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Identification of human pathogenic *Enterocytozoon bieneusi*, *Cyclospora cayetanensis*, and *Cryptosporidium parvum* on the surfaces of vegetables and fruits in Henan, China

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ABSTRACT

Cryptosporidium spp., *Giardia duodenalis*, *Cyclospora cayetanensis*, and *Enterocytozoon bieneusi* are known etiological agents of self-limiting diarrhea, chronic disorders, and severe debilitating illnesses in humans, particularly children and patients with immunodeficiency diseases. To assess the pathogen carriage status of raw vegetables and fruits and the potential transmission routes of the aforementioned parasites in Henan province, China, a total of 1099 vegetables and fruits samples (21 items) were purchased and collected from agricultural farms or open markets. *Cryptosporidium* spp., *E. bieneusi*, *C. cayetanensis* and *G. duodenalis* were screened by employing polymerase chain reaction (PCR) amplification of species-specific genes. Three kinds of human pathogenic agent (*E. bieneusi*, *C. cayetanensis* and *C. parvum*) were identified on the surfaces of the vegetables and fruits (3.7%, 41/1099). *E. bieneusi* was found in 3.5% (38/1099) of the samples, whereas *C. cayetanensis* and *C. parvum* were only identified in two (0.2%) and one (0.1%) of the vegetable and fruit samples, respectively. No *G. duodenalis* contamination was detected in the present study. In total, 12 different *E. bieneusi* ITS genotypes (eight known and four novel) were detected, of which the ten (EbpA, CM8, CHG19, EbpC, CTS3, Henan-IV, and CHV1 to CHV4) that occurred in 20 samples (20/38, 52.6%) clustered into the previously described high potential zoonotic group 1 in the phylogenetic analysis. The remaining two known genotypes (BEB8 and CD6) detected in 18 samples (18/38, 47.4%) belonged to group 2. That *C. cayetanensis*, *C. parvum* and some *E. bieneusi* genotypes have been reported in humans, highlights the possible risk of foodborne related disease outbreaks.

1. Introduction

Cryptosporidium spp., *Giardia duodenalis*, *Cyclospora cayetanensis*, and *Enterocytozoon bieneusi* are important etiological agents of diarrhea in humans, yet the public health risk they pose is often neglected (Certad et al., 2017; Ortega and Sanchez, 2010; Wang et al., 2018a; Wang et al., 2018b). These agents mainly cause self-limiting diarrhea, chronic disorders, or severe debilitating illnesses in individuals, particularly in children and patients with acquired immune deficiency syndrome (Certad et al., 2017; Matos et al., 2012; Ortega and Sanchez, 2010; Wang et al., 2018a). Foodborne or waterborne transmission is the common mode of transmission for the spread of the aforementioned parasites (Åberg et al., 2015; Adam et al., 2016; Cacciò et al., 2005; Decraene et al., 2012; Feng and Xiao, 2011; Ryan et al., 2018).

As a primary source of essential nutrients, vitamins, minerals and fiber for our bodies, vegetables and fruits form a vital part of our daily diet. However, when eaten uncooked, fruits and vegetables can act as vehicles for infection with protozoan parasites, particularly *Cryptosporidium*, *Cyclospora* and *Giardia* (Utaaker et al., 2017). When fruits and vegetables are contaminated with protozoan oocysts or the cysts from fecal material or indirectly from the environment (e.g., from contaminated soil or irrigation water), they pose a persistent threat to public health. Because some parasite life stages like *Cryptosporidium* oocysts are robust, they can survive various processing treatments such as chlorine baths and blast freezing, and they may even fail to be washed away from the leaves or rhizomes of fresh produce when they

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infiltrate leafy vegetables via stomatal openings (Macarisin et al., 2010; Robertson and Chalmers, 2013). So consuming raw vegetables and improperly washed fruits is potentially a prime route for the transmission of human pathogens (Jedrzejewski et al., 2007; Yusof et al., 2017).

