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Cryptosporidium Genotype and Subtype Distribution in Raw Wastewater in Shanghai, China: Evidence for Possible Unique *Cryptosporidium hominis* Transmission▽

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To identify the genotype and subtype distributions of *Cryptosporidium* oocysts in domestic wastewater in Shanghai, China, and to facilitate the characterization of the endemic transmission of cryptosporidiosis, raw domestic wastewater samples were collected from four wastewater treatment plants in Shanghai, China, from December 2006 to April 2007. Genotypes of *Cryptosporidium* species were detected based on PCR-restriction fragment length polymorphism and sequence analyses of the small-subunit rRNA gene. Samples that contained *Cryptosporidium hominis* were further subtyped by DNA sequencing of the 60-kDa glycoprotein gene. Among a total of 90 samples analyzed, 63 were PCR positive, 10 of which had mixed genotypes. Fifty-nine (93.7%) of the PCR-positive samples had *C. hominis*, and 7 (11.1%) had *C. meleagridis*. The other seven *Cryptosporidium* species/genotypes identified included *C. baileyi*, *C. parvum*, *C. suis*, *C. muris*, rat genotype, avian genotype III, and a novel genotype. Forty-seven of the 59 *C. hominis*-positive samples were successfully subtyped, with 29 having subtype family Ib and the remaining belonging to subtype families Ia, Id, Ie, and If. The three Ib subtypes identified, IbA19G2, IbA20G2, and IbA21G2, were very different from the two common Ib subtypes (IbA9G3 and IbA10G2) found in other areas of the world. Likewise, the Ie subtype IeA12G3T3 was also different from the common IeA11G3T3 subtype. Thus, the presence of multiple subtype families and unique Ib, Ie, and If subtypes indicates that there might be endemic transmission of cryptosporidiosis in the study area and that *C. hominis* populations there might be very different from those in other areas.

Cryptosporidium species are a significant cause of diarrheal diseases in both developing and industrialized nations. Recent molecular epidemiologic studies of cryptosporidiosis helped researchers to better understand the transmission of cryptosporidiosis in humans and the public health significance of *Cryptosporidium* spp. in animals and the environment. With the use of genotyping tools, five species of *Cryptosporidium* (*Cryptosporidium hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, and *C. canis*) have been shown to be responsible for most human infections. Of these five species, *C. hominis* and *C. parvum* are the most common (49). Recently, a number of subtyping tools have been developed to characterize the transmission dynamics of *C. parvum* and *C. hominis* (2, 19, 30, 36–38, 40).

Subtyping tools based on sequence analysis of the 60-kDa glycoprotein (gp60) gene have proven to be effective in studying the transmission of human cryptosporidiosis, and the results of such studies were recently reviewed (51). These studies have shown the complexity of *Cryptosporidium* transmission in areas where it is endemic. Among the five common *C. hominis* subtype families, Ia, Ib, Id, Ie, and If, three or four *C. hominis* subtype families were seen in humans in India, Peru, New Orleans, Malawi, South Africa, Kuwait, and Portugal, with only one or two *C. parvum* subtype families usually found in humans in the same area (1, 2, 16, 30, 37, 40, 48–50).

Detection, genotyping, and subtyping of *Cryptosporidium* species in wastewater have served as tools for molecular surveillance and characterization of cryptosporidiosis transmission (51). Although *Cryptosporidium* species were reported to be present in clinical specimens and environmental samples in China (8–10, 13, 32, 44, 53, 55), the genotypes and subtypes involved are not clear. There is only one report on genetic characterization of five *Cryptosporidium* isolates from patients in China (36). Therefore, the transmission route and infection sources of cryptosporidiosis in China are unclear. The objectives of this study were to identify the genotype and subtype distributions of *Cryptosporidium* oocysts in domestic wastewater in Shanghai, China, and to infer the endemic transmission of cryptosporidiosis.

MATERIALS AND METHODS

Wastewater sample collection and processing. A total of 90 raw wastewater samples were obtained from four wastewater treatment plants in Shanghai, China, from December 2006 to April 2007, with an average of two or three samples per week. The raw wastewater was a combination of domestic wastewater and storm water. The sampling period was selected to coincide with the drought period in Shanghai to reduce the influence of rain events on the distributions of species, genotypes, and subtype families of *Cryptosporidium* in wastewater. Grab samples of 500 to 1,000 ml of raw wastewater were collected at the entrances of the treatment plants. *Cryptosporidium* oocysts in samples were concentrated by centrifugation at 6,000 × *g* for 10 min.

