First survey of Cryptosporidium, Giardia and Enterocytozoon in diarrhoeic children from Wuhan, China

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* Note: Nucleotide sequence data reported in this article are publicly available in the GenBank database under accession nos. KY575457 to KY575459.
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ABSTRACT

Intestinal protozoan pathogens cause significant diarrhoeal diseases in children. However, to date, there has been limited genetic study of the intestinal pathogens Cryptosporidium, Giardia and Enterocytozoon in humans in China, with the exception of research in a small number of cities/provinces. In the present study, PCR-based tools were used to detect and characterise these protistan parasites from 500 children with a history of diarrhoea in Wuhan and environs, Hubei province, China. Genomic DNAs from faecal samples were screened for the particular protists by PCR utilising regions in the small subunit (SSU) of the nuclear ribosomal RNA, the 60 kDa glycoprotein (gp60), the internal transcribed spacer of nuclear ribosomal DNA (ITS) and/or the triose phosphate isomerase (tpi) genes as genetic markers. Cryptosporidium meleagridis subtype IIIb (10/500, 2.0%), Giardia duodenalis assemblage A (7/500, 1.4%) and Enterocytozoon bieneusi genotype D (1/500, 0.2%) were identified in small percentages of the 500 samples. No significant gender- or age-associated differences in the prevalence of Cryptosporidium and Giardia infections were found. Future studies might focus on the occurrence of these protists in children as well as animals, with emphasis on Cryptosporidium meleagridis in pets and agriculturally important birds, in different parts of Hubei province.

Keywords:
Cryptosporidium
Giardia
Enterocytozoon
Human
China
PCR-based analyses
Sequencing
1. Introduction

Diarrhoeal diseases are considered to be the second leading cause of morbidity and mortality in young children (<5 years) in developing countries (de Lucio et al., 2016; Kotloff et al., 2013). The protists *Cryptosporidium* spp., *Giardia duodenalis* and *Enterocytozoon bieneusi* are recognised as being particularly important pathogens that cause significant childhood diarrhoeal diseases (Checkley et al., 2015; Feng and Xiao, 2011; Kotloff et al., 2013). All of these parasites have a wide range of hosts, including humans, domestic and wild animals, and are transmitted anthroponotically and/or zoonotically via the faecal-oral route (Feng and Xiao, 2011; Mathis et al., 2005; Ryan and Caccio, 2013; Xiao, 2010).

The accurate detection and characterization of different species, genotypes or assemblages of these parasites is central to determining their potential infection sources and transmission routes (Koehler et al., 2014). Using molecular tools, such as polymerase chain reaction (PCR)-based techniques, *Cryptosporidium hominis, C. parvum, C. meleagridis, C. felis, C. canis, Giardia duodenalis assemblages A and B* as well as *Enterocytozoon bieneusi* genotype D appear to be responsible for most human infections (Ryan and Caccio, 2013; Santín and Fayer, 2009; Xiao, 2010). However, the occurrences of the species, genotypes or assemblages of these parasites are known to vary according to host age (Feng and Xiao, 2011; Xiao, 2010) and geographical location (Feng et al., 2007; Ng et al., 2011). Despite their widespread occurrence (in >20 Chinese cities/provinces), limited molecular epidemiological data of these pathogens in humans are available in China, with the exception of some cities or provinces, including Shanghai (Feng et al., 2012; Liu et al., 2014; Wang et al., 2013a), Tianjin (Peng et al., 2001), Henan (Wang et al., 2013b; Wang et al., 2011; Zhu et al., 2012), Jiangsu (Jiang et al., 2014), Yunnan (Zhang et al., 2016a), Jilin (Zhang et al., 2011), Heilongjiang (Li et al., 2015; Yang et al., 2014). Li and colleagues (2012) conducted a molecular-based survey of the city sewer system from 2006 to 2009, and identified several human-pathogenic species/genotypes/sub-genotypes, including *C. hominis, G. duodenalis* sub-assemblage A-II and *E. bieneusi* genotype D, as being dominant in wastewater from Wuhan, Hubei province (Li et al., 2012). However, to date, there is no published molecular information on these parasites from humans in Hubei province. Here, we conducted the first molecular survey of *Cryptosporidium, Giardia* and *Entercytozoon* in diarrhoeic children in Wuhan, Hubei province, China.

