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Zoonotic *Enterocytozoon bieneusi* in raw wastewater in Zhengzhou, China

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Abstract: Contamination of *Enterocytozoon bieneusi* Desportes, Charpentier, Galian, Bernard, Cochand-Priollet, Laverne, Ravisse, et Modigliani, 1985 in water sources may cause outbreaks of microsporidiosis. To examine the occurrence of *E. bieneusi*, 108 raw wastewater samples were collected from three wastewater treated plants in Zhengzhou, China. In total, 46 samples were PCR positive for *E. bieneusi*. A total of 15 ITS genotypes was identified, including ten known genotypes (D, BEB6, I, J, PigEbIX, PigEBITS5, EbpA, Peru6, Peru8, Type IV) and five novel genotypes (HNWW1, HNWW2, HNWW3, HNWW4, HNWW5). Nine genotypes belonged to a known zoonotic group (group 1) and the other genotypes belonged to potential zoonotic group (group 2). Most of the genotypes had been identified in wildlife or domestic animals in former reports in Zhengzhou. The occurrence of *E. bieneusi* in wastewater was probably related to the rainfall day before sampling. Of 36 sampling days, 20 days had rainfall on the previous day and 16 days had none. As many as 43 of 60 samples were found to be *E. bieneusi*-positive in the 20 days which had rainfall on the previous day. Only three of 48 samples were found to be *E. bieneusi*-positive in the 16 days without rainfall the day before. The significant difference of the occurrence of *E. bieneusi* was observed between wet days and dry days by t-test (43/60 *vs* 3/48, $p < 0.01$). This indicates that the occurrence of *E. bieneusi* in wastewater in Zhengzhou mainly originated from animals and was probably related to rainfall the day before sample collection. Given the zoonotic genotypes detected in wastewater, animal faeces should be treated appropriately before being drained into the water source.

Keywords: rainfall, microsporidiosis, genotypes

Enterocytozoon bieneusi Desportes, Charpentier, Galian, Bernard, Cochand-Priollet, Laverne, Ravisse, et Modigliani, 1985, the dominant member of the human pathogenic microsporidian species, is responsible for 90% of human microsporidiosis (Ghosh and Weiss 2009, Matos et al. 2012). Microsporidiosis is a significant cause of diarrhea and gastrointestinal illness, especially for children and immunosuppressed individuals (Tabatabaie et al. 2015). Based on the molecular tools, over 200 internal transcribed spacer (ITS) genotypes of *E. bieneusi* have been identified, and constitute at least ten distinct genogroups by phylogenetic analysis (group 1 to 9 and the so called outlier in dog) (Karim et al. 2015). The group 1, also named as the zoonotic group, has been widely identified in humans and animals (Matos et al. 2012). Group 1 could be clearly subdivided into 9 major clades named as subgroup 1a–1i. The zoonotic potential of each subgroup was phylogenetically supported by the diversity of host species, which included humans and certain specific animals (Thellier and Breton 2008), while genotypes belonging to other groups have now been commonly considered as animal specific (Santin and Fayer 2011).

Water is an ideal habitat for *E. bieneusi* (see Izquierdo et al. 2011, Guo et al. 2014). A putative waterborne outbreak of microsporidiosis caused by *E. bieneusi* and *Encephalitozoon intestinalis* (Cali, Kotler et Orenstein, 1993) had been reported in Cotte et al. (1999). In one study, *E. bieneusi* was detected in wastewater from four cities in China (Li et al. 2012). A study in Tunisia suggested that genotypes D and IV were the dominant genotypes of *E. bieneusi* contamination in urban wastewater (Ben Ayed et al. 2012). Thus, *E. bieneusi* has frequently been found in wastewater. However, the original source of poullution in wastewater is unclear due to a lack of exact data on *E. bieneusi* genotypes in humans or animals in these cities.

