, and A. Ross. 1996. Longitudinal studies of *Giardia* contamination in two community drinking water supplies: cyst levels, parasite viability, and health impact. *Appl. Environ. Microbiol.* 62: 47-54.
- LeChevallier, M. W., W. D. Norton, and R. G. Lee. 1991. Occurrence of *Giardia* and *Cryptosporidium* in surface water supplies. *Appl. Environ. Microbiol.* 57:2610-2616.
- Limor, J. R., A. A. Lal, and L. Xiao. 2002. Detection and differentiation of *Cryptosporidium* parasites that are pathogenic for humans by real-time PCR. *J. Clin. Microbiol.* 40:2335-2338.
- MacDonald, L. M., K. Sargent, A. Armon, R. C. A. Thompson, and J. A. Reynoldson. 2002. The development of a real-time quantitative-PCR method for characterization of a *Cryptosporidium parvum* in vitro culturing system and assessment of drug efficacy. *Mol. Biochem. Parasitol.* 121:279-282.
- MacKenzie, W. R., W. L. Schell, K. A. Blair, D. G. Addiss, D. E. Peterson, N. J. Hoxie, J. J. Kazmierczak, and J. P. Davis. 1995. Massive outbreak of waterborne *Cryptosporidium* infection in Milwaukee, Wisconsin: recurrence of illness and risk of secondary transmission. *Clin. Infect. Dis.* 21:57-62.
- Mahbubani, M. H., A. K. Bej, M. H. Perlín, F. W. Schaeffer III, W. Jakubowski, and R. M. Atlas. 1991. Detection of *Giardia* cysts by using the polymerase chain reaction and distinguishing live from dead cysts. *Appl. Environ. Microbiol.* 57:3456-3461.
- Mahbubani, M. H., F. W. Schaeffer III, D. D. Jones, and A. K. Bej. 1998. Detection of *Giardia* in environmental waters by immuno-PCR amplification methods. *Curr. Microbiol.* 36:107-113.
- McLauchlin, J., S. Pedraza-Díaz, C. Amac-Hoeteneder, and G. L. Nichols. 1999. Genetic characterization of *Cryptosporidium* strains from 218 patients with diarrhea diagnosed as having sporadic cryptosporidiosis. *J. Clin. Microbiol.* 37:3153-3158.
- Mhlanga, M. M., and L. Malmberg. 2001. Using molecular beacons to detect single-nucleotide polymorphisms with real-time PCR. *Methods* 25:463-471.
- Morgan-Ryan, U. M., A. Fall, L. A. Ward, N. Hjjawi, I. Sulaiman, R. Payer, R. C. A. Thompson, M. Olson, A. Lal, and L. Xiao. 2002. *Cryptosporidium hominis* n. sp. (Apicomplexa: Cryptosporidiidae) from *Homo sapiens*. *J. Eukaryot. Microbiol.* 49:433-440.
- Org, C., W. Moorehead, A. Ross, and J. Isaac-Renton. 1996. Studies of *Giardia* spp. and *Cryptosporidium* spp. in two adjacent watersheds. *Appl. Environ. Microbiol.* 62:2798-2805.
- Ongerth, J. E., G. D. Hunter, and F. B. DeWalle. 1995. Watershed use and *Giardia* cyst presence. *Water Res.* 29:1295-1299.
- Payment, P., A. Berte, M. Prevost, B. Menard, and B. Barbeau. 2000. Occurrence of pathogenic microorganisms in the Saint Lawrence River (Canada) and comparison of health risks for populations using it as their source of drinking water. *Can. J. Microbiol.* 46:565-576.
- Payment, P., R. Plante, and P. Cefka. 2001. Removal of indicator bacteria, human enteric viruses, *Giardia* cysts, and *Cryptosporidium* oocysts at a large wastewater primary treatment facility. *Can. J. Microbiol.* 47:188-193.
