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Occurrence, Source, and Human Infection Potential of *Cryptosporidium* and *Giardia* spp. in Source and Tap Water in Shanghai, China[▽]

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Genotyping studies on the source and human infection potential of *Cryptosporidium* oocysts in water have been almost exclusively conducted in industrialized nations. In this study, 50 source water samples and 30 tap water samples were collected in Shanghai, China, and analyzed by the U.S. Environmental Protection Agency (EPA) Method 1623. To find a cost-effective method to replace the filtration procedure, the water samples were also concentrated by calcium carbonate flocculation (CCF). Of the 50 source water samples, 32% were positive for *Cryptosporidium* and 18% for *Giardia* by Method 1623, whereas 22% were positive for *Cryptosporidium* and 10% for *Giardia* by microscopy of CCF concentrates. When CCF was combined with PCR for detection, the occurrence of *Cryptosporidium* (28%) was similar to that obtained by Method 1623. Genotyping of *Cryptosporidium* in 17 water samples identified the presence of *C. andersoni* in 14 water samples, *C. suis* in 7 water samples, *C. baileyi* in 2 water samples, *C. meleagridis* in 1 water sample, and *C. hominis* in 1 water sample. Therefore, farm animals, especially cattle and pigs, were the major sources of water contamination in Shanghai source water, and most oocysts found in source water in the area were not infectious to humans. *Cryptosporidium* oocysts were found in 2 of 30 tap water samples. The combined use of CCF for concentration and PCR for detection and genotyping provides a less expensive alternative to filtration and fluorescence microscopy for accurate assessment of *Cryptosporidium* contamination in water, although the results from this method are semiquantitative.

Waterborne cryptosporidiosis and giardiasis are significant public health concerns (6, 43). *Cryptosporidium* spp. and *Giardia* spp. are threats to water supplies because they are resistant to chlorine disinfection, have a low infectious dose, and are harbored by many animal species (57). Consequently, numerous waterborne outbreaks of cryptosporidiosis and giardiasis have been documented (62). Therefore, *Cryptosporidium* and *Giardia* are the only major pathogens specifically identified in drinking water regulations in industrialized countries and China (16, 21, 30, 48).

Farm animals, humans, and wild animals have been considered major contributors of *Cryptosporidium* and *Giardia* (oo)cyst contamination in surface water. The relative contribution of each animal species depends on the environmental setting (2, 27, 40, 59, 61). Although oocysts of all *Cryptosporidium* species can potentially appear in water, few of them are important human pathogens (56). Because oocysts of most *Cryptosporidium* spp. are morphologically similar and have the potential to be present in water, genotyping of *Cryptosporidium* oocysts in water is essential for source water management and risk assessment. The same is true for assemblages of *Giardia duodenalis*.

Currently, the identification of *Cryptosporidium* oocysts and *Giardia* cysts in environmental samples is largely performed by using the U.S. Environmental Protection Agency (U.S. EPA) Method 1623 and similar testing methods from the United Kingdom and other countries (54, 61). U.S. EPA Method 1623 requires concentration of (oo)cysts by filtration, isolation of (oo)cysts by immunomagnetic separation (IMS), staining of recovered (oo)cysts with fluorescent antibodies and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), and microscopic detection and enumeration of the stained oocysts or cysts (47). However, because of the high supply cost of this method (>$300), it is rarely used in water works in China. On the other hand, flocculation has been shown to be an inexpensive and convenient method for recovery of *Cryptosporidium*, *Giardia*, *Toxoplasma*, bacteria, and viruses in various water sources (64). In flocculation studies conducted, *Cryptosporidium* oocyst recoveries were 59% for alum when 1,000 oocysts/liter was used in seeding (50) and 64 to 75% for calcium carbonate when 75 to 1,000 oocysts/liter was used in seeding (11, 46, 50). *Giardia* recoveries were 72 to 77% for

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calcium carbonate when 85 cysts/liter were used to seed river or tap water (46).

PCR-based techniques have been used increasingly for detection and characterization of *Cryptosporidium* oocysts and *G. duodenalis* cysts in water (3, 13, 23, 27, 58, 61). Unlike Method 1623, recent PCR methods can differentiate *Cryptosporidium* and *Giardia* species that infect humans from those that do not infect humans. Because most *Cryptosporidium* species and genotypes are host specific, genotyping techniques are also used for contamination source tracking, although it is difficult to differentiate the original infected hosts from the vector hosts which carried the oocyst to the watershed (25). The most commonly used genotyping tool is a small-subunit (SSU) rRNA gene-based PCR-restriction fragment length polymorphism (RFLP) technique, which has been used effectively for genotyping *Cryptosporidium* oocysts in surface water, storm water, finished water, and wastewater samples (3, 13, 18, 27, 29, 45, 58, 61, 65). Similarly, genotyping methods, such as PCR combined with sequencing of the triosephosphate isomerase and glutamate dehydrogenase genes can be used to differentiate *G. duodenalis* assemblages A and B, which infect humans, from assemblages C to H, which do not infect humans, although the broad host ranges of assemblages A and B make the precise tracking of contamination source of *G. duodenalis* in water difficult (19).

