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First identification and coinfection detection of *Enterocytozoon bieneusi*, *Encephalitozoon* spp., *Cryptosporidium* spp. and *Giardia duodenalis* in diarrheic pigs in Southwest China

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Abstract

Background *Enterocytozoon bieneusi*, *Encephalitozoon* spp., *Cryptosporidium* spp., and *Giardia duodenalis* (*G. intestinalis*) are enteric pathogens that cause diarrhea in pigs. This study aimed to determine the prevalence of these enteric parasites and their coinfection with *E. bieneusi* in diarrheic pigs in Southwest China (Chongqing and Sichuan) using nested polymerase chain reaction (nPCR) based methods.

Results A total of 514 fecal samples were collected from diarrheic pigs from 14 pig farms in Chongqing (five farms) and Sichuan (nine farms) Provinces. The prevalence of *Encephalitozoon* spp., *Cryptosporidium* spp. and *G. duodenalis* was 16.14% (83/514), 0% (0/514), and 8.95% (46/514), respectively. Nested PCR revealed 305 mono-infections of *E. bieneusi*, six of *E. cuniculi*, two of *E. hellem*, and nine of *G. duodenalis* and 106 concurrent infections of *E. bieneusi* with the other enteric pathogens. No infections of *E. intestinalis* and *Cryptosporidium* species were detected. The highest coinfection was detected between *E. bieneusi* and *E. cuniculi* (10.5%, 54/514), followed by *E. bieneusi* and *G. duodenalis* (5.8%, 30/514) and *E. bieneusi* and *E. hellem* (2.9%, 15/514). *E. bieneusi* was the most frequently detected enteric pathogen, followed by *E. cuniculi*, *G. duodenalis* and *E. hellem*. There was a significant age-related difference in the prevalence of *E. cuniculi* in fattening pigs ($\chi^2 = 15.266$, $df = 3$, $P = 0.002$) and *G. duodenalis* in suckling pigs ($\chi^2 = 11.92$, $df = 3$, $P = 0.008$) compared with the other age groups. Sequence analysis of the ITS region of *Encephalitozoon* species showed two genotypes (II and III) for *E. cuniculi* and one (TURK1B) for *E. hellem*. Only *G. duodenalis* assemblage A was identified in all nested PCR-positive samples. *E. bieneusi* was found more often than other enteric pathogens.

Conclusions This study showed that *E. bieneusi*, *Encephalitozoon* spp. [*E. cuniculi* and *E. hellem*] and *G. duodenalis* were common enteric parasites in diarrheic pigs in Chongqing and Sichuan Provinces. In case of both mono-infection and

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coinfection, *E. bieneusi* was the most common enteric pathogen in diarrheic pigs. Thus, it may be a significant cause of diarrhea in pigs. Precautions should be taken to prevent the spread of these enteric parasites.

Keywords *Enterocytozoon bieneusi*, *Encephalitozoon* spp., Internal transcribed spacer (ITS), *Cryptosporidium* spp., *Giardia duodenalis*, Diarrheic pigs, Coinfection

Introduction

In pigs, diarrhea is one of the most significant health issues leading to poor productivity and death. Particularly in suckling and weaned pigs, diarrhea results in significant financial losses for the pig industry [1, 2]. Diarrhea disease causes 11.5–29.5% of all pig deaths [3]. It mostly results from a lack of protection of the mother, the environment, and a high infection pressure from enteric pathogens (microsporidia, bacteria, viruses, and parasites) alone or in combination [1, 4]. *Enterocytozoon bieneusi*, *Encephalitozoon* spp., *Giardia duodenalis*, and *Cryptosporidium* spp. are common enteric pathogens that have been found in a wide range of hosts of domestic animals, wild animals and mammals worldwide [5–8].

Microsporidia are single-celled obligate intracellular parasites that form spores and are currently thought to be most closely related to the fungal kingdom [9, 10]. They infect many invertebrates and vertebrates, including humans and pigs [11]. To date, more than 220 genera and 1,700 species of microsporidia have been identified in different hosts [12]. Among them, 17 species were reported in humans, with *Enterocytozoon bieneusi* and *Encephalitozoon* species as the most common species that infect humans, domestic animals and wild animals and cause almost all gastrointestinal infections [9, 13–15].

Encephalitozoon cuniculi is a microsporidian parasite that lives inside cells and produces spores. It can infect numerous mammalian species, including lagomorphs, rodents, dogs, cats, horses, ruminants, wild and exotic carnivores, nonhuman primates, humans, and bird species [16]. *E. cuniculi* was initially detected in pigs in 2007 [17]. This microsporidian species was discovered in one of six samples of swine feces and one of six samples of swine wastewater [17]. According to the number of 5'-GTTT-3' repeats in the ITS sequence of the ribosomal RNA gene, four different genotypes (genotypes I to IV) of *E. cuniculi* can be distinguished [18, 19], and their common names refer to the animal species from which they were first isolated: Genotypes I, II, III, and IV show three-, two-, four-, and five- repeats of the sequence 5'-GTTT-3', respectively [18, 19]. Genotype I is known as the “rabbit strain,” Genotype II as the “mouse strain,” Genotype III as the “dog strain,” and Genotype IV as the “human strain” [19, 20]. Moreover, ITS sequence analysis revealed that *E. hellem* has four genotypes (1 to 4) [21]. However, the ITS sequence of *E. intestinalis* does not appear to vary within the species [22].

Giardia duodenalis and *Cryptosporidium* spp. are common unicellular enteric protozoan parasites and have been found worldwide [23, 24]. They infect a wide variety of vertebrate hosts (such as humans, sheep, pigs, cattle, dogs, and cats), cause diarrhea, and are responsible for many disease outbreaks that spread through water and food in humans and nonhuman animals, particularly those who do not have strong immune systems or are already sick [24, 25]. According to a recent study by Dong et al. [26], the overall prevalence of *Cryptosporidium* worldwide is estimated to be 7.6%, with an average prevalence of 4.3% and 10.4% in developed and developing countries, respectively. However, a prevalence as high as 69.6% has been reported in Mexico [26]. In comparison, giardia infection rates (prevalence) range from 0.4–7.5% and 8–30% in developed and developing countries, respectively [27]. Humans can obtain *Cryptosporidium* oocysts and *Giardia* cysts directly or indirectly through contaminated water, food, and pastures [23]. The Food and Agriculture Organization of the United Nations (FAO) ranked *Cryptosporidium* spp. and *G. duodenalis* fifth and eleventh, respectively, on a list of 24 parasites that can be spread through food [23]. To date, at least 45 *Cryptosporidium* spp. and more than 120 genotypes have been identified; among these 19 species, four genotypes were found in humans. *C. hominis* and *C. parvum* are the most common species that cause cryptosporidiosis in humans [24, 25]. Currently, there are eight confirmed *G. duodenalis* assemblages (A–H) [24]; of these assemblages, A and B commonly infect humans and animals, while the remaining six (C–H) are host-specific [28].