Since the mid-1990s, a very large number of cases and outbreaks reports of foodborne (cryptosporidiosis, cyclosporiasis, giardiasis and microsporidiosis) and waterborne (giardiasis and cryptosporidiosis) parasitic disease have occurred in the world (Åberg et al., 2015; Adam et al., 2016; Döller et al., 2002; Jedrzejewski et al., 2007). There are many other global reports showing that vegetables and fruits can harbor various human pathogens (Giangaspero et al., 2015; Hohweyer et al., 2016; Jedrzejewski et al., 2007; Sim et al., 2017). Therefore, while offering essential nutrients to the human body, fresh fruits and uncooked or undercooked vegetables may occasionally pose a serious threat to public health by acting as mechanical vectors for many human pathogens. In modern-day China, many people are now eating fresh or uncooked vegetables to protect the nutrient content of fresh foods from high temperature cooking. However, no studies have been conducted to evaluate the degree of contamination on vegetables and fruits by pathogens such as *Cryptosporidium* spp., *G. duodenalis*, *C. cayetanensis* and *E. bieneusi* in this country.

In the present study, we screened vegetables and fruits for parasitic contamination in the Henan province of China and report here on the public health aspects of our study results.

2. Materials and methods

2.1. Ethics

Ethical approval for this study was obtained from the Ethics Committee of the Henan Agricultural University. The vegetable and fruit sellers were not engaged in any form of discussion about this study.

2.2. Sample collection

A total of 1099 vegetables and fruit samples (21 items) were purchased and collected from open markets or agricultural produce farms located in Zhengzhou and Kaifeng in Henan Province, China, during June to August of 2014 and 2016 (Table 1). There were a total of 33 sampling sites located in 13 different districts of Henan Province (Fig. 1). All of the food items were produced locally. Each sample of about 200 g was collected in a plastic bag and marked according to the collection site and vegetable or fruit category. The samples were transported to the laboratory on their day of collection and then stored at 4 °C before the next procedure was performed.

2.3. Vegetable and fruit washing

A 25 g sample of each vegetable or fruit was taken for washing, including vegetable outer leaves (lettuce, coriander, celery, baby bok choy, leaf lettuce, water spinach, crown daisy, fennel plant, endive, spinach, schizonepeta, cabbage, leaf mustard, Chinese chive, and chive) and the stripped epidermis of bacca (cucumber, watermelon, potato, bean, green chili). Washing protocols were carried out according to the Bacteriological Analytical Manual (FDA, 2017; Shields et al., 2012). Briefly, 25 grams of produce were weighted and introduced into the filter bag (BagPage®, 400 ml, Interscience Lab. Inc. Boston). A volume of 100 ml of deionized water were added to the filter bag and after sealed, bags were agitated on rocker platform at 100 ppm for 30 min (bags were inverted after 15 min). Finally filter bags were opened, and carefully supernatant of each bag was transferred into two 50 ml conical centrifuge tubes. The eluents obtained were then concentrated by step centrifugation, and four-fifths of the centrifuge tube top volume was discarded each time. The final 1 ml volume of each concentrated solution obtained was used for DNA extraction.

2.4. DNA extraction and PCR amplification

Approximately 200 mg/ml of each sample was used to extract parasite genomic DNA with the E.Z.N.A.R® Stool DNA Kit (Omega Biotek Inc., Norcross, GA, USA), according to the manufacturer's instructions. The extracted DNAs were stored at −20 °C until PCR amplification.

E. bieneusi was identified by PCR amplification of the internal transcribed spacer (ITS) gene (Li et al., 2016), *C. cayetanensis* was identified by PCR amplification of the small subunit ribosomal RNA

Table 1
Pathogens identified on the surfaces of vegetables and fruits.

