Molecular characterization of the waterborne pathogens Cryptosporidium spp., Giardia duodenalis, Enterocytozoon bieneusi, Cyclospora cayetanensis and Eimeria spp. in wastewater and sewage in Guangzhou, China

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Abstract

Background: The waterborne pathogens Cryptosporidium spp., Giardia duodenalis, Enterocytozoon bieneusi and Cyclospora cayetanensis can cause intestinal diseases in humans. An understanding of their occurrence and transport in the environment is essential for accurate quantitative microbial risk assessment.

Methods: A total of 238 influent samples were collected from four wastewater treatment plants (WWTPs) and 88 samples from eight sewer locations in Guangzhou, China. PCR-based tools were used to detect and genetically characterize Cryptosporidium spp., G. duodenalis and E. bieneusi. Eimeria spp. and Cyclospora spp. were also analyzed to assess the sources of Cryptosporidium spp., G. duodenalis and E. bieneusi in wastewater.

Results: The overall occurrence rates in the WWTP and sewer samples were 14.3% (34/238) and 13.6% (12/88) for Cryptosporidium spp., 55.5% (132/238) and 33.0% (29/88) for G. duodenalis, 56.3% (134/238) and 26.1% (23/88) for E. bieneusi and 45.4% (108/238) and 47.7% (42/88) for Eimeria spp., respectively. Altogether, 11 Cryptosporidium species and genotypes, six G. duodenalis genotypes, 11 E. bieneusi genotypes and four C. cayetanensis were found, together with the presence of nine Eimeria species. The common occurrence of Cryptosporidium rat genotype IV, C. muris and Eimeria papillata and E. nieschulzi suggested that rodents were significant sources of the enteric pathogens detected in the wastewater samples.

Conclusions: While the dominant Cryptosporidium spp. detected in the raw wastewater sampled in this study are not pathogenic to humans, the widely detected G. duodenalis assemblage A and E. bieneusi genotypes D and Type IV are well-known zoonotic pathogens. Further studies are needed to monitor the occurrence of these waterborne pathogens in WWTPs to better understand their transmission and environmental transport in China.

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Background
Waterborne parasitic diseases affect humans in both developed and developing countries [1]. Among these, Cryptosporidium spp. and Giardia duodenalis are major parasitic protozoa associated with waterborne outbreaks of illnesses in the USA and Europe [2, 3], and many disease outbreaks caused by these pathogens have been reported [4]. For example, worldwide there were 239 reported outbreaks of cryptosporidiosis with approximately 65,540 cases documented between 2011 and 2016 [1], and 280 million cases of giardiasis were reported annually, mostly in Asia, Africa and South America [5]. Enterocytozoon bieneusi is a microsporidia commonly found in humans and animals that causes diarrhea in both immunocompetent and immunocompromised individuals [6]. It has been implicated in causing outbreaks of diarrhea in humans [7, 8]. Cyclospora cayetanensis is recognized as an emerging food- and waterborne parasite, transmitted by environmentally robust oocysts [9]. These pathogens are unique due to their low infective dose and robust infective stages that are also resistant to many common disinfectants, such as chlorine and chloramines [10, 11].

One illustration of the impact of waterborne parasitic diseases is the massive outbreak of cryptosporidiosis in Milwaukee, Wisconsin in 1993, which caused illness in > 400,000 people and > 100 deaths [12].

Guangzhou, with a population of > 15 million people, is one of the largest cities in China. The subtropical climate and abundant rainfall provide a favorable environment for the transmission of waterborne pathogens. However, a lack of surveillance systems in Guangzhou makes it difficult to investigate the transmission of these pathogens in the major urban environment. Currently, no relevant data are available on the prevalence of enteric parasites in humans and wastewater in the city.