Pigs have been reported to serve as hosts for different zoonotic species of those mentioned above that are enteropathogenic and are considered a possible source of human infections [7, 8]. In our previous work, we addressed the prevalence and genotypic distribution of *E. bieneusi* [29]. However, there is not much information about the coinfection of *E. bieneusi* with other enteric pathogens in diarrheic pigs worldwide. In addition, pigs are a common food source in different parts of the world. Whether a pig is a typical host for enteric pathogens and thus poses human health threats is not yet understood. Therefore, the present study aimed to determine the coinfection of *E. bieneusi* and the prevalence of enteric pathogens in diarrheic pigs, with special emphasis on *Encephalitozoon* species, *G. duodenalis* and *Cryptosporidium* species, in Chongqing and Sichuan Provinces from September 2021 to March 2022.

Methods

Sample collection and DNA extraction

A total of 514 fresh fecal samples were collected from 14 large-scale pig farms in Chongqing (five) and Sichuan (nine) Provinces from September 2021 to March 2022. All fecal specimens were collected directly from the middle part of the feces on the ground after defecation using sterile disposable gloves and placed in individual plastic containers. The pig breeds used in this study were Pig Improvement Company (PIC) pigs. Each pig's collection date, age and identification number were recorded at the time of sampling. The fecal samples of pigs were grouped into four age groups (Table 1). All pigs were in a diarrheic condition during the time of sampling. The piglets were dewormed twice, at 40–50 and 120 days, using albendazole. Sows were dewormed once at approximately four months. The albendazole dosages used for piglets and sows were in the range of 0.07–0.1 g per kg of body weight. All samples were transported immediately to the laboratory on-ice packs and kept at -20 °C until DNA extraction.

All the specimens were washed twice using distilled water to remove any preservatives or impurities and sieved through an 8 cm diameter sieve with a pore size of 45 µm. Then the filtrates were concentrated by centrifugation at 15,000 xg for 10 min. Genomic DNA was extracted from approximately 200 µL (200 mg) of each processed sample using an E.Z.N.A.® Mag-Bind Stool DNA Kit (OMEGA, Biotek Inc., Norcross, GA, USA), as recommended by the manufacturers. DNA was eluted in 50 µL of double-deionized distilled water, and DNA quantification was carried out using a DeNovix DS-11 + spectrophotometer/fluorometer (DeNovix,

Wilmington, United States) instrument. Finally, the extracted DNA was kept at -20 °C in a freezer until PCR analysis was performed.

PCR amplification

Encephalitozoon species were detected by nested PCR amplification of the fragment (approximately 305 bp) of the internal transcribed spacer (ITS) as previously described [30, 31]. In brief, the ITS was amplified by MSP-1 and MSP-2 A and by MSP-3 and MSP-4 A as outer- and inner primer pairs, respectively. The outer primer pair amplifies a large region containing the SSU, ITS, and LSU rRNA genes in several species of microsporidians. In contrast, the inner primer pair amplifies sequences in only *Encephalitozoon* spp. (*E. cuniculi*, *E. intestinalis* and *E. hellem*) [10].

G. duodenalis was screened using nested PCR amplification of the fragment (~500 bp) of the β-giardin (bg) gene as described previously [32]. *Cryptosporidium* spp. were screened using nested PCR amplification of the segment (~830 bp) of the small subunit rRNA (SSU rRNA) gene with previously published primers and PCR cycle settings [33, 34].

All PCRs were carried out in 25 µL reaction mixtures, including 12.5 µL of rTaq PCR Master Mix (Sanger Biotech Co., Ltd., Shanghai, China), 0.5 µL of each primer (0.4 mM), 1 µL of each DNA sample, and 10.5 µL of double distilled water. Positive and negative controls were used in all the PCR tests performed. Finally, the secondary PCR products were subjected to electrophoresis on a 1.5% agarose gel and visualized by the ChemiDoc XRS+Gel Imaging System (Bio-Rad, California, United States). Moreover, separate workplaces were employed

Table 1 Primers and PCR conditions for identifying *Encephalitozoon* spp. by targeting the ITS region, *Cryptosporidium* spp. by targeting the SSU rRNA gene, and *G. duodenalis* by targeting the bg gene

Organisms	Gene	Primer name	Primer sequence(5'-3')	Amplicon size (bp)	Reaction condition	Reference
<i>Encephalitozoon</i> spp.	ITS	MSP-1	TGAATG(G/T)GTCCCTGT	~300	94 °C for 3 min; 35 cycles of 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 1 min; 72 °C for 7 min	[31]
		MSP-2 A	TCACTCGCCGCTACT			
		MSP-3	GGAATTCACACCCGCCGTC(A/G)(C/T)TAT			
		MSP-4 A	CCAAGCTTATGCTTAAGT(C/T)(A/C)AA(A/G)GGGT			
<i>Cryptosporidium</i> spp.	SSU rRNA	F1	TTCTAGAGCTAATACATGCG	830	94 °C for 3 min; 35 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min; 72 °C for 7 min	[33, 34]
		R1	CCCATTTCTTCGAAACAGGA			
		F2	GGAAGGGTTGTATTTATTAGATAAAG			
		R2	AAGGAGTAAGGAACAACCTCCA			
<i>Giardia duodenalis</i>	bg	bg-F1	AAGCCCCGACGACCTCACCCGCACTGC	511	95 °C for 15 min; 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; 72 °C for 7 min	[32]
		bg-R1	GAGGCCGCCCTGGATCTTCGAGACGAC			
		bg-F2	GAACGAGATCGAGGTCGG			
		bg-R2	CTCGACGAGCTTCGTGTT			
<i>E. bieneusi</i>	ITS	EBITS3	GGTCATAGGGATGAAGAG	390	94 °C for 3 min; 35 cycles of 94 °C for 30 s, 57 °C (55 °C for 2nd PCR) for 30 s, and 72 °C for 40 s; 72 °C for 10 min	[35]
		EBITS4	TTCGAGTTCTTTGCGGCTC			
		EBITS1	GCTCTGAATATCTATGGCT			
		EBITS2.4	ATCGCCGACGGATCCAAGTG			

for DNA extraction, PCR preparation and amplification to avoid contamination. The primers and PCR conditions are summarized in Table 2.

