Environmental Transport of Emerging Human-Pathogenic *Cryptosporidium* Species and Subtypes through Combined Sewer Overflow and Wastewater

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ABSTRACT The environmental transport of *Cryptosporidium* spp. through combined sewer overflow (CSO) and the occurrence of several emerging human-pathogenic *Cryptosporidium* species in developing countries remain unclear. In this study, we collected 40 CSO samples and 40 raw wastewater samples from Shanghai, China, and examined them by PCR and DNA sequencing for *Cryptosporidium* species (targeting the small subunit rRNA gene) and *Giardia duodenalis* (targeting the triosephosphate isomerase, β-giardin, and glutamate dehydrogenase genes) and *Enterocytozoon bieneusi* (targeting the ribosomal internal transcribed spacer) genotypes. Human-pathogenic *Cryptosporidium* species were further subtyped by sequence analysis of the 60-kDa glycoprotein gene, with additional multilocus sequence typing on the emerging zoonotic pathogen *Cryptosporidium ubiquitum*. *Cryptosporidium* spp., *G. duodenalis*, and *E. bieneusi* were detected in 12 and 15, 33 and 32, and 37 and 40 CSO and wastewater samples, respectively, including 10 *Cryptosporidium* species, 3 *G. duodenalis* assemblages, and 8 *E. bieneusi* genotypes. In addition to *Cryptosporidium hominis* and *Cryptosporidium parvum*, two new pathogens identified in industrialized nations, *C. ubiquitum* and *Cryptosporidium viatorum*, were frequently detected. The two novel *C. ubiquitum* subtype families identified appeared to be genetic recombinants of known subtype families. Similarly, the dominant group 1 *E. bieneusi* genotypes and *G. duodenalis* subassemblage AII are known human pathogens. The similar distribution of human-pathogenic *Cryptosporidium* species and *E. bieneusi* and *G. duodenalis* genotypes between wastewater and CSO samples reaffirms that storm overflow is potentially a significant contamination source of pathogens in surface water. The frequent identification of *C. ubiquitum* and *C. viatorum* in urban wastewater suggests that these newly identified human pathogens may be endemic in China.

IMPORTANCE *Cryptosporidium* spp., *Giardia duodenalis*, and *Enterocytozoon bieneusi* are major waterborne pathogens. Their transport into surface water through combined sewer overflow, which remains largely untreated in developing countries, has not been examined. In addition, the identification of these pathogens to genotypes and subtypes in urban storm overflow and wastewater is necessary for rapid and accurate assessment of pathogen transmission in humans and transport in the environment. Data from this study suggest that, like untreated urban wastewater, combined sewer overflow is commonly contaminated with human-pathogenic *Cryptosporidium*, *G.

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duodenalis, and *E. bieneusi* genotypes and subtypes, and urban storm overflow potentially plays a significant role in the contamination of drinking source water and recreational water with human pathogens. They also indicate that *Cryptosporidium ubiquitum* and *Cryptosporidium viatorum*, two newly identified human pathogens, may be common in China, and genetic recombination can lead to the emergence of novel *C. ubiquitum* subtype families.

KEYWORDS *Cryptosporidium*, *Giardia duodenalis*, *Enterocytozoon bieneusi*, wastewater, combined sewer overflow, environmental transport, microbial source tracking

W aterborne pathogens, such as *Cryptosporidium* spp., *Giardia duodenalis*, and *Enterocytozoon bieneusi*, are significant causes of diarrhea in children worldwide, especially in developing countries. *Cryptosporidium* spp. and *E. bieneusi* further cause high morbidity and mortality in immunocompromised persons, such as HIV patients, cancer patients, and organ transplant recipients (1, 2). The presence of these pathogens in the environment is a major threat to public health. Humans can be infected with these parasites via contaminated food, water, and fomites or physical contact with infected persons or animals. Nearly 90% of documented outbreaks of these pathogens are associated with water (3).

The environmental ecology of these waterborne parasites remains unclear, especially in developing countries. In industrialized nations, urban wastewater discharge appears to play an important role in their environmental transport, as is well demonstrated in the investigation of the massive cryptosporidiosis outbreak in Milwaukee, WI, in 1993, which was attributed to contamination of drinking source water by untreated urban wastewater due to an extreme weather event (4). The role of wastewater and storm water in environmental transport of waterborne pathogens, especially emerging *Cryptosporidium* species in developing countries, however, has not been thoroughly examined. There is also a need to characterize these pathogens using molecular tools for rapid and accurate assessment of their environmental transport.