Items	Number of samples	Contamination (%)	*E. bieneusi* genotypes	Others
Lettuce	200	14 (7.0%)	CM8 (2); CD6 (7); EbpA (3); Henan-IV (1)	*C. cayetanensis* (1)
Coriander	152	1 (0.7%)	CM8 (1)	
Celery	70	1 (1.4%)	EbpA (1)	
Baby bok choy	59	1 (1.7%)	CHV3 (1)	
Chinese cabbage	47	0		
Leaf lettuce	44	2 (4.5%)	CHG19 (1)	*C. cayetanensis* (1)
Water spinach	28	3 (10.7%)	CD6 (1); BEB8 (1); CTS3 (1)	
Crown daisy	27	0		
Fennel plant	26	1 (3.9%)	EbpC (1)	
Endive	25	1 (4.0%)	Henan-IV (1)	
Spinach	20	0		
Schizonepeta	20	0		
Cabbage	18	0		
Leaf mustard	11	0		
Chinese chive	132	6 (4.5%)	CD6 (1); EbpA (2); EbpC (1); CHV1 (1)	*C. parvum* (1)
Chive	128	4 (1.4%)	CD6 (2); CHV2 (1); CTS3 (1)	
Cucumber	41	1 (2.4%)	CD6 (1)	
Watermelon	15	1 (6.7%)	CD6 (1)	
Potato	3	1 (33.3%)	CHV4 (1)	
Bean (Kidney/French bean)	28	4 (14.3%)	CD6 (4)	
Green chili	5	0		
Total	1099	41 (3.7%)	CD6 (17); EbpA (6); CM8 (3); EbpC (2); Henan-IV (2); CTS3 (2); CHG19 (1); BEB8 (1); CHV1 (1); CHV2 (1); CHV3 (1); CHV4 (1)	*C. cayetanensis* (2); *C. parvum* (1)

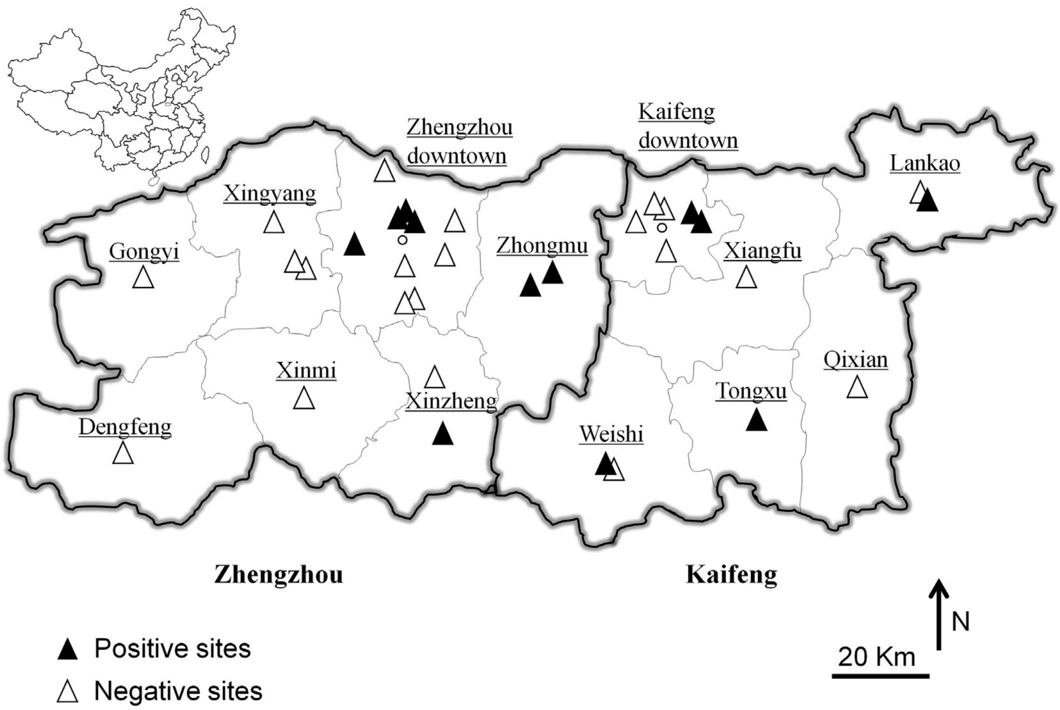

Fig. 1. Study area locations in Zhengzhou and Kaifeng districts of Henan, China. Triangles indicate the sampling sites.

(SSU rRNA) gene (Li et al., 2007), as was as the *Cryptosporidium* spp., and glycoprotein 60 (gp60) gene subtyping also employed (Cui et al., 2014). *G. duodenalis* was identified by PCR amplification of the triose-phosphate isomerase gene (Sulaiman et al., 2003). After electrophoresis on a 1.5% agarose gel stained with nucleic acid dye DNA green, the PCR products were visualized by ultraviolet transillumination. Each sample was divided into three separate specimens, and when any one of them was successfully polymerase chain reaction (PCR)-amplified it was judged to be positive.