Most genotyping studies of *Cryptosporidium* in source water or drinking water were conducted in industrialized nations. In developing countries, there has been only one small-scale study in Kenya, in which *Cryptosporidium* oocysts in nine surface water samples were genotyped (36). Because the stringency of wastewater treatment and discharge and the intensity of animal husbandry are very different between developing countries and industrialized nations, observations made in the latter cannot be applied easily to the former. In China, there have been several studies on the occurrence of *Cryptosporidium* and *Giardia* species using microscopy (9, 51, 52, 55, 63, 66), but a study on molecular characterization of *Cryptosporidium* in source or drinking water has not been done. Therefore, the contamination source and human infection potential of oocysts in water in China and other developing countries are unknown.

The objectives of this study were as follows: (i) to estimate the prevalence of *Cryptosporidium* and *Giardia* spp. in source and tap water in Shanghai, China; (ii) to identify the genotypes of *Cryptosporidium* in water in Shanghai, China; (iii) to determine the source of contamination and human infection potential of *Cryptosporidium* in water; (iv) to find a less expensive method for detecting *Cryptosporidium* and *Giardia* in source water; and (v) to associate recovery of parasites with water quality characteristics.

MATERIALS AND METHODS

Study site. Shanghai is one of the biggest cities in China. It covers 6,341 km^2 and has an estimated population of 20 million. In the city, there is one main river, the Huangpu River, which is 113.4 km long. This river is used for drinking water, irrigation, drainage, shipping, fishery production, and tourism/recreation. The Huangpu River has been providing source water for 70% of Shanghai residents and will provide source water for 50% of the residents after 2012 when a new reservoir with water from the Yangtze River is fully operational. The Huangpu River receives water from Taihu Lake via the Taipu River and from Dianshan Lake via the Dahei River. Although the Dahei River is within the water protection area (1,058 km^2) in Shanghai, which restricts the load and concentration of sewage discharge and farming, along the Taipu River, there are 23 towns and villages with a total population of 1,200,000 persons. Some townships are major suppliers of livestock and poultry for Shanghai. The source water sampling site in the Huangpu River was located upstream of Shanghai, at the intake of the drinking water treatment plants for Shanghai.

Sample collection and processing. A total of 50 source water samples were collected from the end of May 2009 to the middle of January 2010, with an average of one to three samples per week with the exception that no samples were collected in August and October 2009. Each source water sample was split into two 20-liter aliquots. One aliquot was processed according to U.S. EPA Method 1623 (47). Briefly, the aliquot was filtered through Filta-Max xpress filters (IDEXX Laboratories, Inc., Westbrook, ME) and eluted using a Filta-Max xpress wash station (IDEXX Laboratories, Inc.). If only a partial aliquot was filtered because of clogging, the filtered volume was recorded. Nineteen samples (38%) were not fully filtered, and the filtered volume was between 10 to 18 liters, with five samples of more than 15 liters and the remaining samples between 10 and 15 liters. After centrifugation at 1,500 × *g* for 15 min (Eppendorf 5810; Eppendorf, Hamburg, Germany), the pellet was used for immunomagnetic separation (IMS) using an anti-*Cryptosporidium* and anti-*Giardia* IMS kit (Dynabeads GC-Combo; Invitrogen Dynal, A.S., Oslo, Norway). Only 0.5 ml of pellet (1/2 to 1/4 of the pellet volume for water samples) was processed for IMS per sample. Fluorescence staining was performed using a fluorescein isothiocyanate (FITC)-conjugated anti-*Cryptosporidium* and anti-*Giardia* monoclonal antibodies (Aqua-Glo G/C kit; Waterborne, Inc., New Orleans, LA). The internal structure of (oo)cysts was confirmed by 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining. On the basis of the number of (oo)cysts detected, the portion of the pellet for microscopy and the water volume filtered, the number of (oo)cysts was reported as the number of equivalents in 10 liters of water. Another portion of the remaining pellet (0.5 ml) was used for DNA extraction and PCR analysis.