Zhengzhou, in the middle of China, has ~9 million inhabitants and experiences water shortage due to the dry climate. Treated water from wastewater treatment plants (WWTPs) is therefor especially important for the urban water supply. Zhengzhou has three WWTPs which accept and treat most of the raw wastewater in this urban area. Treated wastewater is often discharged into rivers directly and may be used as a source of water for irrigation and rec-

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reation. Contamination of *E. bieneusi* in wastewater could be a potential threat to human health.

Several studies reported that zoonotic genotypes of *E. bieneusi* were widely found in domestic animals in Zhengzhou. Studies in dogs and cats showed that dogs are hosts for not only animal-adapted genotypes but also potential zoonotic genotypes, whereas cats are mainly infected by the zoonotic genotypes (Karim et al. 2014a). A more comprehensive investigation of wildlife at a public zoo in Zhengzhou identified thirteen genotypes of *E. bieneusi*, including a few zoonotic genotypes (Li et al. 2015). However, how the zoonotic *E. bieneusi* is transmitted in the water bodies still remains unknown.

In the present study, a one-year molecular surveillance of *E. bieneusi* in wastewater was conducted in the three WWTPs in Zhengzhou. Genotypes of *E. bieneusi* were detected by ITS sequence analysis. Combined with the former reports in Zhengzhou, the present study suggests that contamination with *E. bieneusi* in wastewater in Zhengzhou was potentially zoonotic, and probably originated from animals faeces and was attributed to wet days before sample collection.

MATERIALS AND METHODS

From November 2014 to October 2015, 108 raw wastewater samples were collected from three WWTPs in 36 days, with three samples per month from each plant. In total, 36 samples were obtained from each WWTP during one year, including 20 samples collected on days just after rainfall and 16 samples collected on days without rainfall on the previous day (Table 1).

The three WWTPs accept 90% of the raw wastewater in Zhengzhou. The domestic wastewater and storm wastewater have not been separated in the study area. Grab samples of 800 ml to 1 000 ml of raw wastewater were collected from the entrance of WWTPs and concentrated by centrifugation at 6 000× g for 10 min to collect spores of *E. bieneusi*.

Half a gram of concentrate was washed twice by distilled water. Genomic DNA was extracted using DNA extraction Kit (Fast DNA SPIN Kit for Soil, BIO 101, Carlsbad, CA) according to the instruction of manufacture. DNA samples were stored at -80 °C until analysed by PCR.

Enterocytozoon bieneusi was detected by nest PCR amplification of a ~390 fragment, including the entire ITS fragment (243 bp) (Sulaiman et al. 2003), by using the primers EBITS3 (5'-GGTCATAGGGATGAAGAG-3') and EBITS4 (5'-TTCGAGTTCTTTCGCGCTC-3') in the primary PCR and the primers EBITS1 (5'-GCTCTGAATATCTATGGCT-3') and EBITS2.4 (5'-ATCGCCGACGGATCCAAGTG-3') in the secondary PCR. Each sample was analysed five times, using 2 µl of DNA per PCR. Positive secondary PCR products were then sent to a commercial company (Sangong Biotech, Shanghai, China) for sequencing. Genotypes of *E. bieneusi* in the wastewater were named according to the established nomenclature (Thellier and Breton 2008). The significant difference of positive samples between rainy days and dry days was compared by the t-test, and significant difference was considered when $p < 0.01$.

Genotypes obtained in this study were compared with reported ITS genotypes by using the Maximum likelihood method in the program Mega 7.0 (http://www.megasoftware.net/). Bootstrap analysis was used to assess the robustness of clusters using 1 000 replicates. The subgroups that each genotype belongs to were also confirmed according to the former research (Karim et al. 2015). New nucleotide sequences generated from the study were deposited in the GenBank database under accession numbers KU885969–KU885973.

Table 1. The genotypes of *E. bieneusi* in three wastewater treated plants (WWTP) in different months.