Shanghai, one of the most populous cities in China, has numerous pump stations to collect raw wastewater and storm water to wastewater treatment plants. In some districts, a combined sewer overflow (CSO) system is used in wastewater disposal, in which runoff from rains is collected using the same network for sewage and other types of wastewater. After heavy rains, the wastewater mixed with storm runoff is discharged directly to a network of streams and creeks, as its volume exceeds the capacity of the reservoirs in pump stations. These streams and creeks are connected to the Huangpu River, which is one of the drinking water sources in Shanghai. Although the city is located near the outlet of the river, storm overflow at the upstream suburbs can potentially contaminate drinking source water and recreational water.

There are many reports of *Cryptosporidium* spp. and *G. duodenalis* in wastewater in recent years, but characterizations of pathogens using molecular methods are scarce. In China, one study characterized *Cryptosporidium* spp., *G. duodenalis*, and *E. bieneusi* in raw wastewater samples from four cities using PCR-sequencing tools (5), whereas another genotyped and subtyped *Cryptosporidium* spp. and *G. duodenalis* in wastewater samples in another city using the same approach (6). The results of the two studies suggest that wastewater may contribute to environmental contamination of waterborne pathogens. The role of CSO in the environmental transport of these major waterborne pathogens in China and elsewhere has not been examined using molecular tools.

This study was conducted to evaluate the transport of human-pathogenic *Cryptosporidium* spp., *G. duodenalis*, and *E. bieneusi* in Shanghai by genotyping and subtyping pathogens in both untreated wastewater and CSO. The identification of pathogens in wastewater and CSO at the genotype and subtype levels provides much needed data on the genetic diversity and environmental transport of major waterborne pathogens as well as the occurrence of some newly identified human-pathogenic *Cryptosporidium*

TABLE 1 Occurrence of *Cryptosporidium* spp. in 40 combined sewer overflow samples and 40 wastewater samples from Shanghai, China

Species/genotype or subtype	No. of positive samples (no. of positive PCR products)	
	CSO	Wastewater
Species in humans		
C. hominis	5 (8)	4 (8)
IaA18R4	1 (5)	1 (1)
IbA19G2	1 (2)	1 (5)
C. viatorum	4 (8)	3 (3)
XVaA6		1 (1)
Species in domestic animals		
C. parvum	2 (4)	4 (4)
IIdA19G1		1 (1)
C. felis		1 (1)
Species in birds		
C. baileyi	1 (1)	3 (5)
C. meleagridis	3 (3)	1 (1)
IIIbA22G1R1c		1 (1)
Species in rodents		
C. ubiquitum	4 (4)	5 (7)
XIIg		3 (4)
XIIh		1 (1)
C. muris	2 (2)	3 (3)
Rat genotype I		1 (1)
Rat genotype IV		1 (1)
Total[a] (%)	12/40 (30.0)[b]	15/40 (37.5)[b]

[a]A sample containing multiple genotypes was counted as one positive case.
[b]$P = 0.637$ for the difference between CSO and wastewater samples.

species, such as *Cryptosporidium ubiquitum* and *Cryptosporidium viatorum*, in developing countries. Results of multilocus sequencing typing (MLST) indicate that genetic recombination may be responsible for the emergence of novel *C. ubiquitum* subtype families.

RESULTS

Occurrence of *Cryptosporidium* spp. PCR analysis revealed that 12 (30.0%) CSO samples and 15 (37.5%) wastewater samples were positive for *Cryptosporidium* spp. ($P = 0.637$ for the difference). Based on sequence analysis of the partial small-subunit (SSU) rRNA gene, seven species were identified in both CSO and wastewater samples, including *Cryptosporidium hominis* (5/40 and 4/40, respectively), *C. ubiquitum* (4/40 and 5/40), *C. viatorum* (4/40 and 3/40), *Cryptosporidium parvum* (2/40 and 4/40), *Cryptosporidium muris* (2/40 and 3/40), *Cryptosporidium meleagridis* (3/40 and 1/40), and *Cryptosporidium baileyi* (1/40 and 3/40). Three other *Cryptosporidium* species or genotypes, including *Cryptosporidium felis* (1/40), rat genotype I (1/40), and rat genotype IV (1/40), were each found in one wastewater sample (Table 1).

Subtype distribution of *Cryptosporidium* spp. Two CSO samples and two wastewater samples containing *C. hominis* and one wastewater sample containing *C. parvum* were successfully amplified at the *gp60* locus. Two distinct *C. hominis* subtypes (IaA18R4 and IbA19G2) and one *C. parvum* subtype (IIdA19G1) were identified (Table 1).