Nucleotide sequencing and analysis

All the samples that produced a positive result with nested PCR amplicons of targeted genes were directly sent for bidirectional sequencing to Sangon Biotech Co., Ltd. (Shanghai, China). The nucleotide sequences obtained in this study were edited using Snap-Gen version 5.1 sequence analysis software (Technelysium Pty Ltd., South Brisbane, Australia). The Basic Local Alignment Search Tool (BLAST) searches were performed to determine whether there were any similarities with the sequences already deposited in GenBank. Then, ClustalX 2.1 [<http://www.clustal.org>] was used to identify the *Encephalitozoon* species and genotypes by comparison with reference sequences of *Encephalitozoon* spp. and genotypes that were downloaded from the National Center for Biotechnology Information [<https://www.ncbi.nlm.nih.gov/>]. The same procedures were followed to determine the species and subtypes of *Cryptosporidium* and *G. duodenalis* assemblages. Representative *E. cuniculi*, *E. hellem* and *G. duodenalis* nucleotide sequences found in diarrheic pigs have been deposited in GenBank at the National Center for Biotechnology Information with the accession numbers OR058746 to OR058756 for *E. cuniculi*, OR058757 for *E. hellem*, and OR091265 to OR091271 for *G. duodenalis*.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) 22.0 (for Windows, Version, IBM Armonk Corp., New York, NY, USA) was used to compare the prevalence of *Encephalitozoon* spp., *Cryptosporidium* spp. and *G. duodenalis* in diarrheic pigs using the χ^2 test. Significance was defined at $p < 0.05$.

Results

Prevalence of enteric pathogens in diarrheic pigs by age group in chongqing and Sichuan Provinces (Southwestern China)

The prevalence of each enteric pathogen in diarrheic pigs in different age groups is described in Table 1. We detected a higher prevalence of *E. bieneusi* infections than of other infections in all age groups (Table 1/ Fig. 1) [29]. According to the age distribution, the highest infection rate of *E. cuniculi* was recorded in fattening pigs (23.1%, 15/65), followed by sows (17%, 18/106) and suckling piglets (8.9%, 29/326). In *E. hellem*, the same prevalence (4.7%) was recorded for suckling piglets and sows. No prevalence was found in weaned pigs for the *Encephalitozoon* species (Table 1/ Fig. 1). The highest rate of *G. duodenalis* infection was found in suckling (10.8%, or 39/362), followed by fattening pigs (7.7%, or 5/65) and sows (2%, or 2/106), and there were no infections in the weaned age group (Fig. 1).

Microsporidian detection in pigs

The overall prevalence of microsporidian species in diarrheic pigs was 96.7% (493/510). The prevalence of *E. bieneusi*, *E. cuniculi*, and *E. hellem* were 79.8% (410/514), 12.0% (62/514) and 4% (21/514), respectively (Table 3). No *E. intestinalis* species were detected in the present study. *Encephalitozoon* spp. were found in all samples collected from Chongqing and Sichuan Provinces except for those from farms 8 and 12. The prevalence of *Encephalitozoon* spp. was highest (69.9%) and lowest (0%) in Sichuan suckling pigs on farms 5 and 8, respectively. The infection rate of *Encephalitozoon* species for the sows was highest (60.0%) and lowest (0.0%) on farms 2 and 8, respectively. In Chongqing, 14.3% (2/14) and 0% (0/1) were recorded as the highest and lowest prevalence rates of *Encephalitozoon* spp. in sows from farms 10 and 12, respectively. In fattening pigs, 7 of 25 (28.0%) and 4 of 20 (20.0%) were recorded as the highest and lowest prevalences of *Encephalitozoon* spp. on farms 13 and 10, respectively. In suckling pigs, 2 of

Table 2 Prevalence of enteric pathogens in pigs with diarrhea by age

Age groups	No. tested	<i>E. bieneusi</i> *	<i>E. cuniculi</i>	<i>E. hellem</i>	<i>G. duodenalis</i>	<i>Cryptosporidium</i> spp.
		No. of Positive (%)	No. of positive (%)	No. of positive (%)	No. of positive (%)	No. of positive (%)
Suckling piglets (Age ≤ 1 month)	326	262 (80.4)	29 (8.9)	15 (4.6)	39 (10.8)	0 (0.0)
Weaned pigs (1 to 3 months)	17	11 (64.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Fattening pigs (4 to 6 months)	65	47 (72.3)	15 (23.1)	1 (1.5)	5 (7.7)	0 (0.0)
Sow (≥ 6 months)	106	90 (84.9)	18 (17.0)	5 (4.7)	2 (2.0)	0 (0.0)
Total	514	410 (79.8)	62 (12.0)	21 (4.0)	46 (8.9)	0 (0.0)

*E. bieneusi** = data from Ghebremichael, Meng [29]