2. Materials and methods

A total of 500 fresh faecal samples (collected between June 2016 and August 2016) from anonymous (individual) children with diarrhoea were donated by the Outpatient Department in Wuhan Pediatric Hospital and Renmin Hospital of Wuhan University, China. Genomic DNA was extracted from 0.2 g of each faecal sample using the PowerSoil DNA Isolation Kit (MoBio, USA), according to the manufacturer's protocol, and stored at -20 °C. This kit was used, as it had been shown to be highly effective at removing components that are inhibitory to PCR (Kosch and Summers, 2013; Nolan et al., 2013; Pontiroli et al., 2011). Aliquots (2 µl) of individual genomic DNA samples were subjected to nested PCR-based amplification and sequencing, employing (individually) four distinct loci of nuclear DNA in separate assays. For *Cryptosporidium*, a portion of the small subunit of the nuclear ribosomal RNA gene (designated pSSU; ~240 bp) was used (Nolan et al., 2010; Xiao et al., 1999), and genotypic/subgenotypic classification was achieved employing a region of the 60 kDa glycoprotein gene (designated pgp60; ~900 bp) (Stensvold et al., 2014). For *Giardia*, a portion of the triose phosphate isomerase gene (designated pipi; ~530 bp) was employed and genetic assignment was to the level of assemblage (Sulaiman et al., 2003). For *Enterocytozoon*, the internal transcribed spacer of nuclear ribosomal DNA gene (designated ITS; ~390 bp, including
125 bp of flanking nuclear ribosomal DNA) was used (Buckholt et al., 2002). The genotype/s of *E. bieneusi* were named according to an established nomenclature (Santin and Fayer, 2009). All primers used in the study are listed in Supplementary Table 1. PCR was carried out in a volume of 50 µl containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl (Promega, Madison, USA), 2.0-3.0 mM of MgCl₂ (depending on the locus), 200 µM of each deoxynucleotide triphosphate, 50 pmol of each primer and 1 U of either GoTaq (Promega, USA) (for pSSU, *ptpi* and *ITS*) or MangoTaq™ (Bioline, USA) (for *pgp60*) DNA polymerase. Known test-positive, test-negative and no-template controls were included in each step of each set of PCRs. All nested PCR products were detected by electrophoresis in 1.5% agarose gels, stained with ethidium bromide before sequencing. For sequencing, aliquots (5 µl) of individual amplicons (undigested) were treated with the enzymes Exo I and a thermosensitive alkaline phosphatase (FastAP, Thermofisher, USA), according to the manufacturer's instructions, and then subjected to direct, automated sequencing (BigDye Terminator v.3.1 chemistry, Applied Biosystems, USA) in both directions using the same primers (separately) as employed in PCR.

Phylogenetic analysis of *pgp60* sequence data for *Cryptosporidium* (including a range of reference sequences; Supplementary Table 2) was conducted by Bayesian inference (BI) using Monte Carlo Markov Chain (MCMC) analysis in MrBayes v.3.2.3. The likelihood parameters set for BI analysis of *pgp60* data were based on the Akaike Information Criteria test (Darriba et al., 2012) in jModeltest v.2.1.7. The number of substitutions (Nst) was set at 6, with a proportion of invariable sites. Posterior probability (pp) values were calculated by running 2,000,000 generations with four simultaneous tree-building chains. Trees were saved every 100th generation. At the end of each run, the standard deviation of split frequencies was <0.01, and the potential scale reduction factor approached one. A 50% majority rule consensus tree for each analysis was constructed based on the final 75% of trees generated by BI. Analyses were run three times to ensure convergence and insensitivity to priors. The outgroup used in phylogenetic analysis was *C. meleagrisidis* subtype IIIId (GenBank accession no. DQ067570.1). Chi-square test was performed using SPSS Statistics 24 software (IBM, USA).