Methods

Specimens
A total of 88 grab samples (500–1000 ml per sample) of raw wastewater were collected at weekly intervals between August 2018 and October 2018 from eight sites of the sewer distribution system located in Guangzhou, China. In addition, 238 raw wastewater samples were collected weekly between November 2018 and February 2019 from four wastewater treatment plants (WWTPs) in the city, including 66 from WWTP1, 28 from WWTP2, 66 from WWTP3 and 78 from WWTP4. All WWTPs examined in the study were located in areas of high population density where a high occurrence of rats and other rodents in the wastewater distribution system had been noted. All wastewater samples were collected into 1000-ml plastic bottles, stored on ice and transported to the laboratory immediately where the pathogens in the samples were concentrated by centrifugation at 1500g for 20 min, following which the sediment was collected and stored at 4°C in 2.5% potassium dichromate solution prior to DNA isolation.

Keywords: Cryptosporidium, Giardia, Enterocytozoon bieneusi, Eimeria/Cyclospora, Wastewater, Sewer system, WWTP influent, China
DNA extraction and pathogen detection by PCR
Following washing of the sample concentrates twice with distilled water to remove the potassium dichromate by centrifugation, genomic DNA was extracted from 0.5 ml of the concentrate using the FastDNA Spin Kit for Soil (MP Biomedical, Santa Ana, CA, USA) and then eluted with 100 μl reagent-grade water. Each sample was analyzed at least three times by nested PCR at each genetic locus, using 2 μl of the genomic DNA as the template for amplification. Positive, negative and no template controls were included in each PCR run. The secondary PCR products were analyzed by 1.5% agarose electrophoresis.

Detection, genotyping and subtyping of Cryptosporidium spp.
A nested PCR targeting an ~830-bp fragment of the SSU rRNA gene was used to detect Cryptosporidium spp. [22]. The Cryptosporidium spp. present in the samples were identified to the species level by sequence analysis of the secondary PCR products. The C. hominis, C. parvum and C. meleagridis thus identified were subtyped by PCR and sequence analysis of an ~850-bp fragment of the gp60 gene as described previously [23, 24].

Detection, genotyping and subtyping of G. duodenalis
The presence of G. duodenalis was determined by running three nested PCR assays targeting a 530-bp fragment of the tpi gene [25], a 599-bp fragment of the gdh gene [26] and a 511-bp fragment of the bg gene [27]. Assemblages and sub-assemblages of G. duodenalis were determined using sequence analysis of the secondary PCR products.

Detection and genotyping of E. bieneusi
A nested PCR analysis of a 392-bp fragment of the rRNA gene containing the entire ITS was used to detect E. bieneusi [28]. Genotypes of E. bieneusi were determined by sequence analysis of the secondary PCR products. The established nomenclature system was used in naming E. bieneusi genotypes [29].

Detection and identification of Eimeria spp. and Cyclospora spp.
Eimeria spp. and Cyclospora spp. were detected in a nested PCR analysis which amplified a 294-bp fragment of the SSU rRNA gene, as well as DNA of the genetically related Isospora spp. [30]. The identification of Eimeria and Cyclospora species was done by sequence analysis of the secondary PCR products.

DNA sequence analysis
All positive secondary PCR products generated from the study were sequenced in both directions on an ABI 3170 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequences were assembled using ChromasPro 1.33 (www.technelysium.com.au/ChromasPro.html), edited using BioEdit 7.0.4 (www.mbio.ncsu.edu/BioEdit/bioedit.html) and aligned using ClustalX 2.1 (www.clustal.org/). The concurrent presence of mixed species or genotypes of the pathogens under analysis was determined by discordant results among the three PCR replicates. To assess the phylogenetic placement of E. bieneusi genotypes found in this study, we constructed a maximum-likelihood tree using MEGA 6.0 (www.megasoftware.net/). The general time-reversible model and gamma distribution were used in the calculation of substitution rates, and 1000 replicates were used in bootstrap analysis of the phylogenetic tree.

Statistical analysis
The frequency of pathogen occurrence between sampling locations was compared using the χ² test implemented in SPSS 19.0 for Windows (IBM Corp., Armonk, NY, USA). Differences with P values of <0.05 were considered to be significant.