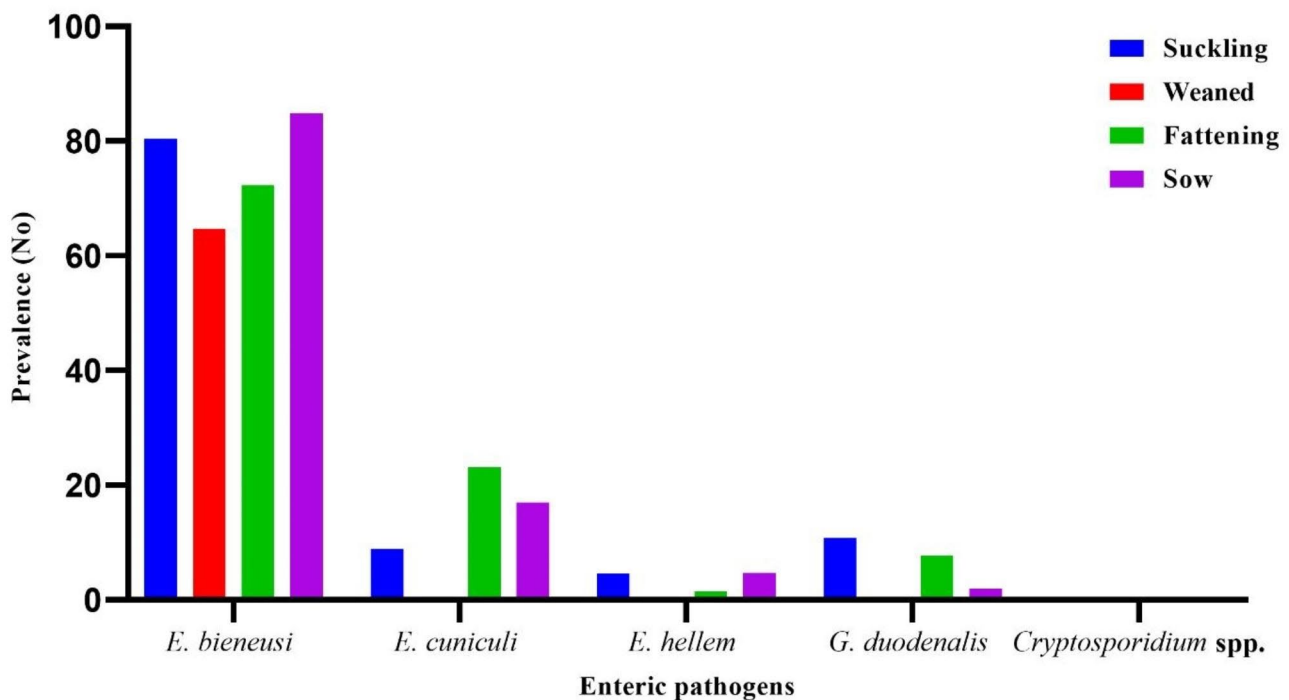


Fig. 1 Prevalence of *E. bieneusi*, *E. cuniculi*, *E. hellem*, *G. duodenalis* and *Cryptosporidium* spp. in age groups of diarrheic pigs from Chongqing and Sichuan Provinces

5 (40.0%) and 0 of 6 (0%) were the highest and lowest prevalences recorded on farms 14 and 12, respectively, for *Encephalitozoon* species. The prevalence of *E. cuniculi* in fattening pigs was significantly higher, 23.1% (15/65), than in sows 17% (18/106) ($\chi^2=15.266$, $df=3$, $P=0.002$). However, there were no statistically significant differences in the prevalence of *E. bieneusi*, and *E. hellem*, even though there was a difference in prevalence among the age groups ($\chi^2=6.437$, $df=3$, $P=0.092$; $\chi^2=2.129$, $df=3$, $P=0.546$) (Table 1). Genotypes II and III of *E. cuniculi* were found at least once on almost all farms except farms 8, 9, and 12. No *E. hellem* were found on six farms [6, 7, 8, 11, 12 and 14] (Table 3).

Genotype distribution of *Encephalitozoon* spp.

Sequence analysis of the ITS region of *E. cuniculi* revealed two distinct genotypes (II and III), and only one genotype (TURK1B) was identified in *E. hellem* in this study. A total of 83 *Encephalitozoon* spp.-positive samples were amplified and sequenced successfully in diarrheic pigs. Furthermore, most farms harbored at least one *Encephalitozoon* spp.-genotype, except farms 8, 9 and 12. For *E. cuniculi*, nine cases of genotype II and fifty-three cases of genotype III were identified. In the present investigation, genotype III was the most prevalent genotype. In both provinces, only genotype TURK1B of *E. hellem* was found (Table 3).

In this study, the sequences of 48 positive samples isolated from diarrheic pigs with genotype III of *E. cuniculi*

were 100% identical to the sequence (KX189630) from *Apodemus agrarius*, a striped field mouse in a study from Poland [36]. At the same time, four samples with genotype III sequences were identical and had the highest degree of similarity (99.5–98.97%) with the *Apodemus agrarius* genotype III (KX189630). A genotype III sequence found in one diarrheic pig sample in this study had 99.65% homology to a genotype III sequence (KJ577583) isolate from *Lagurus lagurus* (steppe lemming) in the Czech Republic [37] (Table 4, Figure S1(B)). In addition, eight positive *E. cuniculi* genotype II samples showed complete sequence identity with genotype II (accession No. KX189632) from yellow-necked mouse [*Apodemus flavicollis*] in Poland [36]. One sample of *E. cuniculi* genotype II had 98.92% homology with isolate from yellow-necked mouse (accession No. KX189632) (Table 4, Figure S1(A)). All 21 positive samples of *E. hellem* showed complete sequence similarity to a positive sample of genotype TURK1B (accession No. MT478448) from a budgerigar [*Melopsittacus undulatus*] in Turkey [38] (Figure S1(C)).

Prevalence of *Cryptosporidium* spp. and *G. duodenalis*

Among the 514 fecal samples collected from pigs, the prevalence of *G. duodenalis* was 8.95% (46/514), and *Cryptosporidium* spp. were not detected (0%, 0/514). This study identified higher infections (10.0%, 41/410) and lower infections (4.4%, 5/114) of *G. duodenalis* the Sichuan and Chongqing Provinces, respectively. The

Table 3 Prevalence and genotypes of *Encephalitozoon* spp. in diarrhetic pigs in Chongqing and Sichuan Provinces

Provinces	Farms	№ tested	Eb*		Enc. spp.		Mono infection of Ec.		Mixed infection of Eb & Ec.		Mono infection of <i>E. hellem</i>		Mixed infection of Eb & Eh	
			No. (%)	No. (%)	Geno-type (No.)	No. (%)	Genotype (No.)	Geno-type (No.)	No. (%)	Genotype (No.)				
Sichuan	1	23	23 (100.0)	5 (21.7)	0	2	PigCE07.01 + III (1), PigCE02.03 + III (1)	0	3	PigCE01.01 + TURK1B (1), PigCE02.01 + TURK1B (1), PigCE08.03 + TURK1B (1)				
	2	48	45 (93.8)	10 (20.8)	0	5	B + III (3), PigCE06.02 + III (1), PigCE08.05 + III (1)	0	5	PigCE01.02 + TURK1B (2), PigCE08.02 + TURK1B (1), PigCE07.05 + TURK1B (1), PigCE06.04 + TURK1B (1)				
	5	5	5 (100.0)	3 (60)	0	3	PigCE07.07 + II (1), PigCE07.05 + II (1), PigCE08.05 + III (1)	0	0					
	3	50	39 (78.0)	7 (14)	III (1)	3	PigCE08.07 + III (2), PigCE08.11 + III (1)	TURK1B (1)	2	B + TURK1B (1), PigCE08.02 + TURK1B (1)				
	4	50	46 (92.0)	16 (32)	0	12	PigCE08.07 + II (1), PigCE07.05 + III (1), PigCE01.04 + III (1), PigCE08.09 + III (1), PigCE08.15 + III (2), PigCE01.06 + III (1), B + III (1), PigCE08.02 + III (3), PigCE08.05 + III (1)		4	PigCE06.01 + TURK1B (1), PigCE07.08 + TURK1B (1), PigCE07.09 + TURK1B (1), B + TURK1B (1)				
	5	23	22 (95.7)	16 (69.6)	III (1)	13	PigCE08.07 + III (1), PigCE01.02 + III (1), PigCE08.17 + III (1), PigCE08.02 + III (6), PigCE07.01 + III (1), PigCE07.09 + III (2), B + III (1)		2	PigCE08.07 + TURK1B (1), PigCE08.04 + TURK1B (1)				
	6	30	28 (93.3)	0 (0)	0	0	0	0	0					
		20	13 (65.0)	1 (5)		1	B + III (1)		0					
	7	40	24 (60.0)	0 (0)	0	0	0	0	0					
		11	7 (63.6)	1 (9.1)		1	PigCE08.02 + III (1)		0					
8	45	34 (75.6)	0 (0)	0	0	0	0	0						
	5	5 (100.0)	0 (0)	0	0	0	0	0						
9	50	35 (70.0)	2 (4)	0	0	0	0	0	2	PigCE08.02 + TURK1B (1), PigCE08.10 + TURK1B (1)				
	Sub total	400	326 (81.5)	61 (15.3)	III (2)	40	II (3), III (37)	1	18					