Four *C. ubiquitum*-positive wastewater samples produced the expected *gp60* PCR product, leading to the identification of two new subtype families that were named as XIIg and XIIh according to the established nomenclature (7). XIIg had 88% to 91% nucleotide sequence similarity to known subtype families XIIa to XIId, whereas XIIh appeared to be a genetic recombinant between XIId and XIIc, with sequence mostly identical to XIId except for the 150-bp sequence at the 3′ end, which was identical to
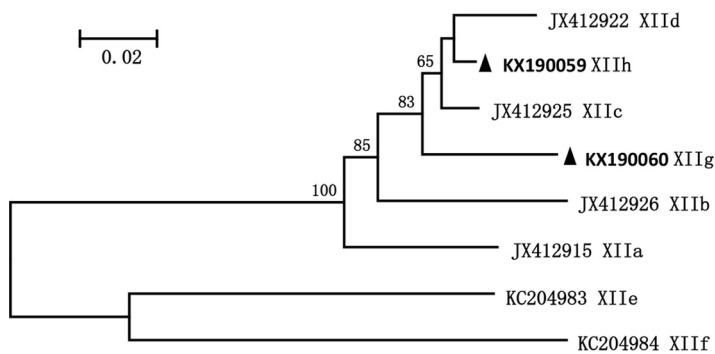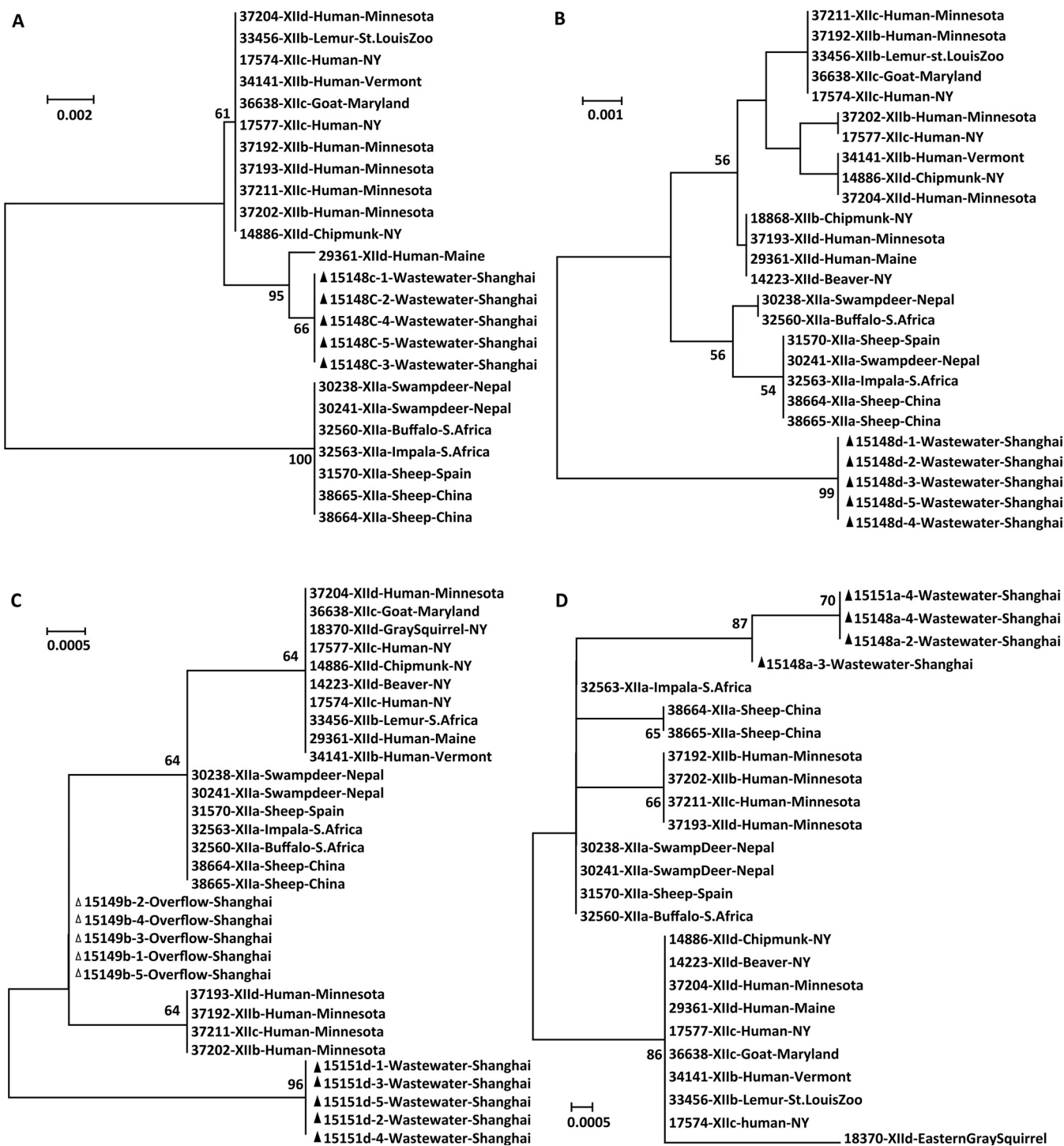
FIG 1 Phylogenetic relationship of novel *Cryptosporidium ubiquitum* subtype families detected in wastewater and combined sewer overflow samples as determined by a neighbor-joining analysis of *gp60* nucleotide sequences based on Kimura 2-parameter distances. Genotypes with ▲ are novel genotypes found in this study.