The other 20-liter aliquot of source water was concentrated by the calcium carbonate flocculation (CCF) method (50). Briefly, a sample was separated into two 10-liter aliquots and stored in flat-bottomed polypropylene barrels. The water was mixed with 100 ml of 1 M calcium chloride ($CaCl_2$) and 100 ml of 1 M sodium hydrogen carbonate ($NaHCO_3$) by continuous stirring on a magnetic stirrer, and the pH was adjusted to 10 by the addition of 5 M sodium hydroxide (NaOH). The water sample was then allowed to rest undisturbed at room temperature overnight. The next day, the supernatant fluid was removed by vacuum aspiration, and the residue was dissolved by adding 200 ml of 10% sulfamic acid and shaking. Another 100 ml of sulfamic acid was added to the container if the flocculate was not fully dissolved. The dissolved flocculate was transferred to centrifuge tubes. The barrels were rinsed twice with 0.01% Tween 80; they were rinsed first with 200 ml of 0.01% Tween 80 and then with 100 ml of 0.01% Tween 80. The rinses were also transferred to the centrifuge tubes. After centrifugation at 3,000 × *g* for 20 min, the supernatant was removed and the pellet volume was recorded. The pellet was washed with Dulbecco's phosphate-buffered saline (DPBS) by centrifugation two or three times until the pH in 10 ml of DPBS was 7.0. A portion (0.5 ml) (1/3 to 1/4 of the pellet volume for the majority of the water samples and 1/9 and 1/12 for two samples) of the pellet was further processed with immunomagnetic separation (IMS), fluorescence staining, and microscopic observation according to U.S. EPA Method 1623 (47). The number of (oo)cysts was reported as the number of equivalents in 10 liters of water as stated above. Another portion of the remaining pellet (0.5 ml) was used for DNA extraction and PCR analysis.

Within the same period, 30 100-liter tap water samples were obtained from mid-June 2009 to mid-January 2010 from a local laboratory, with an average of one or two samples per week except that no samples were collected in August and October 2009. The tap water samples were concentrated by a filtration method using Filta-Max xpress filters (IDEXX Laboratories, Inc.) as stated above and examined for *Cryptosporidium* oocysts and *Giardia* cysts using U.S. EPA Method 1623 (47).

The recoveries of the standard Method 1623 and CCF-modified Method 1623 for *Cryptosporidium* oocysts and *Giardia* cysts in samples was monitored once every 3 months by spiking 10 liters of distilled water with Easyseed containing 100 oocysts and 100 cysts (BTF Pty Ltd., North Ryde, NSW, Australia).

For water quality analyses, total coliform and *Escherichia coli* were enumerated for all water samples using the Quanti-Tray/2000 (IDEXX Laboratories, Inc.) as specified by SM 9223B (16a). Heterotrophic plate count (HPC) was enumerated using the SimPlate for HPC (IDEXX Laboratories, Inc.). The turbidity of samples was measured with a Hach 2100P turbidimeter (Hach Co., Loveland, CO). The total dissolved solids (TDS) and conductivity were measured with a Hach MP-6p meter (Hach Co.).

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TABLE 1. Occurrence of *Cryptosporidium* and *Giardia* spp. in source and tap water samples in Shanghai, China

Water sample and detection technique	*Cryptosporidium*				*Giardia*			
	Filtration		CCF		Filtration		CCF	
	Detection[a]	Intensity (mean ± SD)[b]	Detection	Intensity (mean ± SD)	Detection	Intensity (mean ± SD)	Detection	Intensity (mean ± SD)
Source water								
Microscopy	16/50[c]	5.2 ± 6.5	11/50[c]	4.5 ± 4.3	9/50[d]	3.8 ± 2.1	5/50[d]	2.2 ± 0.5
PCR	6/50	1.8 ± 0.8	14/50	2.1 ± 1.2	ND[e]		ND	
Tap water and microscopy	2/30	0.2 ± 0.0	ND		0		ND	

[a] Number of samples positive for *Cryptosporidium* or *Giardia*/number of samples analyzed.
[b] For microscopy, the intensity is the calculated concentration of *Cryptosporidium* oocysts or *Giardia* cysts for 10 liters of water is shown. For PCR, the intensity is the number of PCR-positive replicates/5 replicates for all positive samples.
[c] Eight samples were positive for *Cryptosporidium* by both methods.
[d] No sample was positive for *Giardia* by both methods.
[e] ND, not done.

DNA extraction and *Cryptosporidium* genotyping. DNA was extracted directly from 0.5-ml water concentrate of each sample from filtration or CCF using the FastDNA spin kit for soil (Bio 101, Carlsbad, CA) and eluted in 100 μl of reagent-grade water as described previously (26). *Cryptosporidium* oocysts present in the samples were genotyped by nested PCR amplification of an approximately 830-bp fragment of the small-subunit (SSU) rRNA gene and restriction fragment length polymorphism (RFLP) analysis of the secondary PCR products, using the restriction enzymes SspI and VspI (27). Each sample was analyzed five times by the PCR-RFLP technique, using 2 μl of the DNA extract per PCR. *Cryptosporidium baileyi* DNA was used as a positive control in all SSU rRNA-based PCR-RFLP analyses. To neutralize residual PCR inhibitors in the extracted DNA, 400 ng of nonacetylated bovine serum albumin (Sigma-Aldrich, St. Louis, MO) per μl was used in the primary PCR. All positive secondary PCR products were sequenced to confirm the genotype identification.