Table 3 (continued)

Provinces	Farms	N ^o tested	Eb [*]	Enc. spp.	Mono infection of Ec.	Mixed infection of Eb & Ec.		Mono infection of <i>E. hellem</i>	Mixed infection of Eb & Eh		
						No. (%)	No. (%)		Geno-type (No.)	No. (%)	Geno-type (No.)
Chongqing	10	20	15 (75.0)	4 (20)	0	4	B + II (1), B + III (2), PigCE02.06 + III (1)	0			
		14	14 (100.0)	2 (14.3)	0	1	PigCE01.01 + III (1)		1	PigCE07.01 + TURK1B (1)	
		11	11 (55.0)	5 (25)	III (2)	3	PigCE08.02 + II (1), PigCE07.02 + II (1), PigCE08.02 + III (1)	0			
		12	9	5 (55.6)	0 (0)	0	0		0		
			6	5 (83.3)	0 (0)	0	0		0		
			1	0 (0.0)	0 (0)	0	0		0		
		13	25	21 (84.0)	7 (28)	0	6	F + II (2), E + III (1), F + III (1), Peru8 + III (1), PigCE05.01 + III (1)	TURK1B (1)	0	
			8	6 (75.0)	0 (0)	0	0		0	0	
			6	5 (83.3)	2 (33.3)	0	2	B + III (1), PigCE08.02 + III (1)	0	0	
		14	5	2 (40.0)	2 (40)	II (1), III (1)	0	0	0	0	
	Sub total	114	84 (73.7)	22 (19.3)	II (1), III (3)	16	II (5), III (11)	1	1		
	Total	514	410 (79.8)	83 (16.1)	II (1), III (5)	56 (10.9)	PigCE07.02 + II (1); PigCE07.05 + II (1); PigCE07.07 + II (1); PigCE08.02 + II (1); PigCE08.07 + II (1); B + II (1); F + II (2); PigCE01.01 + III (1); PigCE01.02 + III (1); PigCE01.04 + III (1); PigCE01.06 + III (1); PigCE02.03 + III (1); PigCE02.06 + III (1); PigCE05.01 + III (1); PigCE06.02 + III (1); PigCE07.01 + III (2); PigCE07.05 + III (1); PigCE07.09 + III (2); PigCE08.02 + III (12); PigCE08.05 + III (3); PigCE08.07 + III (3); PigCE08.09 + III (1); PigCE08.11 + III (1); PigCE08.15 + III (2); PigCE08.17 + III (1); B + III (9); E + III (1); F + III (1), Peru8 + III (1)	TURK1B (2)	19 (3.7)	B + TURK1B (2); PigCE01.01 + TURK1B (1); PigCE01.02 + TURK1B (2); PigCE02.01 + TURK1B (1); PigCE06.01 + TURK1B (1); PigCE06.04 + TURK1B (1); PigCE07.01 + TURK1B (1); PigCE07.05 + TURK1B (1); PigCE07.08 + TURK1B (1); PigCE07.09 + TURK1B (1); PigCE08.02 + TURK1B (3); PigCE08.03 + TURK1B (1); PigCE08.04 + TURK1B (1); PigCE08.07 + TURK1B (1); PigCE08.10 + TURK1B (1)	
Total						62/514 (12%)		21/514 (4%)			

Eb = *E. bieneusi*; Enc. spp. = *Encephalitozoon species*; Ec. = *E. cuniculi*; Eb* = data from [29]

prevalence of *G. duodenalis* on different farms ranged from 0 to 36%. Among the farms, farm 9 had the highest prevalence (36%, 18/50), followed by farm 5 (17.4%, 4/23) and farm 10 (15%, 3/20), while farms 12, 13, and 14 had no *G. duodenalis* (Table 5). The highest rate of *G. duodenalis* infection was found in suckling pigs (10.8%, 39/326), followed by fattening pigs (7.7%, 5/65) and sows (2.0%, 2/106), and no infections were found in weaned pigs. *G. duodenalis* infection rates varied significantly between the age groups ($\chi^2=11.92$, $df=3$, $P=0.008$) (Table 1).

Distribution of *G. duodenalis* assemblages and *Cryptosporidium* spp. genotypes

For isolates of *G. duodenalis*, sequence analysis of the β -giardin (bg) gene revealed that all the positive samples belonged to *G. duodenalis* assemblage A (n=46). Thirty-seven assemblage A sequences shared complete sequence identity with human-, sheep- and cat-derived assemblage A isolated (KP687765, KR075937, KJ027408) from Canada and China, respectively (Table 6). Six assemblage A sequences showed identities of 99.39 to 99.8%, the same as two assemblage A isolates (KP687765, KR075937)

Table 4 *E. cuniculi* genotypes determined on the basis of the small subunit rRNA (rRNA) gene locus

Genotype	Accession no.	Nucleotide position								
		130	158	159						
Genotype II	KX189632 (Ref. seq.)	T	G	G						
	OR058746_PigCE01-II	T	G	G						
	OR058747_PigCE02-II	C	G	G						
	OR058748_PigCE03-II	T	T	T						
		71	86	94	135	150	155	159	197	201
Genotype III	KJ577583 (Ref. seq.)	T	A	A	T	G	T	T	G	T
	OR058749_PigCE04-III	T	A	A	T	G	C	T	G	C
	OR058750_PigCE05-III	T	A	A	T	A	T	T	G	T
	OR058751_PigCE06-III	T	A	A	A	G	T	C	G	T
	OR058752_PigCE07-III	C	A	A	T	G	T	T	G	T
	OR058753_PigCE08-III	T	A	T	T	G	T	T	G	T
	OR058754_PigCE09-III	T	G	A	T	G	T	T	G	T
	OR058755_PigCE10-III	T	A	A	T	G	T	T	A	T
	OR058756_PigCE11-III	T	A	A	T	G	T	T	G	T

derived from humans and sheep in Canada and China, respectively [39, 40] (Table 6, Figure S1(D)). Another three assemblage A sequences were 99.8% identical to a human-derived assemblage A (KP687765) sequence from Canada and a cat-derived isolate (KJ027408) sequence from China.