XIIc. In phylogenetic analysis, XIIh clustered with XIId, whereas XIIg was placed outside the clade containing XIId and XIIc (Fig. 1).

One wastewater sample positive for *C. meleagridis* was successfully amplified and subtyped as IIIbA22G1R1c (GenBank accession number KJ210617) based on DNA sequence analysis. Similarly, one *C. viatorum*-positive wastewater sample was identified as a new subtype, XVaA6, according to the established nomenclature (8).

***Cryptosporidium ubiquitum* MLSTs.** Two wastewater samples and one CSO sample were positive for *C. ubiquitum* as MLST analysis indicated. As they belonged to two new subtype families, these *C. ubiquitum* samples were further compared with data from known subtype families at four genetic loci. (i) At the *cgd6_60* locus, all five sequences obtained from one wastewater sample were similar to the sequence KX28286380 obtained from a human patient infected with the *C. ubiquitum* XIId subtype family in Maine, with two nucleotide substitutions. In phylogenetic analysis, they formed a cluster that is related to other XIIb, XIIc, and XIId subtype families but without clear separation of *cgd6_60* sequences from these subtype families (Fig. 2A). (ii) At the *cgd6_1590* locus, all five sequences obtained from one wastewater sample were identical to each other and had 93% to 98% nucleotide sequence similarity to those from XIIa, XIIb, XIIc, and XIId. In phylogenetic analysis, they formed a cluster that was divergent from clusters formed by *cgd6_1590* sequences from other subtype families. There was no consistent separation of *cgd6_1590* sequences from XIIb, XIIc, and XIId subtype families (Fig. 2B). (iii) At the *cgd2_3690* locus, five sequences obtained from one CSO sample were identical to each other and had only one nucleotide difference from *cgd2_3690* sequences from XIIa, where the other five sequences from a wastewater sample had 5 to 6 nucleotide differences from XIIa and other subtype families. In phylogenetic analysis, both formed their own clusters. There was no consistent separation of *cgd2_3690* sequences from XIIb, XIIc, and XIId subtype families (Fig. 2C). (iv) At the *cgd4_370* locus, the four sequences obtained from two wastewater samples were almost identical to each other, with only 2 to 3 nucleotide differences from the *cgd4_370* sequences previously obtained from XIIa. In phylogenetic analysis, they formed a cluster that was divergent from clusters formed by other subtype families. There was no consistent clustering of *cgd4_370* sequences from subtype families XIIb, XIIc, and XIId (Fig. 2D).

Occurrence of *Giardia duodenalis* assemblages and subtypes. Among the 40 wastewater samples, 32, 30, and 29 were PCR positive at the *tpi*, *gdh*, and *bg* loci, respectively. Similarly, of the 40 CSO samples, 33, 30, and 31 were PCR positive at these loci, respectively. The most commonly detected subtype in wastewater and CSO was A2 at all three genetic loci, indicating that it belonged to subassemblage AII. In addition, assemblage B, assemblage G, and three new assemblage A subtypes related to A2 were seen in a few wastewater and CSO samples (Table 2).

FIG 2 Phylogenetic relationship of *Cryptosporidium ubiquitum* in wastewater and combined sewer overflow samples at the *cgd6_60* (A), *cgd6_1590* (B), *cgd2_3690* (C), and *cgd4_370* (D) loci as determined by neighbor-joining analyses of nucleotide sequences based on Kimura 2-parameter distances. Sequences with △ are from combined sewer overflow samples, and those with ▲ are from wastewater samples.