Results
Occurrence of Cryptosporidium spp. in wastewater
The PCR analysis revealed that 34 (14.3%) wastewater samples from WWTPs and 12 (13.7%) sewer samples from the sewer distribution system were positive for Cryptosporidium spp. The difference in Cryptosporidium occurrence between the samples from the WWTPs and sewer system was not significant (χ² = 0.8821, df = 1, P = 0.3484). Among the samples collected from the four WWTPs, Cryptosporidium spp. were detected in 19.7% of samples from WWTP1, 21.4% from WWTP2, 27.3% from WWTP3 and 11.5% from WWTP4 (χ² = 5.31, df = 1, P = 0.0623). Among samples from the eight sewer sampling sites, the occurrence rates of Cryptosporidium spp. varied from 0 and 27.3%. The difference in Cryptosporidium occurrence among the eight sewer sampling sites was not significant (χ² = 1.54, df = 1, P = 0.218).

Sequence analysis of the PCR products revealed the presence of 11 Cryptosporidium species and genotypes in samples collected from the WWTPs, including C. muris (n = 12), rat genotype IV (n = 10), C. baileyi (n = 8), C. bovis (n = 4), C. occultus (n = 3), C. meleagridis (n = 2), C. parvum (n = 2), C. felis (n = 2), C. serpentis (n = 1), C. canis (n = 1) and rat genotype 1 (n = 1). No concurrent
presence of multiple Cryptosporidium species or genotypes was detected. At least one Cryptosporidium species was detected in each WWTP. The two C. parvum-positive samples from WWTP2 and WWTP3 were identified being subtype IIdA15G1.

Four Cryptosporidium species were identified in samples from the sewer system, including C. felis (n = 5), rat genotype IV (n = 4), C. parvum (n = 2) and C. baileyi (n = 1). For C. parvum, only one of the two positive samples from sewer site 8 was successfully subtyped, yielding IIdA15G1. No concurrent presence of multiple Cryptosporidium species was detected.

**Occurrence of G. duodenalis assemblages and sub-assemblages in wastewater**

Among the 238 wastewater samples collected from the WWTPs, 55.5% (132/238), 52.1% (124/238) and 53.4% (127/238) were PCR positive for G. duodenalis at the gdh, tpi and bg loci, respectively. Similarly, among the 88 samples collected from the sewer system, 31.8% (28/88), 25.0% (22/88) and 25.0% (22/88) were PCR positive at these loci, respectively. The difference in G. duodenalis occurrence between samples from the WWTPs and sewer system was significant ($\chi^2 = 14.371, df = 1, P = 0.0002$). The most commonly detected genotype in the WWTP samples and the sewer system samples was assemblage A (n = 124 and 19 respectively). As subtype A2 was identified at all three genetic loci, the assemblage A belonged to the sub-assemblage AII. In addition, assemblages B (n = 4), G (n = 3) and C (n = 1) were seen in a few WWTP samples. Similarly, assemblages B (n = 5), F (n = 2) and D (n = 1) were detected in some sewer samples. The presence of multiple G. duodenalis assemblages were identified in four samples by identifying different assemblages in replicate PCR analyses of DNA (2 assemblages with A2 + B, 1 with A2 + D and 1 with A2 + F) (Table 2).

**Occurrence of Enterocytozoon bieneusi genotypes in wastewater**

PCR analysis of the ITS locus revealed the presence of E. bieneusi in 56.3% (34/62) of WWTP samples and 26.1% (23/88) of sewer samples (Table 3). The difference between the two groups was significant ($\chi^2 = 23.417, df = 1, P < 0.0001$). Among the samples from the four WWTPs, the highest occurrence was in those collected from WWTP2 (67.9%), followed by WWTP3 (57.6%), WWTP4 (53.9%) and WWTP1 (53.0%). The difference between WWTPs, however, was not significant ($\chi^2 = 1.768, df = 1, P = 0.184$). Among the eight sewer sampling sites, sites 7 and 8 showed the highest occurrence of E. bieneusi (both 63.6%).