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TABLE 1. Prevalence and genotype distribution of *Cryptosporidium* species in raw wastewater samples from Shanghai

Sample location[a]	No. of samples	No. (%) of positive samples	Species and/or genotype(s) (no. of samples)
WWTP 1	23	13 (56.5)	*C. hominis* (9), *C. meleagridis* (1), *C. hominis* and *C. baileyi* (1), *C. hominis* and *C. meleagridis* (1), *C. hominis* and a new genotype (1)
WWTP 2	19	13 (68.4)	*C. hominis* (10), *C. meleagridis* (1), *C. hominis* and *C. baileyi* (1), *C. hominis* and *C. muris* (1)
WWTP 3	22	14 (63.6)	*C. hominis* (11), *C. meleagridis* (2), *C. parvum* (1)
WWTP 4	26	23 (88.5)	*C. hominis* (17), *C. meleagridis* (1), *C. hominis* and *C. baileyi* (1), *C. hominis* and *C. meleagridis* (1), *C. hominis* and *C. suis* (1), *C. hominis* and rat genotype (1), rat genotype and avian genotype III (1)
Total	90	63 (70.0)	Nine species/genotypes

[a] WWTP, wastewater treatment plant.

***Cryptosporidium* genotyping and subtyping.** After washing of the samples twice in distilled water, genomic DNA was extracted from 0.5 ml of concentrate, using a 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. DNA of *Cryptosporidium serpentis* 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/μl of nonacetylated bovine serum albumin (Sigma-Aldrich, St. Louis, MO) was used in the primary PCR. All secondary PCR products for the first 50 samples were sequenced to confirm the genotype identification. PCR products for the remaining 40 samples were sequenced only when RFLP analysis showed banding patterns different from that of *C. hominis*.

Specimens that contained *C. hominis* were further subtyped by DNA sequencing of the gp60 gene amplified by a nested PCR. The primers used were modifications of previously published ones (2), as follows: LX0374 (5′-TTA CTC TCC GTT ATA GTC TCC-3′) and LX0375 (5′-GGA AGG AAC GAT GTA TCT GA-3′) for the primary PCR and AL3532 (5′-TCC GCT GTA TTC TCA GCC-3′) and AL3534 (5′-GCA GAG GAA CCA GCA TC-3′) for the secondary PCR. Nonacetylated bovine serum albumin (final concentration, 400 ng/μl) was used in the primary PCR, and 52°C was used as the annealing temperature in both primary and secondary PCRs. Each sample was amplified at least three times (five times if the three gp60 PCR replicates were all negative) by PCR, using 2 μl of the DNA extract per PCR, and when available, at least two secondary PCR products per sample were sequenced.

Sequence analysis. After purification using Montage PCR filters (Millipore, Bedford, MA), the secondary PCR products of the SSU rRNA or gp60 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 manufacturer-suggested procedures. Sequences were read on an ABI3130 genetic analyzer (Applied Biosystems). Sequence accuracy was confirmed by two-directional sequencing and sequencing of at least two PCR products for each positive sample. Nucleotide sequences obtained were aligned with reference SSU rRNA or gp60 sequences by use of the ClustalX 1.81 package (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) to infer *Cryptosporidium* genotypes and *C. hominis* subtypes.

Nucleotide sequence accession numbers. Unique partial SSU rRNA and gp60 sequences obtained during the study were deposited in the GenBank database under accession numbers FJ153238 to FJ153248, FJ205699, and FJ205700.

RESULTS

***Cryptosporidium* species and genotypes in wastewater.** A total of 90 raw wastewater samples were examined in this study, 63 of which were positive for *Cryptosporidium* species by the SSU rRNA-based PCR-RFLP technique. Restriction analysis and DNA sequencing of PCR products revealed the presence of the following nine species/genotypes of *Cryptosporidium* in the samples (Table 1): *C. hominis*, *C. meleagridis*, *C. parvum*, *C. baileyi*, *C. muris*, *C. suis*, rat genotype, avian genotype III, and a new genotype. *Cryptosporidium hominis* was the most commonly detected species/genotype overall (found in 59 samples, or 93.7% of all PCR-positive samples), followed by *C. meleagridis* (found in 7 samples, or 11.1% of all PCR-positive samples). The other species/genotypes were detected in only one to three samples. Among the 63 positive samples, 10 showed the concurrent presence of two species/genotypes of *Cryptosporidium*, the majority of which were mixed with *C. hominis*, except for one sample, which had both the rat genotype and avian genotype III. The new genotype identified from sample 78 had eight base pair differences from *C. hominis* (GenBank accession no. AF093489) in the partial SSU rRNA gene (Table 2).