Occurrence of *Enterocytozoon bieneusi* genotypes. *E. bieneusi* was detected in all 40 wastewater samples and 37 (92.5%) of the 40 CSO samples. Eight *E. bieneusi* genotypes were detected in wastewater samples, including D, PigEBITS7, Henan V, type IV, Peru 8, Peru 11, and two new genotypes SHW1 and SHW2. Among them, 17 samples had two or more *E. bieneusi* genotypes. These genotypes were also found in CSO samples, except SHW1 (Table 3). Among genotypes detected in this study, D, PigEBITS7,

TABLE 2 Assemblages and subtypes of *Giardia duodenalis* in 40 combined sewer overflow samples and 40 wastewater samples from Shanghai, China, as indicated by sequence analysis of three genetic loci (*tpi*, *gdh*, and *bg*)

Source	Genotype or subtype (accession no.)	No. of positive samples (no. of positive PCR products)		
		tpi	*gdh*	*bg*
CSO	A2 (*tpi*, U57897; *gdh*, AY178737; *bg*, AY072723)	28 (47)	29 (51)	27 (43)
	A3 (FJ971415)			4 (5)
	A2-like (1 SNP compared to KJ888992)	2 (3)		
	B (1 SNP compared to KP687778)		1 (1)	
	G (2 SNPs compared to EU781013)	1 (1)		
	A (AB516350)	2 (2)		
	Subtotal	33	30	31
Wastewater	A2 (*tpi*, U57897; *gdh*, AY178737; *bg*, AY072723)	30 (44)	30 (48)	23 (37)
	A3 (FJ971415)			5 (5)
	B (KP687778)			1 (1)
	A (AB516350)	2 (2)		
	Subtotal	32	30	29

Henan V, Peru 8, Peru 11, and SHW1 belonged to subgroup 1a; SHW2 belonged to subgroup 1b; and type IV belonged to subgroup 1c (Fig. 3).

DISCUSSION

In this study, the transport of waterborne pathogens *Cryptosporidium* spp., *Giardia duodenalis*, and *Enterocytozoon bieneusi* through CSO and the occurrence of several emerging *Cryptosporidium* species in China were examined using molecular diagnostic tools. The genetic characteristics of *C. ubiquitum* and *C. viatorum* were further assessed using newly established subtyping and MLST tools. Data from the study suggest that the same waterborne pathogens presented in urban wastewater are frequently detected in CSO. The most frequently identified *Cryptosporidium* species and *G. duodenalis* and *E. bieneusi* genotypes included *C. hominis*, *C. parvum*, *C. meleagridis*, *G. duodenalis* assemblages A, and *E. bieneusi* group 1 genotypes, which are all recognized human pathogens (9–12). This is similar to the observations of previous studies of wastewater samples from Shanghai and other cities in China (5, 6, 13) but different from other

TABLE 3 Genotype of *Enterocytozoon bieneusi* in 40 combined sewer overflow samples and 40 wastewater samples from Shanghai, China

Genotype[a]	GenBank accession no.[b]	No. of detections in CSO	No. of detections in wastewater
D	DQ683755	17	20
PigEBITS7	AF348475	0	1
Peru 11	AY371286	1	0
Type IV	AF242478	0	1
SHW2	KX190063	0	1
D, PigEBITS7		7	5
D, SHW1		3	1
D, SHW2		0	1
D, Peru 8		0	1
D, Peru 11		0	1
D, Henan V		7	7
D, PigEBITS7, SHW1		0	1
D, Peru 8, type IV		1	0
D, Peru 8, Peru 11, Henan V		1	0
Total		37	40