Sequence analysis. After purification using the MultiScreen PCR plate (Millipore, Bedford, MA), the secondary PCR products of the SSU rRNA gene were sequenced directly with the secondary PCR primers, using an ABI BigDye Terminator v. 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and the procedures suggested by the manufacturer. The sequences were read on an ABI3130 genetic analyzer (Applied Biosystems). Sequence accuracy was confirmed by two-directional sequencing and sequencing of all PCR products for each positive sample. Nucleotide sequences obtained were aligned with reference *Cryptosporidium* SSU rRNA sequences by using the ClustalX 1.81 package (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) to determine the *Cryptosporidium* genotype.

Statistical analysis. The association between detecting *Cryptosporidium* and *Giardia* and physical-chemical (turbidity, conductivity, and TDS) and microbiological parameters (log HPC, log *E. coli* count, and log total coliform count) of water was compared using Student's *t* test. Differences in detecting *Cryptosporidium* by different methods were compared using chi-square analysis. P values of <0.05 were considered statistically significant. All statistical analyses were performed with SPSS 10.0 for Window (SPSS Inc., Somers, NY).

RESULTS

Occurrence of *Cryptosporidium* oocysts and *Giardia* cysts. Of the 50 source water samples, FITC staining indicated that 16 samples (32%) were *Cryptosporidium* positive and nine (18%) were *Giardia* positive using the standard U.S. EPA Method 1623 (Table 1). In the positive samples, the *Cryptosporidium* concentration ranged from 1.8 to 22 with an average of 5.2 oocysts in 10 liters of water; and the *Giardia* concentration ranged from 2 to 8 with an average of 4 cysts in 10 liters of water. DAPI staining indicated that 14 of the 16 *Cryptosporidium*-positive samples were DAPI positive, with the concentration of intact oocysts ranging from 1.8 to 9 and with an average of 3.5 oocysts in 10 liters of water. In contrast, three of nine *Giardia*-positive samples were DAPI positive, with the concentration of intact cysts ranging from 2.5 to 3 and an average of 2.8 cysts in 10 liters of water. No correlation was found between the positive rates of the two parasites and filtered water volume. The recovery efficiencies by the standard method, Method 1623, was 28 to 42% for *Cryptosporidium* oocysts and 25 to 35% for *Giardia* cysts ($n = 9$), and DAPI staining found that more than 96% of the seeded (oo)cysts were DAPI positive.

For the source water samples analyzed by microscopy of concentrates by calcium carbonate flocculation (CCF), FITC staining indicated that 11 samples (22%) and five samples (10%) were positive for *Cryptosporidium* and *Giardia*, respectively (Table 1). In the positive samples, the *Cryptosporidium* concentration ranged from 2 to 16 with an average of 4.5 oocysts in 10 liters of water, whereas the *Giardia* concentration ranged from 1 to 4.5 with an average of 2.2 cysts in 10 liters of water (Table 1). DAPI staining indicated that 10 of the 11 *Cryptosporidium*-positive samples were DAPI positive, with the concentration of intact oocysts ranging from 2 to 16, with an average of 4.4 oocysts in 10 liters of water. In contrast, three of the five *Giardia*-positive samples were DAPI positive, with the concentration of intact cysts ranging from 1 to 2, with an average of 1.7 cysts in 10 liters of water. Eight source water samples were positive for *Cryptosporidium* by both filtration- and flocculation-based methods, while no sample was positive for *Giardia* by both methods. The recovery efficiencies by the CCF-modified Method 1623 was 54 to 58% for *Cryptosporidium* oocysts and 62 to 66% for *Giardia* cysts ($n = 9$), and DAPI staining found that more than 95% seeded (oo)cysts were DAPI positive.

Cryptosporidium was detected by PCR in 17 source water samples, with six PCR-positive samples following concentration by filtration and 14 PCR-positive samples following concentration by CCF. Three samples were positive by PCR of concentrates from both filtration and CCF. One sample concentrated by filtration and one sample concentrated by CCF produced PCR products that were noticeably larger than the expected amplicons (~1,000 bp versus ~830 bp) and therefore were not considered *Cryptosporidium* positive. For the samples concentrated by filtration, all those positive by PCR were negative by microscopy. For the samples concentrated by CCF, two of the samples positive by PCR were also positive by

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TABLE 2. Correlation of total coliform, *E. coli*, and heterotrophic plate count with *Cryptosporidium* and *Giardia* detection in source water samples[a]