3. Results and discussion

Using three separate PCR assays, the 500 individual faecal samples from children with diarrhoea from Wuhan City were screened by PCR for the presence of three intestinal protozoan taxa. In total, 10, 7 and 1 faecal DNA samples were test-positive for *Cryptosporidium* spp., *Giardia duodenalis* and *Enterocytozoon bieneusi*, respectively. No mixed infections of these pathogens were detected. The nucleotide sequences of *Cryptosporidium, Giardia* and *Enterocytozoon* in this study were deposited in GenBank under accession numbers KY575457 to KY575459 (see Supplementary Table 2).

3.1. *Cryptosporidium*

Of the three pathogens tested for in the present study, the infection rate of *Cryptosporidium* spp. was the highest (2.0%) amongst the 500 children. This number was consistent with previous *Cryptosporidium* molecular epidemiological studies of children from other cities/provinces of China (1.6%, Feng et al., 2012; 0.1%, Zhang et al., 2016a). The age and gender distribution of enteric pathogens infection is shown in Table 1. *Cryptosporidium* was found across all age and gender groups of diarrhoeic children (Table 1). Data showed that 2.2% (6/271) of males and 1.7% (4/229) of females were test-positive for *Cryptosporidium*; according to age, 2.4% (5/209) of infants of <2 years of age, 1.7% (4/238) children of 2 to 5 years, and 1.9% (1/53) of children of > 5 years were test-positive for this protist. Nevertheless, no significant differences were detected among different gender or age groups (*P* = 0.710 or
Sequencing of amplicons from Cryptosporidium-positive samples (n = 10) revealed C. meleagridis, which mainly infects birds (e.g., turkeys, chickens, pigeons, parrots, cockatiels and other pet birds) (Qi et al., 2011; Ryan, 2010). C. meleagridis, as a zoonotic Cryptosporidium species, has been mainly reported from children and immuno-compromised or -suppressed persons around the world (Cama et al., 2003, 2007; Gatei et al., 2002; Koehler et al., 2013). Due to its relatively low host specificity, both anthropo-conomic and zoonotic transmission paths of C. meleagridis have been suggested (Xiao, 2010). In China, C. meleagridis has been detected previously in six pediatric patients in three hospitals in Shanghai and five HIV-positive individuals in Henan province (Feng et al., 2012; Wang et al., 2013b). Given the large number of pet birds and more frequent contact of people with their pet birds in China, children and the elderly, which are thought to be more susceptible to C. meleagridis (Qi et al., 2011), could become infected. Interestingly, this is the first study to show a dominance of C. meleagridis in a cohort of children in Wuhan, whereas previous data have indicated that C. hominis is the most frequently identified species in China (Feng et al., 2012; Peng et al., 2001; Wang et al., 2011). A further analysis of pgi60 nucleotide sequence data showed that only the subtype IIIb [IIIbA21G1R1 (n = 1), IIIbA22G1R1 (n = 8) and IIIbA26G1R1 (n = 1)] was represented by C. meleagridis-positive samples (cf. Fig. 1). This subtype has been found mainly in Asian countries including China, India, Indonesia and Thailand (Stensvold et al., 2014). In the present study, it was not possible to acquire detailed epidemiological information relating to the samples tested, such that the source of C. meleagridis infection in children is presently unclear. Therefore, detailed studies (i.e. larger sample sizes, different locations and dynamic time points) are needed to establish the source(s) of C. meleagridis infection in humans in Wuhan, and to conduct more surveys in Hubei province to establish whether domestic or wild birds (e.g., pigeons or sparrows) play a role in transmission.

3.2. Giardia

PCR (ptpi) screening identified seven samples that were test-positive for Giardia. This small percentage (1.4%) of G. duodenalis determined here is in accordance with figures reported for outpatients (1.4-9.5%) in other parts of China (Wang et al., 2011). Similar to results for Cryptosporidium, G. duodenalis was also found across all age and gender groups of diarrhoeic children (Table 1). Data here show that 1.1% (3/271) of male and 1.7% (4/229) of female children were test-positive for Giardia, whereas 1.0% (2/209) infants of <2 years of age, 1.3% (3/238) of children aged between 2 to 5 years and 3.8% (2/53) children of more than 5 years of age were identified as test-positive using the PCR-coupled sequencing approach. The Chi-square test did not show any significant age- or gender-associated difference in the prevalence of G. duodenalis infection (P = 0.287 and 0.544, respectively).