[a]Type IV and SHW2 belong to subgroup 1c and 1b, respectively. The remaining genotypes found in the present study belong to subgroup 1a.
[b]Accession numbers of genotypes in samples with multiple genotypes: SHW1, KX190062; Peru 8, AY371283; Henan V, KJ651439.
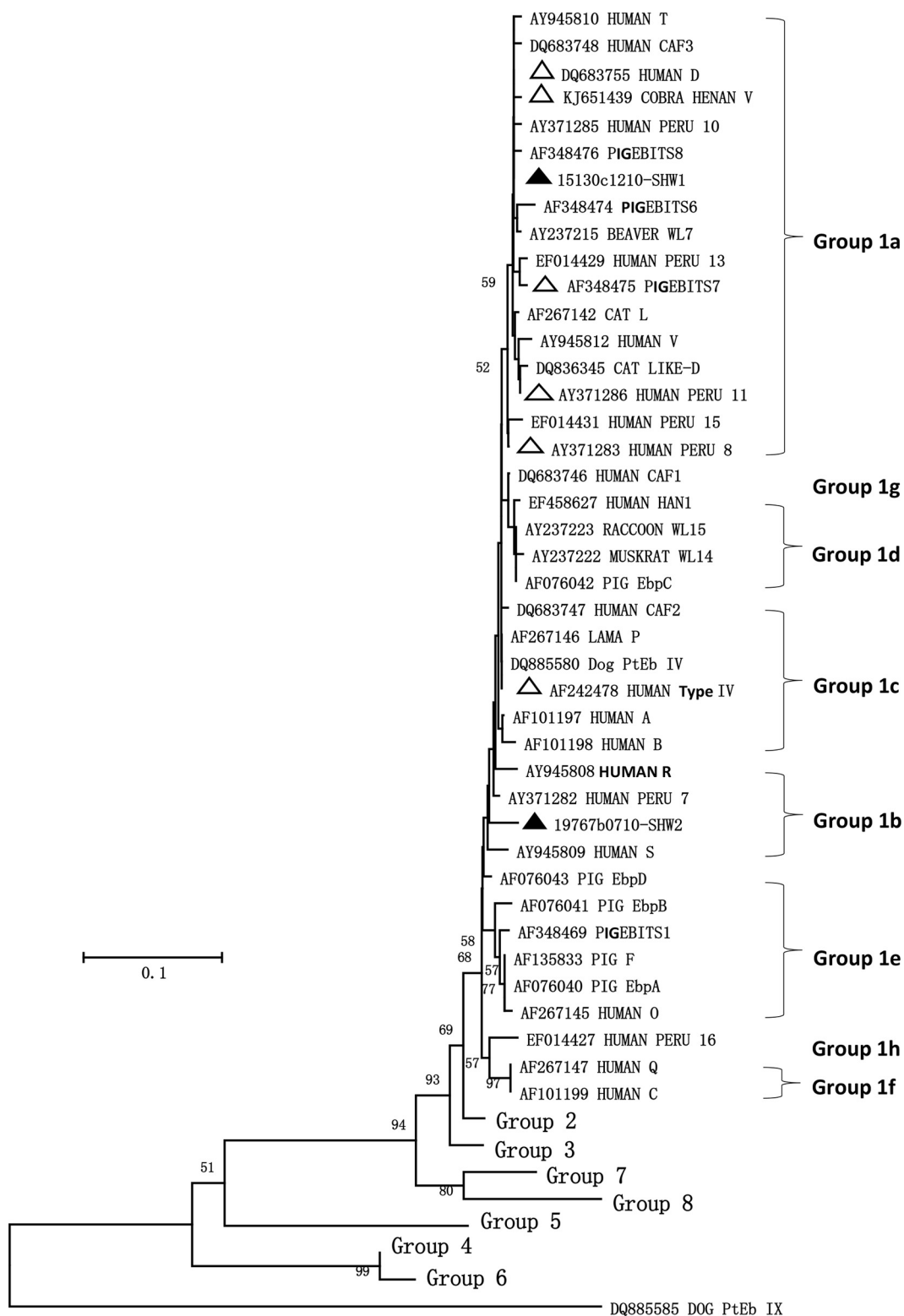
FIG 3 Phylogenetic analysis of *Enterocytozoon bieneusi* genotypes detected in wastewater and combined sewer overflow as determined by a neighbor-joining analysis of ribosomal ITS sequences based on Kimura 2-parameter distances. Genotypes with △ are known genotypes detected in wastewater and combined sewer overflow samples, and those with ▲ are novel genotypes found in this study.

studies conducted in cities with large slaughterhouses, where some *Cryptosporidium* species from farm animals, such as *Cryptosporidium andersoni*, are frequently detected in wastewater samples (14, 15). The human origin of the pathogens in the present study is apparently responsible for the similar distribution of *Cryptosporidium*, *G. duodenalis*, and *E. bieneusi* genotypes between wastewater and CSO. The discharge of CSO into the river system is obviously a public health issue that remains to be addressed fully in urban areas of developing countries.

Subtyping *Cryptosporidium* spp. and *G. duodenalis* supports the human-origin nature of dominant pathogens in CSO. The *C. hominis* (IaA18R4 and IbA19G2), *C. parvum* (IIdA19G1), and *G. duodenalis* (AII) subtypes found in the present study are dominant subtypes of these pathogens in humans in Shanghai (16, 17). In contrast, *G. duodenalis* subassemblage AI, which commonly infects animals, was not detected in both wastewater and CSO.