2.5. Sequence analysis and E. bieneusi phylogenetic analysis

Positive PCR amplicons were sequenced on the ABI PRISM™ 3730 XL DNA Analyzer using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The sequence accuracy was confirmed with bidirectional sequencing, and the sequences obtained were aligned with the ClustalX 2.1 program (http://www.clustal.org/). Reference sequences were downloaded from GenBank to determine the species or genotypes.

The *E. bieneusi* genotypes isolated in this study were compared against known *E. bieneusi* ITS genotypes using a neighbor-joining analysis based on the distances calculated by the Kimura two-parameter model in the Mega 7 program (http://www.megasoftware.net/). Bootstrap analysis was used to assess the robustness of the clusters using 1000 replicates. The nomenclature system established by Thellier and Breton (2008) was used to name the *E. bieneusi* ITS genotypes.

2.6. Nucleotide sequence accession numbers

All novel nucleotide sequences from this study are deposited in GenBank at the National Center for Biotechnology Information under the following accession numbers: MK359051–MK359054.

3. Results

3.1. Pathogen identification on vegetables and fruits

Analysis of the 1099 collected vegetable and fruit samples indicated that three types of human parasite (*E. bieneusi*, *C. cayetanensis* and *C. parvum*) occurred on their surfaces (3.7%, 41/1099). *Giardia duodenalis* was not detected in any of the vegetable or fruit samples. No mixed contamination was found, either. About 66.7% (14/21) of the vegetable and fruit items were positive for the detected pathogens (Table 1). Samples from 12 (36.4%) sites out of the 33 collection sites (Table 1S) and from 7 (53.8%) of the 13 different districts were found to be contaminated with at least one of the three pathogens (Fig. 1).

3.2. Enterocytozoon bieneusi genotype identities

Enterocytozoon bieneusi was distributed across almost all types of vegetables and fruits in Henan, China. *E. bieneusi* was identified in 3.5% (38/1099) of the analyzed samples, and the highest contamination was found in potatoes (1/3, 33.3%), beans (4/28, 14.3%) and water spinach (3/288, 10.7%). *E. bieneusi* was not detected on Chinese cabbage, crown daisy, spinach, schizonepeta, cabbage, leaf mustard or green chili.

Nucleotide sequence analysis of the ITS region showed there was high genetic variability among the *E. bieneusi* isolates detected on the surfaces of the vegetable and fruit samples. A total of 12 different ITS genotypes (eight known and four new) were detected in the 38 successfully sequenced *E. bieneusi* samples. The nucleotide sequences of eight genotypes were identical to the genotypes previously reported; namely, EbpA ($n = 6$), CM8 ($n = 3$), EbpC ($n = 2$), Henan-IV (n = 2), CTS3 (n = 2), BEB8 ($n = 1$), CHG19 (n = 1), and CD6 ($n = 17$). However, the other four genotypes have not been reported previously and are, therefore, designated as novel. We have named them CHV1 (found on the surface of Chinese chive, $n = 1$), CHV2 (found on chive, n = 1), CHV3 (found on baby bok choy, n = 1), and CHV4 (found on potato, n = 1). The most prevalent and widely distributed *E. bieneusi* genotype in this study was CD6 (17/38, 44.7%), which was identified on the surface of lettuce ($n = 7$), beans ($n = 4$), chives ($n = 2$), Chinese chive (n = 1), water spinach (n = 1), cucumber (n = 1) and watermelon (n = 1) (Table 2).

3.3. E. bieneusi phylogenetic analysis

Of the 12 *E. bieneusi* ITS genotypes, ten were detected in 20 samples

Table 2
E. bieneusi ITS genotype distributions by host and location.