Source water sample	No. of samples	Log HPC count (mean ± SD)[b]	*P* value[d]	Log total coliform count (mean ± SD)[c]	*P* value	Log *E. coli* count (mean ± SD)[c]	*P* value
Cryptosporidium							
Positive	30	3.98 ± 0.44	0.24	3.18 ± 0.67	0.003	1.96 ± 1.01	0.02
Negative	20	4.12 ± 0.31		3.65 ± 0.20		2.55 ± 0.34	
Giardia							
Positive	14	3.94 ± 0.50	0.30	3.43 ± 0.68	0.63	2.15 ± 1.00	0.81
Negative	36	4.07 ± 0.35		3.34 ± 0.55		2.21 ± 0.82	

[a] Correlation of total coliform, *E. coli*, and heterotrophic plate count (HPC) with detection of *Cryptosporidium* and *Giardia* in source water samples that were positive by microscopy or PCR analysis of concentrates from filtration or CCF.
[b] Number per 1 ml of sample.
[c] Number per 100 ml of sample.
[d] The *P* values result from a comparison of the values (counts) for source water samples that are positive and negative for *Cryptosporidium* or *Giardia*.

microscopy, and the remaining 12 samples positive by PCR were negative by microscopy. Because only 6 of the 20 cysts observed were DAPI positive and *Giardia* PCR is known to have lower sensitivity, PCR analysis for *Giardia* was not performed.

Two of the 30 tap water samples analyzed by Method 1623 were *Cryptosporidium* positive, each containing two oocysts in 100 liters of tap water. None of the tap water samples were positive for *Giardia* (Table 1).

Comparison of *Cryptosporidium* and *Giardia* detection by the standard and CCF-incorporated Method 1623. Statistically, the differences between filtration and CCF in detecting *Cryptosporidium* and *Giardia* by microscopy were not significant for both *Cryptosporidium* and *Giardia*, although *Cryptosporidium* was detected more often higher detection than *Giardia* was ($P \geq 0.25$). The difference was also not related to the water volume filtered in the standard Method 1623. In comparing *Cryptosporidium* detection by microscopy and PCR, significantly more *Cryptosporidium* were obtained by microscopy than by PCR when the samples were concentrated by filtration ($P = 0.02$), although similar positive rates were obtained by the two methods when the samples were concentrated by CCF ($P = 0.49$) (Table 1). The number of *Cryptosporidium* detected by PCR of CCF concentrates was comparable to that detected by the standard Method 1623 ($P = 0.66$).

Water quality parameters and detection of *Cryptosporidium* and *Giardia* by microscopy and PCR. Of the source water samples, *Cryptosporidium*-positive samples (by microscopy or PCR) had a significantly lower mean total coliform count ($P = 0.003$) and mean *E. coli* count than *Cryptosporidium*-negative samples ($P = 0.02$) (Table 2). In contrast, *Cryptosporidium*-positive samples had significantly higher conductivity ($P = 0.0001$) and total dissolved solids (TDS) ($P = 0.001$) than *Cryptosporidium*-negative samples (Table 3). We did not find any significant correlation between the detection of *Giardia* and water quality parameters.

Occurrence of *Cryptosporidium* species by PCR. RFLP analysis of the PCR products using restriction enzymes SspI and VspI indicated the presence of at least five different *Cryptosporidium* species/genotypes. DNA sequencing of all PCR products confirmed the results of the RFLP analysis. The five *Cryptosporidium* species were *C. andersoni*, *C. suis*, *C. baileyi*, *C. hominis*, and *C. meleagridis* (Tables 4 and 5). The most common species in source water samples was *C. andersoni*, which was found in 26 PCR products from 14 samples (Table 5). In contrast, *C. suis* was found in nine PCR products from seven samples, followed by *C. baileyi* in three PCR products from two samples, *C. hominis* in one PCR product from one sample, and *C. meleagridis* in two PCR products from one sample (Table 5). Of the 17 PCR-positive samples, seven (41.2%) contained more than one species, including six samples containing two species and one sample containing three species (Table 4).

Source water samples concentrated by CCF produced more

TABLE 3. Correlation of turbidity, conductivity, and total dissolved solids (TDS) with *Cryptosporidium* and *Giardia* detection in source water samples[a]

Source water sample	No. of samples	Turbidity (NTU[b]) (mean ± SD)	*P* value[c]	Conductivity (μS/cm) (mean ± SD)	*P* value	TDS (mg/liter) (Mean ± SD)	*P* value
Cryptosporidium							
Positive	30	38.20 ± 25.63	0.71	965.23 ± 218.42	0.0001	509.33 ± 154.21	0.001
Negative	20	35.37 ± 26.31		759.60 ± 122.69		380.15 ± 62.02	
Giardia							
Positive	14	41.79 ± 34.52	0.42	879.71 ± 218.85	0.95	498.36 ± 204.02	0.20
Negative	36	35.24 ± 21.63		884.25 ± 210.72		441.83 ± 105.52	

[a] Correlation of turbidity, conductivity, and total dissolved solids (TDS) with *Cryptosporidium* and *Giardia* detection in source water samples that were positive by microscopy or PCR analysis of concentrates from filtration or CCF.
[b] NTU, nephelometric turbidity unit.
[c] The *P* values result from a comparison of the values (turbidity, conductivity, and TDS) for source water samples that are positive and negative for *Cryptosporidium* or *Giardia*.