Phylogenetic analysis of *E. bieneusi* sequences lends further support to the human-origin nature of common pathogens in wastewater and CSO. Six known genotypes of *E. bieneusi* (i.e., D, PigEBITS7, Henan V, Peru 8, Peru 11, and type IV) were found in this study. Among them, Peru 8 and Peru 11 have been reported only in humans (18), whereas others infect humans as well as animals. Within group 1, except for type IV, which is zoonotic and belongs to subgroup 1c, all of the known genotypes in this study belong to subgroup 1a. One of the two novel genotypes, SHW1, also belonged to subgroup 1a, whereas the other one, SHW2, belonged to subgroup 1b. Genotypes in subgroups 1a, 1b, and 1c are well-known *E. bieneusi* genotypes that infect humans, even though they are also found in some animals (19). In contrast, some host-adapted group 1 genotypes, such as those in subgroup 1e, which are common pathogens in pigs, were not found in this study. The dominant *E. bieneusi* genotype in wastewater and CSO in this study, genotype D, thus far has not been identified in humans in China because of the lack of studies. However, it was identified as the dominant *E. bieneusi* genotype in raw wastewater in Shanghai and other cities and in nonhuman primates in China, indicating that it may also be a major human pathogen in China (5, 20).

Two emerging human-pathogenic *Cryptosporidium* species, including *C. ubiquitum* and *C. viatorum*, were found in both wastewater and CSO samples at a higher frequency than other species. *C. ubiquitum* is a common parasite of small ruminants and rodents. However, it is becoming an important *Cryptosporidium* species in humans in industrialized nations (21, 22), although it has been reported in only a few human cases in developing countries (21, 23, 24). In contrast, *C. viatorum* is known as a human pathogen in developing countries, as human infections in the United Kingdom and Sweden have been linked to travels to India, Pakistan, Bangladesh, Kenya, Barbados, and Guatemala (8). However, its presence in developing countries has been confirmed only in Ethiopia and Nigeria (25, 26). In the present study, *C. viatorum* was detected at a frequency similar to those of *C. hominis*, *C. parvum*, and *C. ubiquitum*. The detection of *C. ubiquitum* and *C. viatorum* in wastewater and CSO suggests that these two *Cryptosporidium* species are probably circulating in humans in China, and waterborne transmission plays a potential role in their epidemiology. Previously, they were not detected in raw wastewater in Shanghai and three other major Chinese cities (5). As there have been increased population exchanges between China and African nations in recent years, further studies are needed to monitor their environmental transport and human infection in China.

Subtype analysis of *C. ubiquitum* and *C. viatorum* has shown the genetic uniqueness of these two *Cryptosporidium* species in China. The two *C. ubiquitum* subtypes found in this study belong to two novel subtype families (21), and the *C. viatorum* subtype (XVaA6) found in this study has not been reported elsewhere (8). In phylogenetic analysis of *gp60* sequences, the new *C. ubiquitum* subtype families formed a cluster with the rodent subtype families XIIb, XIIc, and XIId rather than the ruminant subtype family XIIa. The genetic uniqueness of the new *C. ubiquitum* subtype families has been confirmed by data from a newly developed MLST analysis, with sequences from China

clustered together at each of the four polymorphic loci in addition to *gp60*. Both *gp60* sequence and MLST analyses suggest that the two new *C. ubiquitum* subtype families are probably genetic recombinants. Previously, phylogenetic analysis of sequences from these MLST loci also showed no consistent cluster formations among XIIb, XIIc, and XIId isolates (27). Thus, unlike the ruminant-adapted XIIa subtype family, XIIb, XIIc, and XIId from rodents and the two novel subtype families identified in China are all genetic recombinants and different subtype families identified in rodents at the *gp60* locus are in fact members of the same host-adapted group. The MLST data further suggest that human *C. ubiquitum* infection in China may be caused by pathogens of rodent rather than ruminant origin as seen previously in the United States (21).

In summary, data from this study suggest that molecular diagnostic tools can be used effectively in characterizing the transport of human-pathogenic *Cryptosporidium* spp., *G. duodenalis*, and *E. bieneusi* through CSO, and the distribution of species and genotypes of these pathogens in CSO from Shanghai, China, is similar to that in wastewater. They confirm CSO as a threat to contamination of drinking source water in developing countries and suggest that it may be a vehicle for environmental transport of emerging human-pathogenic *Cryptosporidium* species, such as *C. viatorum* and *C. ubiquitum*. Genetic recombination appears to be a driving force for the emergence of novel *C. ubiquitum gp60* subtype families. Efforts should be made to confirm the occurrence of these pathogens in humans in China and elucidate their environmental ecology.