Species/ genotypes	Identified in this study	Host	Accession no.	Locations
EbpC	Chinese chive (1); Fennel plant (1)	Human	KX905207	China
		Human	AY371279	USA
		Cattle	KU531575	China
		Pig	AB470283	Japan
		Pig	AF135832	Germany
		Pig	JF927956	USA
		Goat	KP262380	China
		Dog	KJ668723	China
		Dog	KX869923	China
		Deer	KX383620	China
		Horse	KU194596	China
		Muskrat	AY237221	USA
EbpA	Lettuce (3); Chinese chive (2); Celery (1)	Cattle	KU245702	China
		Pig	KP318001	Brazil
		Pig	KJ668725	Germany
		Goat	KP262383	China
		Dog	KJ668725	China
		Deer	KX383623	China
		wild Boar	KX670588	China
		Horse	KU194597	China
CHG19	Leaf lettuce (1)	Goat	KP262374	China
		Horse	KU194592	China
		wild Boar	KX670587	China
CM8	Lettuce (2); Coriander (1)	Cattle	KU245698	China
		Horse	KU194591	China
		Monkey	KJ728801	China
Henan-IV	Lettuce (1); Endive (1)	Monkey	KJ728794	China
CTS3	Chive (1); Water spinach (1)	Sheep	MH817462	China
CD6	Lettuce (7); Bean (4); Chive (2); Cucumber (1);	Cattle	KU245697	China
		Goat	KP262368	China
	Chinese chive (1);	Dog	KJ668733	China
	Water spinach (1); Watermelon (1)	Monkey	KU604934	China
BEB8	Water spinach (1)	Cattle	KT984487	Brazil
		Monkey	KT984487	China

(20/38, 52.6%). These genotypes belong to the previously described high-potential zoonotic group 1 according to the phylogenetic analysis. The genotypes included six known ones (EbpA, CM8, CHG19, EbpC, CTS3, and Henan-IV) and four novel genotypes (CHV1 to CHV4). The other two known genotypes (BEB8 and CD6) were detected in 19 specimens (19/39, 48.7%) clustered in previously thought to be cattle-specific cluster group 2 (Fig. 2).

Nucleotide sequence analysis revealed some genetic diversity among the newly identified CHV1–CHV4 genotypes: CHV1 has three substitutions at positions 62 (G to A), 63 (C to G), and 104 (T to A) compared with genotype CM8 (KJ728801); CHV2 has one substitution at position 350 (G to A) compared with genotype CM8 (KJ728801); CHV3 has one substitution at position 215 (A to G) compared with genotype Henan-IV (KJ728794); and, CHV4 has five substitutions at positions 88 (A to G), 107 (G to A), 171 (G to T), 212 (G to A), and 236 (G to A) compared with genotype Henan-IV (KJ728794) (Fig. 1S).

3.4. *Cyclospora cayetanensis* and *C. parvum* on vegetables and fruits

Cyclospora cayetanensis and *C. parvum* were rarely identified on the surfaces of the vegetables and fruits we screened. *Cyclospora cayetanensis* was identified in two (0.2%) samples of lettuce and leaf lettuce, and *C. parvum* in one (0.1%) sample of Chinese chive (Table 1).

The sequences of two SSU rRNA genes from *C. cayetanensis* were identical to the human isolate from Shanghai, China (KJ569533). The SSU rRNA gene sequence obtained from the single *C. parvum*-positive sample shared 100% similarity with several other previously reported sequences, including those from human isolates in the UK (KM012045), Lebanon (KU311869), Tanzania (KU892559), and from cattle (KT884499; KP793008), horse (KU200956), chinchillas (KM819102), and macaca (KJ917579) in China. *Cryptosporidium parvum* gp60 gene subtyping of the *C. parvum* isolate was unsuccessful.

4. Discussion

The present study aimed to evaluate the level of contamination of human parasitic agents on the surfaces of the fresh vegetables and fruits in Henan, Central China. Three kinds of pathogen (*C. parvum*, *C. cayetanensis*, and *E. bieneusi*) were identified with a 3.7% (41/1099) contamination. Similar studies have been conducted to assess the contamination status of parasites in raw vegetables and fruits in different countries, including India, Canada, Nigeria, Iran, Ethiopia, Italy, Korea, and Nigeria, among others (Bekele et al., 2017; Caradonna et al., 2017; Karshima, 2018; Lalonde and Gajadhar, 2016; Maikai et al., 2013; Ranjbar-Bahadori et al., 2013; Sim et al., 2017; Utaaker et al., 2017).