- Pedraza-Díaz, S., C. Amar, and J. McLauchlin. 2002. The identification and characterization of an unusual genotype of *Cryptosporidium* from human faeces as *Cryptosporidium meleagridis*. *FEMS Microbiol. Lett.* 189:189-194.
- Rintala-Ekine, R., P. Rönkäinen, and M.-L. Hänninen. 2001. Simultaneous detection of *Cryptosporidium parvum* and *Giardia* in sewage sludge by IC-PCR. *J. Appl. Microbiol.* 91:1030-1035.
- Robertson, L. J., and B. Gjerde. 2001. Occurrence of parasites on fruits and vegetables in Norway. *J. Food Prot.* 64:1793-1798.
- Robertson, L. J., C. A. Paton, A. T. Campbell, P. G. Smith, M. H. Jackson, R. A. Gilmour, S. E. Black, D. A. Stevenson, and H. V. Smith. 2000. *Giardia* cysts and *Cryptosporidium* oocysts at sewage treatment works in Scotland. *UK. Water Res.* 34:2310-2322.
- Rose, J. B., and T. R. Shifka. 1999. *Giardia*, *Cryptosporidium*, and *Cyclospora* and their impact on foods: a review. *J. Food Prot.* 62:1059-1070.
- Rossan, L., P. Nørskov, K. Holmström, and O. F. Rasmussen. 1992. Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. *Int. J. Food Microbiol.* 17:37-45.
- Spano, F., C. Puri, L. Ranucci, L. Putignani, and A. Crisanti. 1997. Cloning of the entire COWP gene of *Cryptosporidium parvum* and ultrastructural localization of the protein during sexual parasite development. *Parasitology* 114:427-437.
- Tanriverdi, S., A. Tanyeli, F. Balamisli, R. Koksul, F. Kilinc, X. Feng, G. Balzer, S. Tzipori, and G. Widmer. 2002. Detection and genotyping of oocysts of *Cryptosporidium parvum* by real-time PCR and melting curve analysis. *J. Clin. Microbiol.* 40:3237-3244.
- Thompson, R. C. A. 2000. *Giardiasis* as a re-emerging infectious disease and its zoonotic potential. *Int. J. Parasitol.* 30:1259-1267.
- Thoss, V., M. S. Baird, M. A. Lock, and P. Y. Courty. 2002. Quantifying the phenolic content of freshwaters using simple assays with different underlying reaction mechanisms. *J. Environ. Monit.* 4:270-275.
- Walker, N. J. 2002. Tech.Sight. A technique whose time has come. *Science* 296:557-559.
- Wallis, P. M., S. L. Erlandsen, J. L. Isaac-Renton, M. E. Olson, W. J. Robertson, and H. van Heulen. 1996. Prevalence of *Giardia* cysts and *Cryptosporidium* oocysts and characterization of *Giardia* spp. isolated from drinking water in Canada. *Appl. Environ. Microbiol.* 62:2789-2797.
- Wallis, P. M., D. Matson, M. Jones, and J. Jamieson. 2001. Application of monitoring data for *Giardia* and *Cryptosporidium* to boil water advisories. *Risk Anal.* 21:1077-1085.
- Widmer, G., J. Lin, V. Kapur, X. Feng, and M. S. Abrahamsen. 2002. Genomics and genetics of *Cryptosporidium parvum*: the key to understanding cryptosporidiosis. *Microb. Infect.* 4:1081-1090.
- Wilson, I. G. 1997. Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.* 63:3741-3751.
- Xiao, L., J. Limor, U. M. Morgan, I. M. Sulaiman, R. C. A. Thompson, and A. A. Lal. 2000. Sequence differences in the diagnostic target region of the oocyst wall protein gene of *Cryptosporidium* parasites. *Appl. Environ. Microbiol.* 66:5499-5502.