MATERIALS AND METHODS

Study sites. Two wastewater pump stations located in the Yangpu and Hongkou districts in Shanghai were studied. The population densities in the two districts are 21,747 people/km² and 36,014 people/km², respectively. The areas studied adopt a CSO system in wastewater disposal, in which storm water and wastewater are not separated as in other districts of the inner city. In days with heavy rains, storm water mixed with sewage is discharged into a network of streams, which flow into the Huangpu River.

Sample collection and processing. Grab samples of untreated wastewater and CSO were used in this study. A total of 80 samples were collected from the two pump stations from June through September in 2012 and 2014. Among the 80 samples, 40 were CSO samples collected in days with heavy rains and 40 samples were wastewater samples collected from the same sites 1 day after rain events, with one sample at each site each time. Each sample was collected using a 3-liter plastic container. The 3-liter samples were transported to the laboratory immediately, and pathogens in them were concentrated by centrifugation at $1,500 \times g$ for 20 min. After the process, 600 to 1,000 μl of pellet was generated. Half (300 to 500 μl) of the pellet from each sample was used in DNA extraction immediately or stored at $-80°C$ for less than 2 weeks.

DNA extraction and pathogen detection by PCR. DNA was extracted from the sample concentrates using a FastDNA SPIN kit for soil (BIO 101, Carlsbad, CA) and eluted into 100 μl of reagent-grade water. The extracted DNA was kept at $-20°C$ before being used in PCR analysis. Each sample was analyzed in quintuplicate using 2 μl of DNA per PCR, with the inclusion of two positive PCR controls and one negative PCR control. No measurement of the DNA concentration was made prior to PCR analysis.

To detect and identify *Cryptosporidium* spp., an ~580-bp fragment of the small-subunit (SSU) rRNA gene was amplified by nested PCR (28). Specimens that were positive for *C. hominis* or *C. parvum* were subtyped by nested PCR analysis of the 60-kDa glycoprotein (*gp60*) gene (7). Those containing *C. meleagridis*, *C. ubiquitum*, and *C. viatorum* were subtyped at the *gp60* locus using PCR methods developed specifically for these human pathogens (8, 21, 29). *C. ubiquitum* was further characterized by a newly established MLST method (27). Only four polymorphic loci, including the *cgd2_3690*, *cgd4_370*, *cgd6_60*, and *cgd6_1590* genes, were targeted.

The detection of *G. duodenalis* was conducted using nested PCR analysis of the triosephosphate isomerase (*tpi*), β-giardin (*bg*), and glutamate dehydrogenase (*gdh*) genes as described previously (30). Nested PCR analysis of an ~500-bp fragment of the rRNA gene containing the entire internal transcribed spacer (ITS) was used in the detection of *E. bieneusi* (5).

Genotyping and subtyping of pathogens by sequence analysis. To identify genotypes and subtypes of pathogens, all positive secondary PCR products from the genetic loci described above were sequenced directly using the BigDye Terminator v3.1 cycle sequencing kits (Applied Biosystems, Foster City, CA). The sequence accuracy was confirmed by bidirectional sequencing. The sequences were read on an ABI3170 genetic analyzer (Applied Biosystems) and assembled using ChromasPro (v1.33) software (http://technelysium.com.au). Nucleotide sequences obtained were aligned with references of each gene downloaded from GenBank using ClustalX (http://www.clustal.org) to determine the genotype or subtype of these pathogens. The accepted terminology was used in naming *Cryptosporidium* subtypes and *E. bieneusi* genotypes (31, 32). Neighbor-joining analysis implemented in Mega 6.0 (http://www.megasoftware.net) was used to assess the phylogenetic positions of new *C. ubiquitum* subtype families

and *E. bieneusi* genotypes found in this study based on genetic distances calculated using the Kimura 2-parameter model.

Statistical analysis. The frequency of *Cryptosporidium* occurrence between wastewater and overflow was compared using the χ^2 test implemented in SPSS 19.0 for Windows (SPSS, Inc., Chicago, IL, USA). Differences with a *P* value of <0.05 were considered significant.

Accession number(s). Representative nucleotide sequences generated from the study were submitted to GenBank under accession numbers KX190059 to KX190063 and KY676886 to KY676890.

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