Enterocytozoon bieneusi DNA was amplifiable in 38/1099 samples (3.5%) of vegetables and fruits in the present study, the genotypes of which have previously been reported in humans, various other animals (Wang et al., 2018b), and in berries, sprouts, and curly lettuce (Jedrzejewski et al., 2007). *Enterocytozoon bieneusi* has been reported to cause foodborne outbreaks in Sweden where it was linked with consuming cheese sandwiches and salad where both contained pre-washed cucumber slices (Decraene et al., 2012). *Enterocytozoon bieneusi* was also reported as present in Chinese wastewater in Shanghai, Nanjing, Wuhan, Qingdao and Zhengzhou (Li et al., 2012; Ye et al., 2017).

Molecular analyses provide important information for tracing the sources of pathogen contamination of food items. Twelve different *E. bieneusi* ITS genotypes, of which eight are known and four are novel, were associated with vegetables and fruits in the present study. The *E. bieneusi* ITS genotypes we identified have previously been mainly reported in humans, farm animals, and pets animals (Table 2). Among the identified genotypes, the most prevalent associated with the surfaces of vegetables and fruits is genotype CD6 (44.7%), which was previously mainly reported in cattle, goat, and cat in China (Wang et al., 2018b). Some genotypes such as EbpA, EbpC and Henan-IV have also been reported in humans (Table 2). Although other genotypes and the novel genotypes (CHV1 to CHV4) we identified herein have not been found in humans, the phylogenetic analysis showed that they cluster into the previously described high potential zoonotic group 1 (Fig. 2).

Cyclospora cayetanensis and *C. parvum* were rarely identified on the surfaces of the vegetables and fruits in this study, with *C. cayetanensis* being found in only two (0.2%) samples, *C. parvum* in only one (0.1%), and *G. duodenalis* in none of the samples. In contrast, a high incidence of *C. cayetanensis* and *C. parvum* was reported for vegetable and fruit samples in Korea (Sim et al., 2017), Ghana (Duedu et al., 2014) and Ethiopia (Bekele et al., 2017). Here, *C. cayetanensis* and *C. parvum* were only identified on the surfaces of lettuce, leaf lettuce, and Chinese chive. Similarly, *Cryptosporidium* and *Cyclospora* had the highest detection rates in chives and winter-grown cabbages, respectively, in Korea (Sim et al., 2017). In another study, *Cryptosporidium* oocysts were identified in a wide variety of foods including fruits, vegetables, dairy products, meat and various species of shellfish (Ryan et al., 2018). *Giardia duodenalis* was also identified in vegetables and fruits in Sudan (22.9%, 8/35) (Mohamed et al., 2016), Ethiopia (10.0%, 36/360) (Bekele et al., 2017), Ghana (5.6%, 22/395) (Duedu et al., 2014), and India (4.9%, 14/284) (Utaaker et al., 2017), etc.

Of note, human and livestock feces are usually used as fertilizer for cultivated land soil in Henan, China. All the eight known *E. bieneusi* genotypes we identified on the surfaces of vegetables and fruits have previously been reported in humans and domestic animals, such as cattle, goats, pigs, and dogs, for example (Table 2). Likewise, *C. parvum* was reported to commonly occur in humans and various other animals, especially cattle (Ryan et al., 2014), whereas *C. cayetanensis* has only been reported in humans (Ortega and Sanchez, 2010; Zhou et al., 2011). Similarly, *C. parvum* oocyst-contaminated foods reportedly