Prevalence of single infection and coinfection of *E. bienersi*, *Encephalitozoon* spp. [*E. cuniculi* and *E. hellem*], *Cryptosporidium* spp. and *G. duodenalis* in diarrheic pigs in Southwestern China

Table 7 displays the occurrence of single- and mixed infections of enteric pathogens detected in diarrheic pigs. The nested PCR revealed 305 *E. bienersi* mono-infections, six *E. cuniculi* mono-infections, two *E. hellem* mono-infections, and nine *G. duodenalis* mono-infections (Table 7). However, no infections with *E. intestinalis* and *Cryptosporidium* spp. were detected. Most of the diarrheic pigs that tested positive, 62.6% (322/514), had only one pathogen, and 20.6% (106/514) tested positive for two or three pathogens. Regarding mono-infection, *E. bienersi* was the most commonly found agent, followed by *G. duodenalis*, *E. cuniculi*, and *E. hellem*.

The present study revealed eight and forty-eight positive isolates as coinfections between Genotypes II and III of *E. cuniculi* and *E. bienersi*, respectively. The highest mixed infection rate was recorded for *E. bienersi* and Genotype III of *E. cuniculi* with 12 positive isolates of Genotype PigCE08.02, followed by genotypes PigCE08.05 and PigCE08.07, each with three positive isolates, whereas PigCE07.01, PigCE07.09, and PigCE08.15 each had two positive isolates. Two positive isolates had *E. bienersi* Genotype F and *E. cuniculi* Genotype II. All the remaining genotypes of *E. bienersi* and *E. cuniculi* genotypes II and III had only one positive isolate each (Table 3). Between *E. bienersi* and *G.*

duodenalis assemblage A, the highest mixed infection rate was recorded for PigCE08.02 with nine positive isolates, followed by Genotype B in five positive isolates, PigCE08.07 with four positive isolates, and PigCE08.01 and PigCE08.10, each with two positive isolates. The remaining positive isolates had one genotype each of *E. bienersi* and *G. duodenalis* (Table 5).

In total, the highest mixed infection rate was detected between *E. bienersi* and *E. cuniculi* (10.5%, 54/514), followed by *E. bienersi* and *G. duodenalis* (5.8%, 30/514) and *E. bienersi* and *E. hellem* (2.9%, 15/514). *E. bienersi* was the most frequently detected enteric pathogen, followed by *E. cuniculi*, *G. duodenalis* and *E. hellem*.

Discussion

Pork is one of the most widely consumed meats in China, and its quality and demand are rising. Pigs are considered one of the most important reservoirs for enteric pathogens (*E. bienersi*, *E. cuniculi*, *E. hellem*, *Cryptosporidium*, and *G. duodenalis*) [41]. An epidemiological investigation is one of the best ways to learn about the molecular characteristics and diversity of enteric pathogens. Therefore, we examined enteric pathogen prevalence and mixed infection in diarrheic pigs from Chongqing and Sichuan Provinces in Southwestern China. This study represents the first report of the occurrence and mixed infections of *E. bienersi*, *E. cuniculi*, *E. hellem*, *Cryptosporidium*, and *G. duodenalis* in diarrheic pigs in Chongqing and Sichuan Provinces. The prevalence of *E. bienersi*, *E. cuniculi*, *E. hellem*, *Cryptosporidium*, and *G. duodenalis* was 79.8% (410/514) [29], 12% (62/514), 4% (21/514), 0% (0/514) and 8.95% (46/514) among fecal samples, respectively. The present study results show that enteric pathogens are common in diarrheic pigs in Chongqing and Sichuan Provinces. On the 14 farms studied, fecal samples from diarrheic pigs harbored at least one enteric pathogen. *E.*

Table 5 Farm-specific prevalence and genetic characterizations of *G. duodenalis* and *Cryptosporidium* spp. in diarrheic pigs in Chongqing and Sichuan Provinces, China

Provinces	Farms	N° tested	<i>G. duodenalis</i>					<i>Cryptosporidium</i> spp. No. (%)
			Overall infection		Mono infection	Mixed infection of Eb & <i>G. duodenalis</i>		
			No. (%)	As-semblage (No.)		No. (%)	No.	
Sichuan	1	23	1(4.4%)	A (1)	0	1	PigCE07.02 + A (1)	0
	2	48	7 (14.6%)	A (7)	0	7	PigCE01.01 + A (2), PigCE01.02 + A (1), PigCE01.03 + A (1), PigCE08.02 + A (1), PigCE07.05 + A (1), B + A (1)	0
		5	0	0	0	0	0	0
	3	50	5 (10.0%)	A (5)	0	5	PigCE06.01 + A (1), PigCE08.02 + A (1), PigCE08.09 + A (1), PigCE08.10 + A (1), PigCE08.12 + A (1),	0
	4	50	0	0	0	0	0	0
	5	23	4 (17.4%)	A (4)	0	4	PigCE01.02 + A (1), PigCE08.07 + A (1), PigCE08.16 + A (1), B + A (1)	0
	6	30	0	0	0	0	0	0
		20	2 (10.0%)	A (1)	1	1	B + A (1)	0
	7	40	2 (5.0%)	A (2)	1	1	B + A (1)	0
		11	0	0	0	0	0	0
8	45	2 (4.4%)	A (2)	0	2	PigCE08.02 + A (1), PigCE08.07 + A (1)	0	
	5	0	0	0	0	0	0	
9	50	18 (36.0%)	A (18)	6	12	PigCE02.01 + A (1), PigCE07.01 + A (1), PigCE08.02 + A (6), PigCE08.07 + A (2), PigCE08.10 + A (1), B + A (1),	0	
	Subtotal	400	41 (10.3%)	A (41)	8 (2)	33 (8.3)	B + A (4), PigCE01.01 + A (2), PigCE01.02 + A (2), PigCE01.03 + A (1), PigCE02.01 + A (1), PigCE06.01 + A (1), PigCE07.01 + A (1), PigCE07.02 + A (1), PigCE07.05 + A (1), PigCE08.02 + A (9), PigCE08.07 + A (4), PigCE08.09 + A (1), PigCE08.10 + A (2), PigCE08.12 + A (1), PigCE08.16 + A (1)	0
Chongqing	10	20	3 (15.0%)	A (3)	1	2	PigCE07.04 + A (1), PigCE08.03 + A (1)	0
		14	0	0	0	0	0	0
	11	20	2 (10.0%)	A (2)	0	1	B + A (1), III + A (1)	0
	12	9	0	0	0	0	0	0
		6	0	0	0	0	0	0
		1	0	0	0	0	0	0
	13	25	0	0	0	0	0	0
		8	0	0	0	0	0	0
		6	0	0	0	0	0	0
	14	5	0	0	0	0	0	0
	Subtotal	114	5 (4.4%)	A (5)	2	3	B + A (1), PigCE07.04 + A (1), PigCE08.03 + A (1),	0
	Total	514	46 (8.95%)	A (46)	9 (1.75)	37 (7.2)	B + A (5), PigCE01.01 + A (2) PigCE01.02 + A (2), PigCE01.03 + A (1), PigCE02.01 + A (1), PigCE06.01 + A (1), PigCE07.01 + A (1), PigCE07.02 + A (1), PigCE07.04 + A (1), PigCE07.05 + A (1), PigCE08.02 + A (9), PigCE08.03 + A (1), PigCE08.07 + A (4), PigCE08.09 + A (1), PigCE08.10 + A (2), PigCE08.12 + A (1), PigCE08.16 + A (1), III + A (1)	0