Sampling Time	Genotypes			Rainfall[a] (mm)	Heavy rainy[b]	Total positive+
	WWTP1	WWTP2	WWTP3			
2014/11/05	-	-	-	0	No	
2014/11/15	-	HNWW1	BEB6	0.51	No	4
2014/11/24	D	I	-	NR	No	
2014/12/05	HNWW1	-	-	0	No	
2014/12/15	-	-	-	0	No	1
2014/12/24	-	-	-	0	No	
2015/01/04	Peru6	-	-	0	No	
2015/01/14	-	-	-	0	No	3
2015/01/25	BEB6	-	PigEBITS5	4.06	Yes	
2015/02/05	-	-	-	0	No	
2015/02/15	PigEbIX	BEB6	-	NR	No	3
2015/02/27	-	-	BEB6	NR	No	
2015/03/07	-	-	-	0	No	
2015/03/20	D	BEB6	D	5.08	Yes	3
2015/03/30	-	-	-	0	No	
2015/04/07	-	-	-	0	No	
2015/04/19	BEB6	EbpA	D	6.10	Yes	3
2015/04/29	-	-	-	0	No	
2015/05/07	HNWW2	BEB6	D	17.02	Yes	
2015/05/18	-	-	-	0	No	3
2015/05/30	-	-	-	NR	No	
2015/06/05	-	-	HNWW3	NR	No	
2015/06/16	HNWW2	PigEBITS5		1.02	No	6
2015/06/28	PigEbIX	EbpA	BEB6	7.11	Yes	
2015/07/06	-	-	-	0	No	
2015/07/16	BEB6	PigEBITS5	D	6.0	Yes	5
2015/07/31	BEB6	PigEBITS5	-	5.0*	Yes	
2015/08/09	EbpA	BEB6	HNWW4	6.0*	Yes	
2015/08/20	-	-	-	0.7	No	6
2015/08/30	J	HNWW2	D	18.0	Yes	
2015/09/10	BEB6	HNWW5	-	0.3	No	
2015/09/20	-	-	EbpA	0	No	6
2015/09/25	BEB6	Peru 6	HNWW4	4.1	Yes	
2015/10/09	-	-	-	0	No	
2015/10/19	-	-	-	0	No	3
2015/10/26	Peru8	Type IV	D	18.03	Yes	
Total positive	17	15	14	-	31	46

[a] rainfall in the day before sample collected; [b] heavy rain > 4 mm/day in the day before sample collected; * rain storm day; NR – rainy day, but without rainfall recorded in the website, weather data from the website: www.wunderground.com; + total positive sample in each month.

RESULTS

Enterocytozoon bieneusi was commonly identified in WWTPs in Zhengzhou. In total, 43% (46/108) of wastewater specimens were positive for *E. bieneusi* ITS PCR. The number of positive specimens was close between three different WWTPs, with 17 in WWTP1, 15 in WWTP2 and