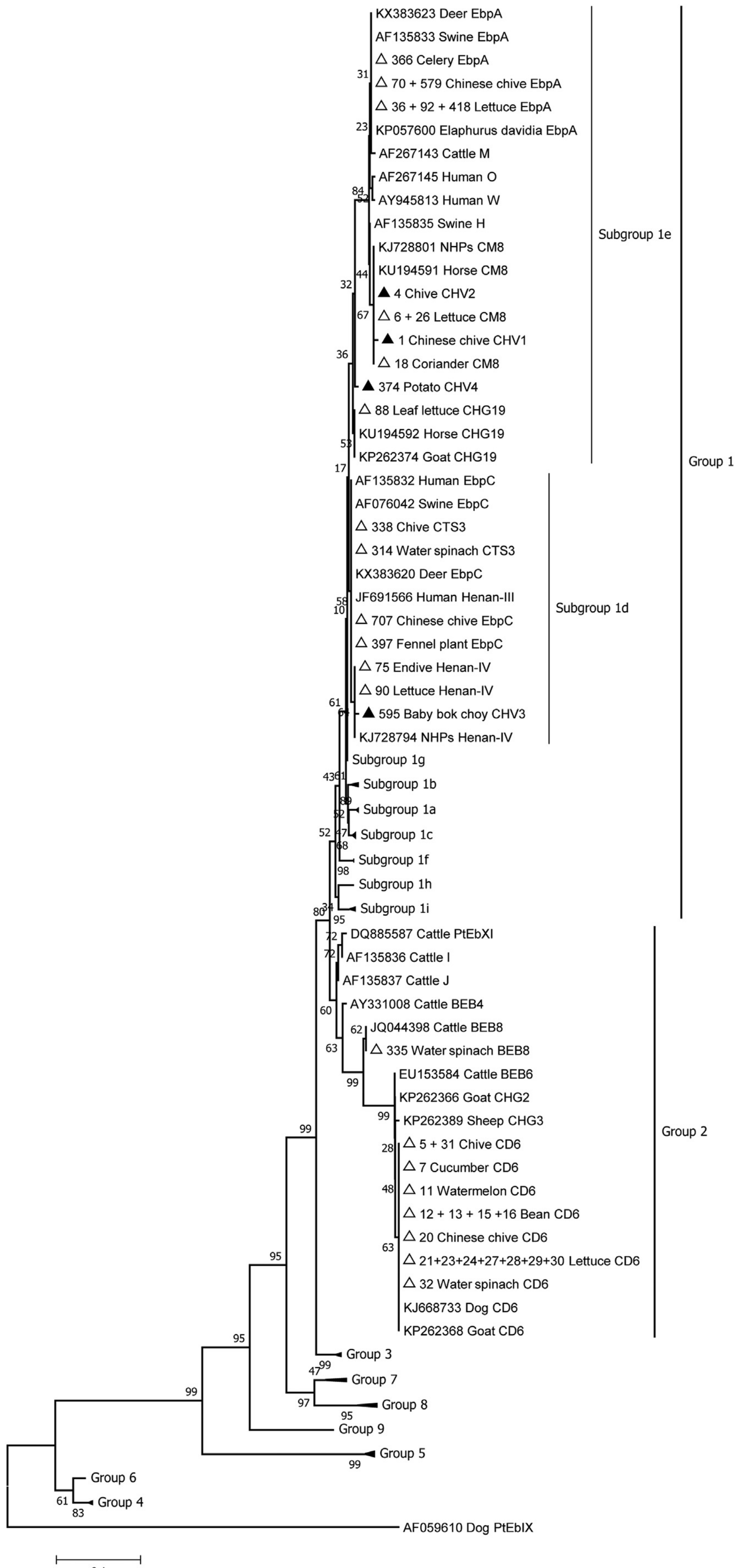

Fig. 2. Phylogenetic relationships of the *Enterocytozoon bieneusi* genotypes identified in this study and other reported genotypes. The phylogeny was inferred from a neighbor-joining analysis of the ITS sequences based on the distances calculated using the Kimura two-parameter model. Bootstrap values above 50% from 1000 replicates are shown on the nodes. The genotypes detected in this study are represented by triangles. Previously known genotypes are indicated by open triangles and novel genotypes are indicated by filled triangles. The serial numbers and sources of the isolates are shown with a plus sign (+) to connect them when there was more than one.

originated from districts with the highest numbers of homesteads possessing cattle herds in Poland (Rzezutka et al., 2010). Areas practicing wastewater irrigation with little or no wastewater treatment indicate that contaminated irrigation water might be a source of transmission of waterborne protozoa (Faour-Klingbeil and Todd, 2018).

In conclusion, this is the first report on the presence and prevalence of *E. bieneusi*, *C. cayetanensis* and *C. parvum* on vegetables and fruits in Henan, China. Some human parasitic agents were commonly found on the vegetables and fruits we screened. Identifying these parasites highlights the risk of a future disease outbreak occurring from the consumption of contaminated undercooked or uncooked vegetables and unwashed fruits.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.108292.

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Declaration of competing interest

No conflicts of interest are declared.

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