water-storage tanks (Pozio et al., 1997). The results probably represent underestimates of the concentrations that caused the outbreaks. For the 1993 Milwaukee outbreak, however, Haas & Rose (1994) showed (with certain assumptions) that the measured concentration in drinking-water was close to the value predicted on the basis of the attack rate, water consumption, and dose-response relationship.

Low oocyst concentrations in drinking-water have also been found in situations where there was no evidence for the occurrence of an outbreak (LeChevallier, Norton & Lee, 1991; Karanis & Seitz, 1996; Rose, Lisle & LeChevallier, 1997; McClellan, 1998). Current detection methods do not allow the determination of pathogenicity of oocysts in water. Detection of oocysts in treated water should therefore, wherever possible, prompt further tests to confirm the presence of (viable) *C. parvum* oocysts. Whether or not the capacity for such tests exists, every effort should be made to thoroughly examine other water quality parameters that may point to faecal contamination and to trace both the source of contamination and the operational conditions that resulted in the presence of oocysts in the drinking-water. If the presence of *C. parvum* oocysts is confirmed, control measures (improved source protection and/or water treatment) should be instituted; ideally, an epidemiological study should also be undertaken to determine whether there has been significant waterborne transmission. Lack of capacity to confirm the pathogenicity or viability of oocysts should not delay or prevent further investigation or remedial action if a significant threat to public health is suspected.

Giardia intestinalis

Taxonomy

Giardia is a flagellated protozoan the taxonomy and host specificity of which remain the subject of considerable debate. The organism has been found in more than 40 animal species (Meyer, 1994). Nowadays, five species of *Giardia* are established in the scientific literature, including the three species proposed by Filice (1952)—*G. muris* in rodents, birds, and reptiles, *G. intestinalis* (syn: *duodenalis*, syn: *lamblia*) in mammals (including man), rodents, reptiles, and possibly birds, *G. agilis* in amphibians—*G. ardae* in the great blue heron (Erlandsen et al., 1990), and *G. psittaci* in the budgerigar (Erlandsen & Bemrick, 1987). A morphologically distinct *Giardia* isolated from the straw-necked ibis (Forshaw, 1992) was later suggested to be a strain of *G. ardae* (McRoberts et al., 1996).

Giardia is thought to be predominantly asexual, and the species concept—defined on the basis of sexually reproductive compatibility—is therefore difficult to apply. The high degree of genetic heterogeneity found in human and animal isolates (Nash et al., 1985; Andrews et al., 1989; Meloni, Lymbery & Thompson, 1989; Morgan et al., 1994) makes speciation uncertain and suggests that

coliform level as an indicator for microbiological safety of drinking-water. Additional means of safeguarding drinking-water are therefore imperative.

Cryptosporidium parvum

Taxonomy

Members of the genus *Cryptosporidium* (Apicomplexa, Cryptosporidiidae) are small coccidian protozoan parasites that infect the microvillous region of epithelial cells in the digestive and respiratory tracts of vertebrates. Several species of *Cryptosporidium* have been described and appear to be specific for a class of vertebrates: *C. parvum*, *C. muris*, *C. felis*, and *C. wairi* infect mammals, *C. baileyi* and *C. meleagridis* infect birds, *C. serpentis* infects reptiles, and *C. nasorum* infects tropical fish. Infections in humans are almost exclusively caused by *C. parvum*, although this species is also frequently found in infections of cattle and sheep and causes infections in many other mammal species.

Life cycle

Oocysts, the environmentally resistant transmission stage of the parasite, are shed by infected hosts with their faeces (Fayer & Ungar, 1986; Fayer, Speer & Dubey, 1997) and are immediately infectious. They may remain in the environment for very long periods without loss of infectivity: a very robust oocyst wall protects the sporozoites inside against physical and chemical damage. When an oocyst is ingested by a new host, excystation—opening of the suture in the oocyst wall—is triggered by the body temperature and the interaction with stomach acid and bile salts. Four motile sporozoites are released, which infect the epithelial cells of the small intestine, mainly in the jejunum and ileum. The parasite infects the apex of the epithelial cells, residing beneath the cell membrane but outside the cytoplasm. The sporozoites undergo several transformations in an asexual (merogony) and a sexual (gametogony) reproduction cycle; it is the latter that generates the oocysts.

Oocysts of *C. parvum* are spherical, with a diameter of 4–6 µm, and may be either thick- or thin-walled oocysts. Thin-walled oocysts may excyst within the same host and start a new life cycle (autoinfection). This can lead to heavily infected intestinal epithelia and result in malabsorptive or secretory diarrhoea. Thick-walled oocysts are excreted with the faeces.

Pathogenicity

Infection studies in healthy human volunteers demonstrated a clear relationship between probability of infection and the ingested oocyst dose of a bovine *C. parvum* strain (Dupont et al., 1995). At the lowest dose (30 oocysts), the probability of infection was 20%; at a dose of 1000 oocysts, probability increased