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TABLE 4. *Cryptosporidium* species in water samples in Shanghai, China

Sample(s)	Species (no. of positive PCR results[a])	
	Filtration-based method	Flocculation-based method
29765		*C. andersoni* (1)
29412		*C. andersoni* (1)
29416		*C. baileyi* (2) and *C. andersoni* (1)
29418		*C. suis* (1)
29426		*C. andersoni* (2)
29427	*C. suis* (1)	
29430		*C. andersoni* (1)
29432		*C. andersoni* (3)
29433, 29434	*C. andersoni* (2)	*C. suis* (1)
29436		*C. andersoni* (3) and *C. suis* (1)
29437, 29438	*C. andersoni* (2) and *C. suis* (1)	*C. andersoni* (4)
29439	*C. suis* (1) and *C. andersoni* (1)	
29441	*C. hominis* (1)	
29443, 29444	*C. suis* (2)	*C. suis* (1) and *C. andersoni* (1)
29446		*C. andersoni* (1)
29448		*C. andersoni* (2)
29452		*C. meleagridis* (2), *C. andersoni* (1), and *C. baileyi* (1)
Total positive	6	14
Total species	*C. andersoni* (5), *C. suis* (5), and *C. hominis* (1)	*C. andersoni* (21), *C. suis* (4), *C. baileyi* (3), and *C. meleagridis* (2)

[a] Number of positive PCR results in five replicate analyses of each sample.

PCR-positive results than those concentrated by filtration (14 samples versus 6 samples or 30 PCR replicates versus 11 replicates) (Tables 4 and 5). As a result, more *Cryptosporidium* species were found in water samples concentrated by CCF (*C. andersoni*, *C. suis*, *C. baileyi*, and *C. meleagridis*) than in samples concentrated by filtration (*C. andersoni*, *C. suis*, and *C. hominis*). In water samples concentrated by CCF, there were 12 samples with *C. andersoni*, 4 samples with *C. suis*, 2 samples with *C. baileyi*, and 1 sample with *C. meleagridis*. In contrast, in samples concentrated by filtration, there were three samples with *C. andersoni*, three samples with *C. suis*, and one sample with *C. hominis* (Table 4).

DISCUSSION

Using the standard U.S. EPA Method 1623, 32% of the 50 source water samples were positive for *Cryptosporidium* and 18% were positive for *Giardia*. Finding *Cryptosporidium* and *Giardia* (oo)cysts in source water samples was expected because the study area was adjacent to highly populated and developed land areas. The prevalence of *Cryptosporidium* in this study was within the range obtained in other studies conducted in China, although the prevalence of *Giardia* was lower (34, 51, 52). The rate of *Cryptosporidium*-positive samples in source water was similar to data (20 to 40%) presented in recent studies in France, Finland, Hungary, Portugal, and Spain (4, 15, 17, 22, 40, 41, 59). Likewise, the prevalence of *Giardia* in this study was also similar to rates reported in Finland, Norway, and Portugal (11.8% to 16.7%) (3, 22, 42).

A higher occurrence of *Cryptosporidium* and *Giardia* in source water was obtained by microscopy of concentrates from filtration than from calcium carbonate flocculation (CCF), although the differences were not significant ($P \geq 0.25$). Because the recovery rates for *Cryptosporidium* oocysts and *Giardia* cysts in this study were similar to data previously reported for CCF (64) or the standard Method 1623 (47), this difference could be associated with the high turbidity of source water samples. The turbidity of the source water samples in this study was between 12 and 157 nephelometric turbidity units (NTU). Previously, recovery rates of *Cryptosporidium* oocysts and *Giardia* cysts by filtration were positively correlated with the turbidity of samples (20, 24), whereas those by CCF were negatively correlated with the turbidity (64). In addition, a higher packed pellet volume was obtained by the CCF method (2 to 4 ml) than the standard filtration method (1 to 2.5 ml), which resulted in a higher proportion of the pellet being processed for immunomagnetic separation (IMS) and microscopy for filtration than for CCF. In addition, the flocculants could have some inhibitory effect on the IMS isolation of oocysts and cysts, although this issue has not been studied in this study or in earlier studies.