Subtypes of *C. hominis* in wastewater. Wastewater samples positive for *C. hominis* (59 samples) were subtyped by gp60 sequence analysis. Forty-seven (79.7%) of the 59 samples were positive by gp60 PCR. The reduced detection rate by gp60 PCR was probably due to the single-copy nature of the target gene (instead of five copies for the SSU rRNA gene) and the small numbers and random distribution of oocysts in water samples. The obtained nucleotide sequences were compared with the known subtype sequences deposited in GenBank. This analysis suggested that 43 samples had only one subtype and 4 samples had two subtypes. Altogether, there were 10 subtypes in five subtype families in the 47 positive wastewater samples, including Ia (two subtypes in 6 samples), Ib (three subtypes in 29 samples), Id (two subtypes in 4 samples), Ie (one subtype in 6 samples), and If (two subtypes in 5 samples) (Table 3). For the Ib subtype family, 22 of 29 (75.9%) positive samples had the IbA21G2 subtype (Table 3).

DISCUSSION

There are only a few reports available on genotyping of *Cryptosporidium* species in raw wastewater, and the results of

TABLE 2. Nucleotide differences in the partial SSU rRNA gene between the new *Cryptosporidium* genotype and *C. hominis*

Genotype	Nucleotide at position[a]:							
	263	686	687	690	691	695	696	795
C. hominis (AF093489)	T	A	T	T	T	T	T	T
New genotype	A	G	A	A	A			C

[a] Nucleotide position numbers are according to the sequence under GenBank accession no. AF093498, with the beginning of the sequence as position 1.

TABLE 3. *Cryptosporidium hominis* subtypes in raw wastewater in Shanghai

Subtype family	No. of samples	Subtype	No. of samples
Ia	6	IaA18R4	3
		IaA19R4	3
Ib	27	IbA19G2	4
		IbA20G2	2
		IbA21G2	20
		IbA19G2 and IbA21G2	1
Id	4	IdA14	2
		IdA16	2
Ie	5	IeA12G3T3	5
If	2	IfA20G1	1
		IfA22G1	1
Ib plus If	2	IbA20G2 and IfA22G1	1
		IbA21G2 and IfA22G1	1
Ie plus If	1	IeA12G3T3 and IfA22G1	1

these studies are summarized in Table 4. The results of this study support previous findings of the complexity of *Cryptosporidium* populations in raw urban wastewater (22–24, 45, 46, 52, 54). However, the compositions of the populations were quite different (Table 4). Nine *Cryptosporidium* species or genotypes were found in raw wastewater samples in Shanghai, with *C. hominis* being the dominant species. Because of the host adaptation nature of *Cryptosporidium* spp., the results of this study indicate that humans (*C. hominis*, *C. parvum*, *C. meleagridis*, and *C. suis*), farm animals (*C. parvum* and *C. suis*), rodents (*C. muris* and rat genotype), and birds (*C. meleagridis*, *C. baileyi*, and avian genotype III) all contributed to *Cryptosporidium* contamination in wastewater in this study. A noticeable difference was the absence of *C. andersoni*, which was seen in some of the earlier studies in other areas (Table 4). This is consistent with the absence of cattle slaughterhouses in the studied area. Previously, it was shown that humans, slaughtered farm animals, rodents, and birds all contributed to *Cryptosporidium* contamination in urban wastewater (22–24, 45, 46, 52, 54).

The predominance of *C. hominis* in wastewater in Shanghai indicates that anthroponotic transmission is important in cryptosporidiosis epidemiology in this area. This is consistent with the finding by Peng et al. (36), who reported that all five specimens from patients in China belonged to *C. hominis*. Thus far, *C. hominis* is responsible for far more infections than *C. parvum* in humans in developing countries where genotyping studies have been conducted (3, 4, 12, 17, 18, 30, 34, 37, 39, 41–43, 47). In contrast, in the United Kingdom, other parts of Europe, and New Zealand, *C. parvum* is as common as *C. hominis* in humans (2, 6, 14, 20, 21, 25, 29, 31, 33). The differences in distribution of *Cryptosporidium* genotypes in humans are considered an indication of differences in infection sources (28, 29, 33).

The results of subtype analysis indicate that *C. hominis* transmission in Shanghai, China, is probably unique. The following are some features of *C. hominis* in wastewater in Shanghai.

(i) There are many subtype families and many subtypes within most families. In this study, there were five *C. hominis*

TABLE 4. *Cryptosporidium* genotypes in raw urban wastewater by PCR-based techniques