Table 6 *G. duodenalis* assemblage A subtypes defined on the basis of the beta-giardin (bg) gene locus

Accession no.	Nucleotide position							
	83	99	330	358	362	409	453	470
KR075937 (Ref. seq.)	A	G	A	A	A	C	G	A
OR091265_PigCE01_Gbg	A	G	A	A	A	C	G	A
OR091266_PigCE02_Gbg	G	G	A	A	A	C	G	A
OR091267_PigCE03_Gbg.	A	G	A	A	A	T	G	A
OR091268_PigCE04_Gbg	A	G	A	A	A	C	A	A
OR091269_PigCE05_Gbg	A	A	G	A	G	C	G	A
OR091270_PigCE06_Gbg	A	G	A	G	A	C	G	A
OR091271_PigCE07_Gbg	A	G	A	A	A	C	G	T

Table 7 Single and mixed infections of *E. bienersi*, *Encephalitozoon* spp. [*E. cuniculi* and *E. hellem*], *Cryptosporidium* spp. and *G. duodenalis* in diarrheic pigs (total samples n = 514)

Enteric pathogens	No. positive	%
<i>Enterocytozoon bienersi</i>	305	59.3
<i>Encephalitozoon cuniculi</i>	6	1.2
<i>Encephalitozoon hellem</i>	2	0.4
<i>Cryptosporidium</i> spp.	0	0.0
<i>G. duodenalis</i>	9	1.8
Total with single infection	322	62.6
<i>E. bienersi</i> + <i>E. cuniculi</i>	54	10.5
<i>E. bienersi</i> + <i>E. hellem</i>	15	2.9
<i>E. bienersi</i> + <i>Cryptosporidium</i> spp.	0	0.0
<i>E. bienersi</i> + <i>G. duodenalis</i>	30	5.8
<i>E. bienersi</i> + <i>E. cuniculi</i> + <i>G. duodenalis</i>	2	0.4
<i>E. bienersi</i> + <i>E. hellem</i> + <i>G. duodenalis</i>	4	0.8
<i>E. bienersi</i> + <i>E. hellem</i> + <i>Cryptosporidium</i> spp.	0	0.0
<i>E. cuniculi</i> + <i>G. duodenalis</i>	1	0.2
Total with mixed infection	106	20.6
Negative	86	16.7

biensersi, *E. cuniculi*, *E. hellem* and *G. duodenalis* assemblage A were the common pathogens causing diarrhea in pigs.

E. bienersi, *E. intestinalis*, *E. hellem*, and *E. cuniculi* are the most common causes of microsporidiosis in humans [14, 42]. The overall prevalence of *E. cuniculi* and *E. hellem* were 12% (62/514) and 4% (21/514), respectively. However, no infection of *E. intestinalis* was detected in the diarrheic pigs in our study. The prevalence of *E. cuniculi* detected in our study is higher than that reported by Nemeji et al. [43] 3.5% (16/460), in wild boards from Central Europe and by Reetz et al. [44] 8.8% (3/34) in domestic pigs from Germany. A recent study performed by Pekmezci et al. [38] on budgerigar from Turkey revealed a 14.7% (21/143) prevalence of *E. hellem*, much higher than that detected in our study.

In this study, the sequences of amplicons from *E. cuniculi*-positive samples were determined to belong to Genotypes II, and III, with Genotype III showing dominance (77.4%, 48/62) among the detected genotypes. A study on wild boars in Central Europe revealed a higher prevalence of *E. cuniculi* genotype II (61.9%, 13/21) [43].

Regarding the *Encephalitozoon* spp. infection of pigs, this study is the first report from Southwestern China, and a similar genotype of *E. hellem* (TURK1B) was identified in budgerigar from Turkey [38], but there are numerous reports of *E. hellem* in various bird hosts and mammals [45]. To the researchers' knowledge, this is the first report of the TURK1B genotype of *E. hellem* in pigs, and further study is needed to investigate the relationship between budgerigars and pigs.

Different *Cryptosporidium* species cause cryptosporidiosis in pigs. At least 42 species and 70 genotypes of *Cryptosporidium* spp. have been found in different hosts. Twenty *Cryptosporidium* species have been found in humans and eight in pigs. Six of the eight *Cryptosporidium* species found in pigs [*C. scrofarum*, *C. suis*, *C. parvum*, *C. muris*, *C. andersoni*, and *C. tyzzeri*] can infect pigs naturally. In contrast, the other two species [*C. hominis* and *C. meleagridis*] can infect pigs only by human intervention [41]. In addition, infections with *Cryptosporidium* have been identified in pigs of all age groups in multiple developed and developing countries [46, 47]. The present study detected no *Cryptosporidium* species-positive samples upon amplification of the SSU rRNA genes using nested PCR. Our results were similar to that in previous reports from Hunan [41], Ezhou, Xiaogan, Xiangyang [48], Zhengzhou (farms 2,3 and 4), Zhoukou and Luohe [49]. The lack of detection may be attributed to the well-managed breeding practices of farms [41]. Furthermore, previous studies indicated that *Cryptosporidium* spp. were associated with asymptomatic pigs, *Cryptosporidium* spp. might not be a direct cause of diarrhea in pigs [50, 51], while it is associated with diarrhea in alpacas and calves [52, 53]. However, a recent meta-analysis study by Wang and colleagues showed a 10.5% and 8.2% prevalence in pigs in Chongqing and Sichuan Provinces, respectively [54]. In addition, different prevalences of *Cryptosporidium* spp. in pigs have been reported in different provinces of China, with the highest in Heilongjiang Province (55.8%, 63/113) [55] and the lowest in the Tibet Autonomous Region (0.49%, 3/614) [56].