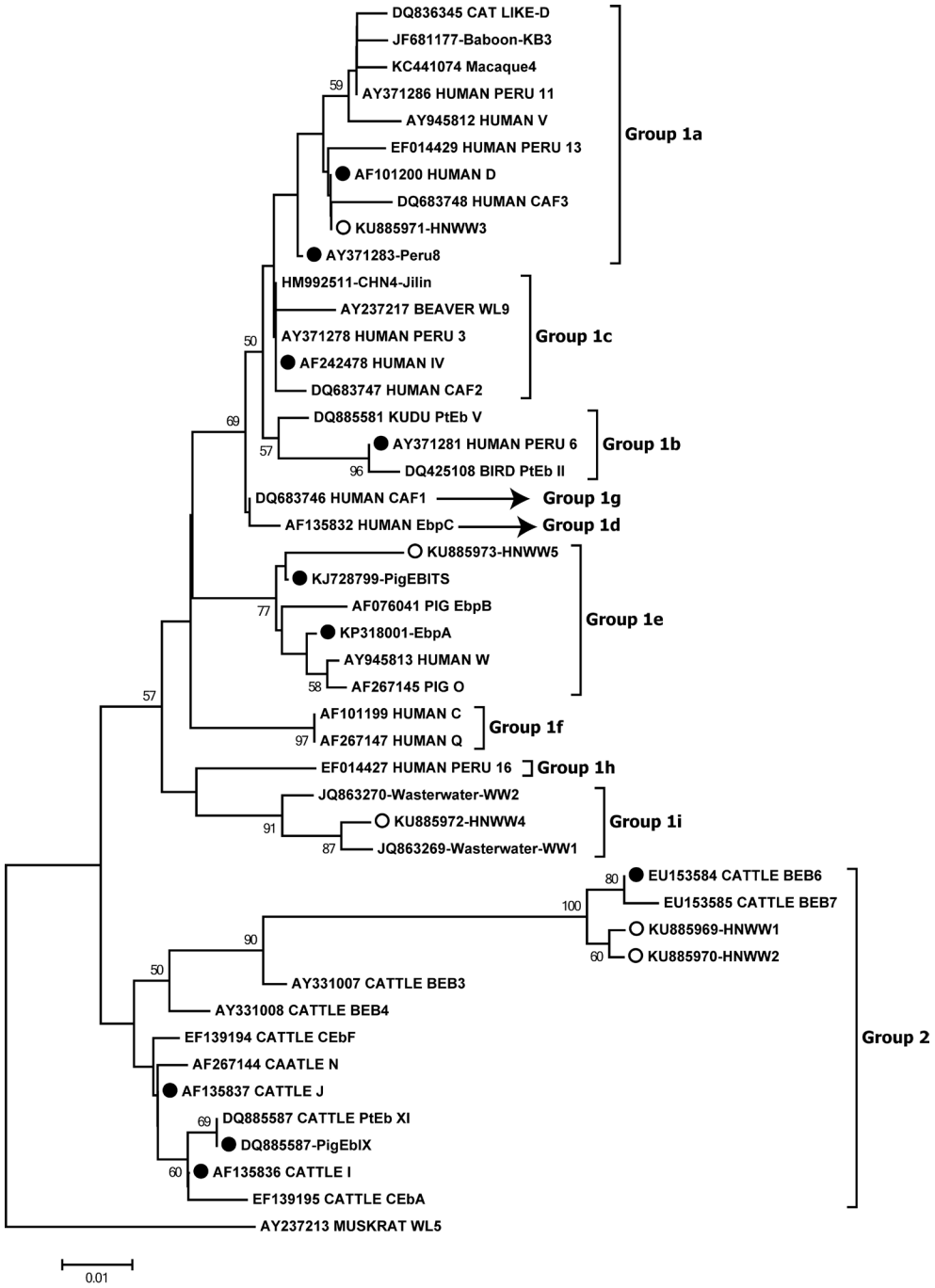

Fig. 1. Phylogenetic relationships of genotypes of *Enterocytozoon bieneusi* identified in this study and other reported genotypes. The phylogeny was inferred with a Maximum Likelihood analysis of the ITS sequences based on distances calculated with the Kimura two-parameter model. Bootstrap values greater than 50% from 1 000 replicates are shown on the nodes. The genotypes detected in this study are shown with circle known genotypes are marked with solid circles and the new genotypes are indicated by open circles. The ITS tree was rooted with GenBank sequence AY237213 WL15 (an *E. bieneusi* genotype from group 3). The subgroups in group 1 were named according to Thellier and Breton (2008) and Karim et al. (2015).

14 in WWTP3. The prevalence (46/108, 43%) of *E. bieneusi* in Zhengzhou was lower than that of other cities in China in a previous survey (Li et al. 2012).

Different distribution patterns of genotypes of *E. bieneusi* were observed between WWTPs. In WWTP1, BEB6 (6/17) was the dominant genotype (Table 1). In WWTP2, four isolates of BEB6 and three isolates of PigEBITS5 were found, accounting for 47% of all positive samples. However, genotype D (6/14) was the dominant *E. bieneusi* in WWTP3, followed by BEB6 (3/15) and novel genotype HNWW4 (2/15).