Sample source	Sampling period (mo/yr)	No. of positive samples/total no. of samples	Species or genotype(s) (no. of samples)	Reference
Milwaukee, WI	04/2000–07/2000	12/49	*C. andersoni* (5), *C. canis* (1), *C. muris* (1), *C. felis* (1), cervine genotype (1), *C. andersoni* and *C. hominis* (1), *C. andersoni* and *C. parvum* (1), *C. andersoni* and *C. muris* (1)	52
Switzerland and Germany	05/2000–10/2000	6/8	*C. hominis* (3), *C. parvum* (2), *C. muris* (1)	45
Milwaukee, WI	08/2000–07/2001 and 03/2002	50/179	*C. hominis* (24), *C. andersoni* (23), *C. parvum* (5), *C. muris* (4), mouse genotype (1), cervine genotype (6)	54
Vantaa River Basin, Finland	10/2001–11/2001	8/36	*C. parvum* (8), unknown (1)[a]	22
Tokyo, Japan	05/2003–08/2003	148/239	*C. hominis* (78), *C. parvum* (16), *C. meleagridis* (13), *C. suis* (5), pig genotype II (2), mouse genotype (1), unknown (27), new genotype (1)	23, 24
Milwaukee, WI	10/2002–02/2003	51/55	*C. hominis* (32), *C. andersoni* (13), *C. parvum* (8), *C. muris* (14), *C. meleagridis* (3), *C. felis* (3), *C. canis* (2), cervine genotype (9), squirrel genotype (1), W16-like (1), W19 (1), new genotype (3)	46
Galicia, Spain	01/2007–12/2007	12/12	*C. parvum* (4), *C. andersoni* (3), *C. hominis* (5)	5
Shanghai, China	12/2006–04/2007	63/90	*C. hominis* (47), *C. meleagridis* (5), *C. parvum* (1), *C. hominis* and *C. baileyi* (3), *C. hominis* and *C. meleagridis* (2), *C. hominis* and *C. muris* (1), *C. hominis* and *C. suis* (1), *C. hominis* and rat genotype (1), rat genotype and avian genotype III (1), *C. hominis* and new genotype (1)	This study

[a] The methods used detect only *C. parvum*, *C. hominis*, *C. meleagridis*, and closely related *Cryptosporidium* species and genotypes.

subtype families, Ia, Ib, Id, Ie, and If, and two or three subtypes in each family except family Ie. This is similar to the complexity of *Cryptosporidium* populations in other developing countries, such as India, Malawi, and South Africa, where many subtype families and many subtypes within the subtype families Ia and Id were reported (16, 30, 37). In contrast, in developed countries, *C. hominis* heterogeneity is generally small, as reflected by the common occurrence of subtype family Ib and/or the low heterogeneity in subtype families Ia and Id (1, 2, 7, 19, 49). In this study, many subtypes were detected in not only families Ia and Id but also families Ib and If. Thus, like in other developing countries, the high *C. hominis* heterogeneity is also likely an indicator of endemic cryptosporidiosis transmission in the studied area.

(ii) In this study, there were three unique Ib subtypes, namely, IbA19G2, IbA20G2, and IbA21G2. *Cryptosporidium hominis* family Ib is a common subtype family, and IbA9G3 and IbA10G2 are the two common subtypes within this subtype family. IbA9G3 is commonly seen in humans in Malawi, Kenya, India, and Australia, and IbA10G2 is commonly seen in South Africa, Botswana, Jamaica, Peru, the United States, Canada, Australia, and European countries (1, 2, 7, 16, 18, 19, 30, 49). In addition, IbA10G2 is responsible for more than half of the waterborne outbreaks in the United States, United Kingdom, Canada, and France (11, 19, 35, 51). The *C. hominis* IbA19G2, IbA20G2, and IbA21G2 subtypes identified in this study are very different from the two common Ib subtypes found in other areas of the world.

(iii) The presence of subtype IeA12G3T3 was observed in this study. Within the *C. hominis* subtype family Ie, humans in most areas are infected with IeA11G3T3, with the exception of Kingston, Jamaica, New Orleans, LA, and Adelaide, Australia, where IeA12G3T3 is seen (7, 15, 49).

(iv) The presence of the If subtype family was observed. *Cryptosporidium hominis* family If was detected initially in children in South Africa (30). This family was not seen in most other studies but was occasionally seen in human immunodeficiency virus-positive adults in Portugal (1). It was identified in humans in India (34), but this was based on RFLP analysis of gp60 PCR products and its identity was not established (1, 34). The common occurrence of If subtypes (5/47 samples) in this study further supports the presence of unique transmission of *C. hominis* in Shanghai, China.

In summary, the results of this study indicate that there is probably extensive transmission of cryptosporidiosis between humans in Shanghai, that anthroponotic transmission may play an important role in cryptosporidiosis epidemiology, and that the *C. hominis* populations in China may be very different from those in other areas. Nevertheless, these hypotheses are based on molecular surveillance of *Cryptosporidium* species/genotypes and subtypes in urban wastewater and suffer from the intrinsic difficulty associated with interpreting cryptosporidiosis transmission data based on oocyst shedding at the community level, as some oocysts of *Cryptosporidium* species or subtypes may be shed in larger numbers than others, resulting in the overrepresentation of these species and subtypes in domestic wastewater. Therefore, further molecular epidemiologic studies of humans in diverse areas are needed to confirm these hypotheses and to characterize *Cryptosporidium* populations, transmission dynamics, and infection sources in China.

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