Among the 134 E. bieneusi-positive samples from the WWTPs, nine genotypes of E. bieneusi were identified,
Table 2  *Giardia duodenalis* assemblages in samples collected from wastewater treatment plants and in sewer samples, Guangzhou, China

| Sample location | No. of samples | No. of positive samples (%) | Assemblage (no. of samples) |
|-----------------|----------------|-----------------------------|-----------------------------|
|                 | gdh tpi bg     | gdh tpi bg                  | gdh tpi bg                  |
| WWTP1           | 66             | 42 37 37                     | A (37); G (3); B (2)        |
| WWTP2           | 28             | 19 19 19                     | A (19)                      |
| WWTP3           | 66             | 29 29 32                     | A (27); C (1); B (1)        |
| WWTP4           | 78             | 42 39 39                     | A (41); B (1)               |
| Sub-total       | 238            | 124 (52.1%) 127 (53.4%)     | A (124); B (4); G (3); C (1)|
|                 |                |                             | A (117); B (3); C (2); A+B (1)|
|                 |                |                             | A (117); B (3); C (1); A+B (1)|
| Sewer 1         | 11             | 5 5 5                        | A (4); B (1)                |
| Sewer 2         | 11             | 0 0 0                        | A (5)                       |
| Sewer 3         | 11             | 0 0 0                        | A (5)                       |
| Sewer 4         | 11             | 0 0 0                        | A (5)                       |
| Sewer 5         | 11             | 1 0 1                        | A (6)                       |
| Sewer 6         | 11             | 6 5 4                        | A (3); B (2); A+B (1)       |
| Sewer 7         | 11             | 6 5 5                        | A (3); B (2); A+B (1)       |
| Sewer 8         | 11             | 10 7 7                       | A (6); B (1); A+B (1); A+D (1); A+F (1)|
| Sub-total       | 88             | 28 (31.8%) 22 (25.0%)        | A (19); B (4); A+B (2); D (1); A+D (1); A+F (1)|
|                 |                | 22 (25.0%)                   | A (19); B (5); F (2); A+B (1) |
|                 |                |                             | A (17); B (3); D (2)        |
|                 |                |                             | A (134); B (6); C (1); D (2); A+B (1) |
| Total           | 326            | 160 (49.1%) 146 (44.8%)      | A (143); B (8); G (3); C (1); D (1); A+B (2); A+D (1); A+F (1)|
|                 |                | 149 (45.7%)                  | A (132); B (6); C (2); F (2); A+B (2) |
|                 |                |                             | A (134); B (6); C (1); D (2); A+B (1)|

bg β-Giardin gene; gdh, glutamate dehydrogenase gene; gp60, 60-kDa glycoprotein gene

Table 3 Internal transcribed spacer genotypes of *Enterocytozoon bieneusi* in samples collected from wastewater treatment plants and in sewer samples, Guangzhou, China

| Sample location | No. of samples | No. of positive samples (%) | Genotypes (no. of samples) |
|-----------------|----------------|-----------------------------|----------------------------|
|                 | gdh tpi bg     | gdh tpi bg                  | gdh tpi bg                  |
| WWTP1           | 66             | 35 (53.0%)                  | D (27); Peru8 (4); Type IV + D (2); GZW1a (1); Ebpc (1) |
| WWTP2           | 28             | 19 (67.9%)                  | D (11); Type IV (5); Type IV + D (2); Peru8 + Type IV (1) |
| WWTP3           | 66             | 38 (57.6%)                  | D (22); Type IV (11); Type IV + D (1); Peru8 (2); Peru6 (1); PteB IX (1) |
| WWTP4           | 78             | 42 (53.9%)                  | D (17); Type IV (14); Peru8 (4); Peru11 (2); Type IV + D (2); Ebpc (1); Type IV + Peru11 (1); MWC-m1 (1) |
| Sub-total       | 238            | 134 (56.3%)                 | D (77); Type IV (30); Peru8 (10); Peru11 (2); Ebpc (2); Peru6 (1); MWC-m1 (1); GZW1a (1); PteB IX (1); Type IV + D (7); Type IV + Peru11 (1); Peru8 + Type IV (1) |
|                 |                | 134 (45.7%)                 | A (132); B (6); C (2); F (2); A+B (2) |
|                 |                |                             | A (134); B (6); C (1); D (2); A+B (1) |
|                 |                |                             | A (134); B (6); C (1); D (2); A+B (1) |

* Novel genotype found in this study
including D \( (n=77) \), Type IV \( (n=30) \), Peru8 \( (n=10) \), Peru11 \( (n=2) \), EbpC \( (n=2) \), Peru6 \( (n=1) \), MWCm1 \( (n=1) \) and PtEb IX \( (n=1) \). A novel genotype was detected and named GZW1 \( (n=1) \). In addition, nine samples were found to have concurrent presence of two genotypes, including Type IV + Peru11 in seven samples, Peru8 + Type IV in one sample and Type IV + D in one sample (Table 3).