Although G. duodenalis assemblage B has been described previously as being more common in children in other countries, such as Cambodia, Ethiopia, Lebanon and Morocco (de Lucio et al., 2016; El Fatni et al., 2014; Lobo et al., 2014; Moore et al., 2016; Osman et al., 2016), all seven ptpi sequences (490 bp) determined here were the same as that with GenBank accession no. GU564278, which belongs to sub-assemblage A-II (Wang et al., 2011). According to limited genotypic studies of G. duodenalis in China, except for a recent study which identified 16 canid-specific assemblage C from 17 Giardia-positive diarrheal outpatients in Shanghai (Liu et al., 2014), both subtypes A-I and A-II of assemblage A, as well as assemblage B, were found previously in Chinese outpatients and inpatients (Wang et al., 2013a; Wang et al., 2011; Yong et al., 2000). In Anhui province, the four test-positive samples from individuals were either assemblage A or B (Yong et al., 2000); in Hebei province, three patients were diagnosed as having G. duodenalis sub-assemblage A-II infection (Cheng et al.,
217 2001); in Henan province, assemblages A and B were identified in 12 and six patients, 218 respectively (Wang et al., 2011); in Shanghai, G. duodenalis assemblages A and B were 219 reported in seven and 11 patients, respectively, during a cryptosporidiosis outbreak in a 220 pediatric hospital (Wang et al., 2013a). Both assemblages A and B typically follow an 221 anthropoontic rather than a zoonotic transmission route (Ryan and Caccio, 2013). This 222 information suggests that humans are probably the major source for giardiasis in Wuhan, Hubei 223 province, but this proposal requires testing.

3.3. Enterocytozoon

E. bieneusi was identified in one faecal sample from a one year-old boy. An analysis 227 revealed that the sequence derived from this sample was the same as that with GenBank 228 accession no. JQ029731 (Wang et al., 2013b). This sequence represents genotype D, which is 229 presently the predominate zoonotic genotype (Santín and Fayer, 2009). Due to the fact that 230 there was only one test-positive sample, it was not possible to conduct any statistical analyses.

Although E. bieneusi causes ~90% of reported human cases of microsporidiosis, the 233 transmission route/s of this pathogen is/are unclear (Matos et al., 2012). A previous study 234 indicated that the release of spores from E. bieneusi into the environment via stool and 235 respiratory secretions is probably the principal source of contamination/infection (Matias et al., 236 2005). In China, E. bieneusi was first reported in diarrhoeic children (prevalence: 9/40, 22.5%), 237 pigs (prevalence: 10/61, 16.4%), dogs (prevalence: 2/26, 7.8%) and cows (prevalence: 35/93, 238 37.6%) in Jilin province in 2011 (Zhang et al., 2011). Subsequently, E. bieneusi infection was 239 reported in humans from other cities/provinces (Liu et al., 2014; Wang et al., 2013a, 2013b; 240 Yang et al., 2014) as well as in other host species, including buffaloes (Bubalus bubalis) (Ma et 241 al., 2015), cattle (Bos taurus) (Li et al., 2016a; Ma et al., 2015), goats (Capra aegagrus hircus) 242 (Peng et al., 2016), horses (Equus ferus caballus) (Qi et al., 2016), cats (Felis catus) (Li et al., 243 2015), rabbits (Oryctolagus cuniculus) (Yang et al., 2016), birds (Anas platyrhynchos 244 domesticus, Anser anser domesticus, Columba livia, Gallus gallus domesticus, Grus japonensis, 245 Gr. grus, Gr. vipio and Gr. leucogeranus) (Li et al., 2014; Zhao et al., 2016), bears (Helarctos 246 malayanus, Ursus arctos pruinosus and U. thibetanus) (Li et al., 2016b), deer (Axis porcinus, 247 Cervus elaphus, Ce. nippon and Elaphurus davidianus) (Li et al., 2016b; Zhang et al., 2015), 248 foxes (Alopex lagopus, Vulpes vulpes and V. lagopus) (Yang et al., 2015; Zhang et al., 2016b; 249 Zhao et al., 2015), non-human primates (i.e. Macaca fascicularis, M. mulatta, M. fuscata, 250 Papio anubis, Presbytis leucocephalus and Rhinopithecus roxellana) (Karim et al., 2014a, 251 2014b; Li et al., 2016b), raccoon dogs (Nyctereutes procyonoides) (Yang et al., 2015; Zhao et al., 252 2015), reptiles (Naja naja and P. mocusus) (Karim et al., 2014c), rodents (Chinchilla 253 lanigera) (Qi et al., 2015), squirrels (Callosciurus erythraeus) (Deng et al., 2016), red pandas 254 (Ailurus fulgens) and giant pandas (Ailuropoda melanoleuca) (Tian et al., 2015), in China. 255 Notably, high sequence diversity in ITS is known to exist within E. bieneusi in China (Wang et 256 al., 2013b; Yang et al., 2014), indicating that more in-depth studies are needed to establish the 257 sub-structuring of E. bieneusi populations and the epidemiological significance of 258 Enterocytozoon genotypes.