Based on the β -giardin (bg) genes of *G. duodenalis*, an overall prevalence of 8.95% (46/514) was detected in diarrheic pigs in Southwest China. The infection rates of *G. duodenalis* in pigs were lower than those previously reported in other provinces of China, i.e., Shanghai (26.88%, 25/93) [57] and Zhejiang (10.5%, 13/124) [58], and other parts of the world, i.e., in western Australia (31.1%, 90/289) [59], northwestern of England (57.1%, 4/7) [60], and Ontario Canada (66.4%, 81/122) [61]. However, they were higher than those reported in Hubei (0.97%, 8/826), Xinjiang (2.6%, 21/801), Henan (1.7%, 15/897), Yunnan (2.5%, 5/200), and Guangdong (4.2%, 3/72) [23, 49, 58, 62]. Many factors can affect the prevalence of *G. duodenalis* in pigs, such as the age groups, methods of breeding, management system, detection methods, water supply, farm hygiene, animal stocking density, and pig health status [49, 57].

Giardia duodenalis infections occur in all age groups of pigs, from nursing piglets to adult sows [63]. In this study, *G. duodenalis* had the highest prevalence in suckling pigs (10.8%, 39/326) and the lowest in weaned pigs (0%, 0/17). Our rates were lower than those revealed in a study in Australia (18.7%, 23/123) [59] but higher than those in Denmark (2%, 3/152) [64], Zambia (6.3%, 2/32) [65], southern China (2.3%, 2/87) [58], and Shaanxi province (6.5%, 10/155) [66]. More studies from Xinjiang and three Provinces of southern China (Yunnan, Zhejiang and Guangdong) found a higher prevalence in fattening pigs (5.4%, 7/129) and (12.3, 9/73), respectively [58, 62]. In contrast, the highest *G. duodenalis* prevalence was found in weaned pigs in Denmark (27.4%, 64/234) [64] and Australia (41.0%, 64/156) [59]. Moreover, other studies detected a higher prevalence in sows in Hubei Province of China (1.38%, 5/362) [23], Shaanxi Province of China (10.5%, 6/57) [66], and Zambia (40.0%, 6/15) [65]. Such variation may be due differences into sample size.

A previous review [67] showed that *G. duodenalis* assemblage A is reported to infect domestic animals, wild animals (including beavers, cats, lemurs, cows, sheep, dogs, chinchillas, alpacas, horses, and pigs), and humans. In this study, analysis of the DNA sequence of the β -giardin (bg) gene showed that all isolates belonged to *G. duodenalis* assemblage A and subassemblage A1, consistent with previous reports in humans and calves [32]. However, a higher prevalence of *G. duodenalis* assemblage E was reported in Shaanxi Province (80%, 36/45) [66], Henan Province (60%, 9/15) [49], Hubei Province (87.5%, 7/8), Denmark (84.6%, 11/67) [64] and Australia (12.8%, 37/289) [59]. The higher prevalence of *G. duodenalis* assemblage A in Chongqing and Sichuan Provinces is worthy of further study. The present results, in line with those of previous reports, suggest that pigs are a potential source of environmental contamination and infection for humans.

Out of the 514 fecal samples, considering all five enteric pathogens assessed in this study, 322 diarrheic pigs had mono-infections, accounting for 62.6% of the overall prevalence. In all of these cases, *E. bienewisi*, *E. cuniculi*, *E. hellem* and *G. duodenalis* were present. However, no infection with *E. intestinalis* or *Cryptosporidium* species was detected in this study. In addition to single infections, we found mixed infections in 106 samples, which accounted for 20.6% of the total positive samples. *E. bienewisi* was found more often than the other enteric pathogens, which is consistent with the results of a study performed on wild boar [43], pet rabbits [22], and horses [68]. The cause for multiple infections may be contaminated sewage. To the best of our knowledge, this is the first report of coinfection of *E. bienewisi* with other enteric pathogens in China. Therefore, further investigation should be conducted to confirm whether *E. bienewisi* is the main cause of diarrhea in pigs.

Conclusions

This study detected the occurrence of mono-infection and concurrent infection of *E. bienewisi*, *Encephalitozoon* spp., *Cryptosporidium* spp. and *G. duodenalis* in diarrheic pigs in China. Mono-infections and coinfections of *E. bienewisi*, *Encephalitozoon* spp., and *G. duodenalis* were detected in 62.6% and 20.6% of fecal samples, respectively. No infections of *E. intestinalis* or *Cryptosporidium* species were detected in any of our samples. The highest rate of coinfection was detected between *E. bienewisi* and *E. cuniculi* (10.5%, 54/514), followed by *E. bienewisi* and *G. duodenalis* (5.8%, 30/514) and *E. bienewisi* and *E. hellem* (2.9%, 15/514). *E. bienewisi* is a prevalent pathogen in diarrheic pigs and may be a significant source of diarrheal disease. Therefore, farmers, veterinary workers, health workers, and people who have close contact with pigs should take care to avoid being infected by these enteric pathogens. Moreover, our data showed that enteric pathogens were present and likely common in diarrheic pigs in Chongqing and Sichuan Provinces and could be a potential source for zoonotic transmission in humans and other animals and environmental contamination. Further investigation is needed on farms with diverse breeding systems and animals of various ages and both sexes to determine whether *E. bienewisi* is the main cause of diarrhea in pigs. In addition, a long-term study, including the viruses and bacterial infecting our samples, is needed.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12866-023-03070-x>.

Supplementary Material 1

Acknowledgements

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Authors' contributions

The experiments were conceived and designed by GQP, STG, ZYZ, and XZM. YJY collected the specimens. JHW, STG, and ZRW prepared the materials and performed the experiments. STG, AKA and TL analyzed the data. The manuscript first draft was written by STG. GQP, STG, AKA, JLB, XZM, and JC critically revised and edited the manuscript. The final manuscript was read and approved by all authors for publication.

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Data Availability

The datasets generated for this study can be found in GenBank under the accession numbers OR058746 to OR058757 for *Encephalitozoon* spp. and OR091265 to OR091271 *Giardia duodenalis*.

Declarations

Ethics approval and consent to participate

This research was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Southwest University (approval no. IACUC-20220420-04). All fecal samples were collected based on the accessibility of the animals for sampling and the owner's or farm manager's willingness to participate in the study.

Consent for publication

Not applicable.

Competing of interests

The authors declare that they have no competing interests.

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