Among 23 \( E. bieneusi \)-positive sewer samples, six genotypes were detected, including D \( (n=11) \), Type IV \( (n=5) \), PtEb IX \( (n=2) \), EbpC \( (n=1) \) and two novel genotypes named GZW2 \( (n=1) \) and GZW3 \( (n=1) \). In addition, the concurrent presence of two genotypes (Type IV + D) was detected in two sewer samples.

In the maximum likelihood analysis of the ITS sequences obtained, genotypes D Type IV, Peru11, EbpC, Peru6 and Peru8 were clustered within Group 1, and PtEb IX within Group 11, while the three novel genotypes GZW1, GZW2 and GZW3 formed a new clade between Groups 1 and 2 (Fig. 1).

**Occurrence of Eimeria spp. and Cyclospora spp. in wastewater**

For *Eimeria* spp. or *Cyclospora* spp., the occurrence rates were 45.4\% (108/238) and 47.7\% (42/88) in WWTP and sewer samples, respectively. The difference in occurrence rates between these was not significant (Table 4; \( \chi^2=0.143, df=1, P=0.7053 \)). The majority of the PCR products (141/150, 94.0\%) in the WWTP samples were from *Eimeria* spp., including *E. papillata* \( (n=54) \), *E. nieschulzi* \( (n=30) \), *E. necatrix* \( (n=6) \), *E. falciformis* \( (n=6) \), *E. polita* \( (n=1) \), *E. mitis* \( (n=1) \), *E. acerminina* \( (n=1) \) and *E. polita* \( (n=1) \). In addition, five samples showed concurrent presence of two *Eimeria* species; these *Eimeria* species were also found in sewer samples (Table 4). *Cyclospora cayetanensis*, however, was detected in three sewer samples and one WWTP sample (4/150; 2.7\%). In addition, *Isospora* spp. were found using the PCR in three sewer samples and two WWTPs (Table 4).

**Discussion**

The results of the present study provide some preliminary data on the occurrence of zoonotic waterborne pathogens (i.e. *Cryptosporidium* spp., *G. duodenalis* and *E. bieneusi*) in the wastewater of Guangzhou, China. Prior to this study, data on the occurrence of these pathogens in WWTP samples were available from Harbin, Qingdao, Nanjing, Shanghai and Wuhan [21, 31–33]. In this study, we detected *Cryptosporidium* spp., *G. duodenalis*, *E. bieneusi* and *Eimeria* spp. in 14.1, 49.1, 49.7 and 43.3\%, respectively, of all wastewater samples collected. Overall, the occurrence rates of these pathogens were lower than those reported from the studies previously conducted in China [21, 31]. The reasons for this difference are as yet clear, but differences in the number of samples analyzed, geography and habitat, wastewater treatment practices and health status of local residents may be contributing factors.

As seen in this study, 11 *Cryptosporidium* species and genotypes were detected in samples collected from WWTPs. Among these, *C. muris* (which infects a range of rodents, other mammals and humans) was the most common species (in 33\% or 11 of the 34 *Cryptosporidium*-positive samples). This result is in agreement with the frequent detection of rat genotype IV (10 of 34 *Cryptosporidium*-positive samples), suggesting that rodents contribute significantly to the occurrence of *Cryptosporidium* spp. in wastewater systems in Guangzhou. In previous studies in China [32], Greece [34] and Brazil [35], rodents were identified as a major source of *Cryptosporidium* oocysts in wastewater. Nevertheless, the predominance of *C. muris* and rat genotype IV in the present study contrasts with the dominance of *C. hominis* in wastewater reported in previous studies conducted in China [21, 31, 36]. This difference may either be due to differences in the sampling scheme or differences in the transmission of *Cryptosporidium* spp. among areas in China. It should be mentioned that there was no concurrent presence of multiple *Cryptosporidium* species/genotypes detected in the present study, possibly due to the relatively lower occurrence rates of these pathogens. The direct Sanger sequencing used in the analysis of PCR products, however, is not ideal for the detection of mixed *Cryptosporidium* spp. in samples. Next-generation sequencing techniques have been demonstrated to have better capability of detecting mixed populations of *Cryptosporidium* spp. in water samples [37].