DISCUSSION

According to previous reports from Zhengzhou (Karim et al. 2014a, 2015, Li et al. 2015, Qi et al. 2015), *E. bieneusi* in wastewater mainly originated from animals faeces

and was a potential zoonotic risk. Phylogenetic analysis revealed that all of the 15 genotypes clustered into either group 1 (zoonotic group) or group 2 (potential zoonotic group) (Fig. 1) in the present study. Twenty-four out of 46 positive specimens were clustered in group 1, with genotype D (8) as the dominant one. Genotype D was also been found in animals in Zhengzhou, including captive wildlife and pet animals (Li et al. 2015, Qi et al. 2015). It is worth noting that genotype D has been identified in humans as well as various animals, and is considered a zoonotic genotype (Yang et al. 2015). The other zoonotic genotypes (PigEBITS5, EbpA, Peru8, Type IV) were also detected in animals in Zhengzhou (Karim et al. 2014a, 2015, Li et al. 2015, Qi et al. 2015). Beside these, the remaining genotypes of positive samples (22/46) belonged to the potential zoonotic group (group 2). BEB6 was the most frequently found genotype, accounting for 28% (13/46) of all positive samples. To date, BEB6 has been usually derived from animals (especially sheep and cattle) (Ye et al. 2015, Zhao et al. 2015), but also identified in human and nonhuman primates in Henan Province (Wang et al. 2013, Karim et al. 2014b). Genotypes I and J reported in captive wildlife in Zhengzhou (Li et al. 2015) had also been detected in humans in Jilin, China (Zhang et al. 2011). The novel genotypes (HNWW1 to HNWW5) showed only difference 1–3 bases from known zoonotic or potential zoonotic genotypes. Given that actual zoonotic and potential zoonotic genotypes present in wastewater had also been detected in humans and animals in Zhengzhou and other cities, attention should be paid to contamination with *E. bieneusi* in wastewater in this area.

Occurrence of *E. bieneusi* in wastewater was probably related to rainfall before sampling days. Here, we recorded wet days during samples surveillance, and rainfall sampling days were obtained from a website (www.wunderground.com). Of 36 sampling days, 20 were recorded as wet the previous day. In total, 43/60 (72%) samples were found to be *E. bieneusi*-positive on those 20 days for all three WWTPs. Only 2 of 20 days were clear of *E. bieneusi*-positive samples. Meanwhile, when the rainfall the previous day was more than 6.0 mm/day, samples collected from three WWTPs were all found to be *E. bieneusi*-positive the next day. However, only 3 (3/48, 6%) *E. bieneusi*-positive samples were detected on the other 16 days without rainfall before sampling (Table 1). The t-test analysis showed that significant difference was observed (43/60 *vs* 3/48, $p < 0.01$), which indicated that wet days more easily facilitated contamination with *E. bieneusi* in wastewater in the following next days. To elucidate the relationship between rainfall and the occurrence of *E. bieneusi*, samples collected after days with more than 4.0 mm/day rainfall were classified as heavy rain group (11 days, with 33 samples, indicated as 'Yes' in Table 1), whereas the other samples were classified as little or no rain group (25 days, with 75 samples, indicated as 'No' in Table 1). The t-test also indicated a significant difference between these two groups (31/33 in heavy rainy group *vs* 15/75 in small or no rainy group, $p < 0.01$). Thus, the occurrence of zoonotic *E. bieneusi* in wastewater in this study could be related to rainfall the day before sampling. This may be explained by contaminated animal faeces being flushed into the water source after rainfall, as most of the genotypes identified in the present study had been previously reported in animals in this area. Contamination by *E. bieneusi* in water sources had been reported before (Ben Ayed et al. 2012, Li et al. 2012, Guo et al. 2014), but the factors responsible was investigated. More information on the prevalence of *E. bieneusi* in wastewater, especially during the wastewater treatment process, is required. Strategies to prevent the draining of animal faeces directly into the water source should be developed.

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