In contrast to observations by microscopy, when PCR was used, a lower detection rate (12% versus 28%) for *Cryptosporidium* was obtained in the source water samples concentrated by filtration than in the samples concentrated by CCF (Table 1). Even though the recommended FastDNA spin kit for soil for DNA extraction and 400 ng/μl of bovine serum albumin in

TABLE 5. *Cryptosporidium* species in water concentrates by both filtration and flocculation-based methods

Cryptosporidium species	Major known host	Pathogenic in humans?	No. of samples positive	No. of positive PCR results[a]	
				Filtration	CCF
C. andersoni	Cattle	Rarely	14	5	21
C. suis	Pigs	Rarely	7	5	4
C. baileyi	Birds	No	2	0	3
C. hominis	Humans	Yes	1	1	0
C. meleagridis	Birds	Yes	1	0	2

[a] Total number of positive PCR results in five replicate analyses of each sample.

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the PCR were used to reduce PCR inhibition, this strategy is known to have a lower sensitivity than DNA extraction of *Cryptosporidium* oocysts isolated from water concentrates by IMS (26). It is unclear whether some of the chemical treatments in the process of flocculation can lead to better neutralization of PCR inhibitors, thus improving PCR detection of *Cryptosporidium* oocysts in water samples.

In this study, most samples that were positive for *Cryptosporidium* by microscopy were negative by PCR. Multiple reasons could have contributed to the inconsistent results obtained by PCR and microscopy for the same samples. The number of *Cryptosporidium* oocysts in water samples was low. Only a portion of the pellet was examined by microscopy or PCR, and only one oocyst was detected in the majority of microscopy-positive portions. It is possible that the stochastic distribution of oocysts in samples led to the absence of oocysts in many aliquots analyzed by PCR or microscopy. Furthermore, the number of empty *Cryptosporidium* oocysts in environmental samples can be high (31). In this study, 15 of the 37 oocysts and 3 of the 25 oocysts detected in pellets concentrated by filtration and CCF, respectively, were DAPI negative. The presence of more empty oocysts (ghosts) in the filtration method was possibly also partially responsible for the lower occurrence of *Cryptosporidium* oocysts detected by PCR in this study, because the ghosts could still be detected by microscopy but not by PCR. In a recent study in Luxembourg, only 25% of the samples positive by microscopy were confirmed by PCR (23).

The combination of CCF and PCR was shown to be a cost-effective method for detection of *Cryptosporidium* in water and for contamination source tracking. Although both *Cryptosporidium* and *Giardia* are listed in the water quality standards of China (16), the high cost of using standard Method 1623 ($300 per sample for the material used, including a filter, chemicals, and IMS and staining reagents) has restricted its usage in routine detection of these pathogens. As a result, the expensive set of Method 1623 equipment ($75,000 for a centrifuge, a filtration and elution system, IMS equipment, and a fluorescence microscope) purchased by many laboratories in China is seldom used because of the high running cost. In this study, although the number of *Cryptosporidium* detected by microscopy of concentrates by CCF was lower than that by filtration in Method 1623, when CCF was combined with PCR, *Cryptosporidium* detection was similar to that of the standard Method 1623 ($P = 0.66$). Currently, regulatory agencies and the water industry are trying to develop appropriate *Cryptosporidium* genotyping procedures downstream of Method 1623. Flocculation combined with PCR may be a viable option. A shortcoming of the CCF method combined with PCR may be its inability to quantify *Cryptosporidium* oocyst as Method 1623 does. This can be partially overcome by estimating the contamination intensity by the number of positive PCR results in multiple replicates (Table 1) (37, 60). Since many studies have shown that Method 1623 overestimates the human infection potential of *Cryptosporidium* in water because of its inability to differentiate *Cryptosporidium* species that are pathogenic in humans from *Cryptosporidium* species and genotypes that are not pathogenic in humans (27, 45, 61), CCF combined with PCR has some advantage because of its genotyping capability. Compared to conventional detection by filtration, IMS, and immunofluorescence microscopy, the low cost of CCF combined with PCR ($100 per sample for the material used, including chemicals, PCR reagents, and sequencing and $25,000 for equipment, including a centrifuge and a PCR machine) makes it cost-effective for detecting *Cryptosporidium* in source water in China. A drawback of the CCF technique is its inapplicability to finished water samples because of their low turbidity and requirement for processing much larger volume (100 liters or more) of samples. Like filtration-based methods, they also cannot be used in processing of source water with extremely high turbidity.