*Cryptosporidium muris* was identified in the present study. Although it is not a major zoonotic pathogen, the species has gained increased attention in recent years. Several recent studies have documented the occurrence of *C. muris* in humans in developing countries [38–40]. In addition, macaque monkeys in China are commonly infected with *C. muris* [41]. Experimental infection with *C. muris* can cause persistent diarrhea in humans [42]. These findings on *C. muris* and the occurrence of other well-known human pathogen species, such as *C. parvum*, *C. meleagris*, *C. canis* and *C. felis*, indicate that *Cryptosporidium* spp. wastewater constitute a possible public health issue.

The results of this study show that assemblage A was the dominant *G. duodenalis* genotype in the wastewater samples. This result is consistent with observations in previous studies in Shanghai and elsewhere in China [21, 33, 43], USA, Tunisia and Hungary [44–46], which all reported that this assemblage was the dominant *G.
Fig. 1 Phylogenetic relationship of *Enterocytozoon bieneusi* genotypes detected in samples collected from wastewater treatment plants and sewer samples as determined by a maximum-likelihood analysis of the ribosomal internal transcribed spacer based on substitution rates calculated using the general time-reversible model and gamma distribution. Known and novel genotypes identified in this study are indicated by blue and red triangles, respectively. Bootstrap values of < 50% from 1000 replicate analysis are not shown.
The results of the genetic characterization of *Eimeria* spp. support the contribution of rodents to the occurrence of enteric pathogens in wastewater [21]. *Eimeria* spp. are host specific and form host-associated clusters in the phylogenetic analysis of the SSU rRNA sequences, which make it possible to track the source of pathogens in environmental samples [21]. In the present study, several rodent *Eimeria* spp., such as *E. papillata* (*n* = 64) and *E. nieschulzi* (*n* = 51), were commonly detected in wastewater samples, corroborating the contribution of rodents to the occurrence of *Cryptosporidium* spp. and *E. bieneusi*.

Although the detection of *C. cayetanensis* in wastewater samples in the present study was sporadic, the presence of this species represents a significant finding. *Cyclospora cayetanensis* is an emerging parasitic pathogen responsible for numerous foodborne outbreaks of cyclosporiasis in humans in industrialized countries [53]. Thus far, it has only been occasionally reported in the USA and Peru [16, 50]. Nevertheless, humans cannot be excluded as a source of contamination with these genotypes [51, 52]. As these *E. bieneusi* genotypes are major zoonotic genotypes, the presence of *E. bieneusi* in wastewater represents a potentially high public health hazard.

**Table 4** *Eimeria* species and related parasites in samples collected from wastewater treatment plants and in sewer samples, Guangzhou, China