4. Concluding remarks

In the present study, for the first time, we have reported and characterised genotypes or 261 assemblages of Cryptosporidium, Giardia and Enterocytozoon in humans in Hubei province, 262 China. It is noteworthy that, although no new genotypes or assemblages of Cryptosporidium, 263 Giardia or Enterocytozoon were discovered, C. meleagridis (mainly infecting avian hosts) was 264 found to dominate in the children studied in Wuhan. Clearly, this finding encourages future
detailed, molecular-based studies of humans and animals (especially pet birds and poultry) in an effort to better understand the transmission of this enteric pathogen in humans in China.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/XXXXXXX

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Table 1
Age and gender distributions of Cryptosporidium meleagridis, Giardia duodenalis and Enterocytozoon bieneusi detected using PCR-based methods in faecal samples from diarrhoeic children from Wuhan in Hubei province (between June and August 2016)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Test-positive/total no. tested (%) for Cryptosporidium meleagridis</th>
<th>Test-positive/total no. tested (%) for Giardia duodenalis</th>
<th>Test-positive/total no. tested (%) for Enterocytozoon bieneusi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2</td>
<td>5/209 (2.4)</td>
<td>2/209 (1.0)</td>
<td>1/209 (0.5)</td>
</tr>
<tr>
<td>2-5</td>
<td>4/238 (1.7)</td>
<td>3/238 (1.3)</td>
<td>0/238 (0)</td>
</tr>
<tr>
<td>&gt;5</td>
<td>1/53 (1.9)</td>
<td>2/53 (3.8)</td>
<td>0/53 (0)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>6/271 (2.2)</td>
<td>3/271 (1.1)</td>
<td>1/271 (0.4)</td>
</tr>
<tr>
<td>Female</td>
<td>4/229 (1.7)</td>
<td>4/229 (1.7)</td>
<td>0/229 (0)</td>
</tr>
<tr>
<td>Totals</td>
<td>10/500 (2.0)</td>
<td>7/500 (1.4)</td>
<td>1/500 (0.2)</td>
</tr>
</tbody>
</table>
Fig. 1. Phylogenetic relationship of *Cryptosporidium meleagridis* from children in the present study (bold-type, followed by GenBank accession number) with other *Cryptosporidium* taxa based on analysis of *pgp60* sequence data by Bayesian inference. Reference sequences for the *Cryptosporidium* taxa are listed in Supplementary Table 2; *Cryptosporidium meleagridis* subtype IIId (GenBank accession no. DQ067570.1) was used as the outgroup. Posterior probabilities are indicated at the nodes.
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