In this study, *Cryptosporidium*-positive samples detected by microscopy or PCR were associated with higher conductivity and TDS but lower total coliform and *E. coli* counts than *Cryptosporidium*-negative samples. The biological or environmental basis for this observation, however, is not clear, and the small sample size of the study could have contributed to some of the observed associations. Nevertheless, the correlation between the occurrence of *Cryptosporidium* and *Giardia* and conductivity was reported in samples of recreational rivers in Malaysia (5) and in a drinking water reservoir in Luxembourg (23). In addition, organic matter was found to be significantly correlated with *Cryptosporidium* and *Giardia* occurrence in surface water in Argentina (1). Total coliform, fecal coliform, or *E. coli* was reported to correlate with *Cryptosporidium* and *Giardia* occurrence in source water samples in several studies in Italy, Luxembourg, Spain, United States, Canada, and Argentina (1, 12, 23, 31, 39, 49). In some other studies, no correlation between total coliform or *E. coli* and *Cryptosporidium* was reported (7, 8). However, the negative correlation between microbiological parameters and *Cryptosporidium* occurrence as shown in this study was unexpected. The high turbidity of the source water in this study and small sample size could have contributed somehow to the discrepancy between this study and previous studies.

Genotyping results suggested that *Cryptosporidium* oocyst contamination of source water in Shanghai, China, was mainly from animal farms, especially cattle farms. In this study, *C. andersoni* was the dominant species, as it was found in more than 80% (14/17) of the *Cryptosporidium*-positive samples detected by PCR. *Cryptosporidium andersoni* is predominantly a parasite of juvenile and adult cattle and has been found only on a few occasions in small numbers of other farm animals, such as sheep, pigs, and horses (56). Interestingly, several other common bovine *Cryptosporidium* genotypes, such as *C. parvum* in preweaned calves and *C. bovis* and *C. ryanae* in older calves, were not found in water samples, indicating that adult cattle contributed to the contamination more than juvenile cattle and calves. Similar to this study, a predominance of *C. andersoni* was also found in the Potomac River and other watersheds in the United States (59, 61) and in other studies in industrialized nations (3, 14, 38, 44).

Genotyping results also suggested that pigs and birds were also important contamination sources in the source water in Shanghai, China. *Cryptosporidium suis* found in this study is a dominant species in pigs, whereas *C. baileyi* and *C. meleagridis* are mainly parasites of birds (57). Previously, *C. suis* has not been found in source water, whereas *C. baileyi* was found in one sample each in Canada (45) and Germany (53), and *C. meleagridis* was found in two samples in Japan (28). Finding *Cryptosporidium* from farm animals in a short river spanning

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mostly urban and suburban areas in one of the world's largest metropolitan areas was unexpected. Nevertheless, this result is in agreement with the fact that several upstream townships and villages are major producers of farm animals.

Surprisingly, urban wastewater discharge was not a significant contributor to *Cryptosporidium* oocyst contamination in this study. Although *C. hominis* is the most common *Cryptosporidium* genotype in untreated urban wastewater in the study area (18), only one source water sample was positive for *C. hominis* in this study. This is possibly because the sampling point is within the source water protection area of Shanghai, China. As expected, *Cryptosporidium* genotypes from wildlife were not found in this study, indicating that unlike source water supplying some major metropolitan areas in industrialized nations (25, 27, 33, 45, 53, 61), wild animals do not appear to be a source for *Cryptosporidium* contamination in source water in the Shanghai area. The river studied, the Huangpu River, is located in one of the highly developed regions in China with a high population density and intensive animal husbandry.

Among the five *Cryptosporidium* species found in water, only *C. hominis* and *C. meleagridis* are major human pathogens. The majority of human *Cryptosporidium* infections are caused by *C. hominis* and *C. parvum* (56). Even though *C. meleagridis* is also responsible for some human infections, it was found in this study at a very low prevalence. Human infections with *C. andersoni* and *C. suis* have each been reported in only two cases and three cases, respectively (10, 32, 35). Therefore, most the *Cryptosporidium* oocysts detected in source water in this study were not from major species that infect humans. On the other hand, finding *Cryptosporidium* oocysts in two drinking water samples highlights the need for more stringent treatment of drinking water in Shanghai.

In conclusion, the occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in source water and the presence of *Cryptosporidium* in tap water in Shanghai, China, represent some potential water quality and public health problems. They also suggest the need for regular monitoring of the two pathogens in source water and treated water, which is currently impractical because of the expense of using Method 1623. The data generated by Method 1623 are also not very useful in accurate risk assessment of contamination. In contrast, the combined use of CCF for pathogen concentration and PCR for pathogen detection and genotyping can provide data that are useful in assessing the extent, source, and human infection potential of *Cryptosporidium* oocysts in water samples. The use of CCF and PCR in this study led to the identification of the farm animal origin and non-human-pathogenic nature of *Cryptosporidium* oocysts in source water in Shanghai. With further development, this technique would be a less expensive alternative to the standard Method 1623 for monitoring *Cryptosporidium* and *Giardia* contamination in raw source water in developing countries.

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