| Sample location | No. of samples | No. of positive samples | Species (no. of samples) |
|-----------------|----------------|-------------------------|--------------------------|
| WWTP1           | 66             | 36 (54.6%)              | *E. papillata* (21); *E. nieschulzi* (13); *E. falciformis* (2) |
| WWTP2           | 28             | 7 (50.0%)               | *E. papillata* (4); *E. polita* (1); *E. nieschulzi* (1); *Isospora* sp. (1) |
| WWTP3           | 66             | 26 (39.4%)              | *E. papillata* (13); *E. nieschulzi* (5); *E. falciformis* (2); *E. necatrix* (1); *E. mitis* (1); *E. acermlina* (1); *E. nieschulzi* + *E. falciformis* (1); *E. nieschulzi* + *E. papillata* (1); *C. cayetanensis* (1) |
| WWTP4           | 78             | 39 (50.0%)              | *E. papillata* (16); *E. nieschulzi* (11); *E. necatrix* (5); *E. papillata* + *E. necatrix* (3); *E. falciformis* (2); *E. polita* (1); *Isospora* sp. (1) |
| Sub-total       | 238            | 108 (45.4%)             | *E. papillata* (54); *E. nieschulzi* (30); *E. necatrix* (6); *E. falciformis* (6); *E. papillata* + *E. necatrix* (4); *E. nieschulzi* + *E. falciformis* (1); *Isospora* sp. (2); *E. polita* (1); *E. mitis* (1); *E. acermlina* (1); *E. polita* (1); *C. cayetanensis* (1) |
| Sewer 1         | 11             | 6 (54.6%)               | *E. nieschulzi* (4); *E. papillata* (1); *Isospora* sp. (1) |
| Sewer 2         | 11             | 2 (18.2%)               | *E. nieschulzi* (1); *E. falciformis* (1) |
| Sewer 3         | 11             | 1 (9.1%)                | *E. nieschulzi* (1) |
| Sewer 4         | 11             | 9 (81.8%)               | *E. nieschulzi* (6); *E. papillata* (1); *Isospora* sp. (1); *E. falciformis* (1) |
| Sewer 5         | 11             | 3 (27.3%)               | *E. nieschulzi* (1); *E. papillata* (1); *Isospora* sp. (1) |
| Sewer 6         | 11             | 8 (72.7%)               | *C. cayetanensis* (3); *E. nieschulzi* (2); *E. papillata* (2); *E. falciformis* (1) |
| Sewer 7         | 11             | 6 (54.6%)               | *E. nieschulzi* (3); *E. papillata* (3) |
| Sewer 8         | 11             | 7 (63.6%)               | *E. nieschulzi* (4); *E. papillata* (2) |
| Sub-total       | 88             | 42 (47.7%)              | *E. nieschulzi* (22); *E. papillata* (10); *C. cayetanensis* (3); *E. falciformis* (3); *Isospora* sp. (3) |
| Total           | 326            | 150 (46.0%)             | *E. papillata* (64); *E. nieschulzi* (52); *E. falciformis* (9); *E. necatrix* (6); *Isospora* sp. (5); *E. polita* (1); *E. mitis* (1); *E. acermlina* (1); *E. polita* (1); *E. papillata* + *E. necatrix* (4); *E. nieschulzi* + *E. falciformis* (1); *C. cayetanensis* (4) |
in humans in Anhui and Henan, China [54, 55]. The finding of \( C. \) \textit{cayetanensis} in some samples in the study reinforces the contribution of humans to the occurrence of enteric parasites in wastewater.

**Conclusions**

The results of the present study provide some insight into the genetic make-up of \textit{Cryptosporidium} spp., \textit{Giardia duodenalis}, \textit{Enterocytozoon bieneusi}, \textit{Eimeria} spp. and \textit{Cyclospora} spp. in the wastewater of Guangzhou, China. With the exception of \textit{G. duodenalis}, \textit{E. bieneusi} genotypes Type IV and D, and \textit{C. cayetanensis}, some of the pathogens detected do not appear to originate from humans. Rodents in particular probably contribute significantly to the occurrence of these enteric pathogens in wastewater. Given the public health risk to humans that these parasites represent, guidelines on the discharge and reuse of wastewater are needed to generate key information needed for an improved understanding of the transmission and environmental transport of enteric pathogens in urban communities.

**Abbreviations**

bg: \( \beta \)-Gardin gene; gdh: Glutamate dehydrogenase gene; gp60: 60-kDa glycoprotein gene; ITS: Internal transcribed spacer; PCR: Polymerase chain reaction; SSU rRNA: Small subunit ribosomal RNA gene; tpi: Triosephosphate isomerase.

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**Authors’ contributions**

LX and YF conceived and designed the experiments. YF, XW, RY and WZ performed the experiments. YF, RY and XW analyzed the data. YF, LX and YF wrote the paper. All authors read and approved the final manuscript.

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**Availability of data and materials**

Data supporting the conclusions of this article are included within the article.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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