

Cryptosporidium suis Infection in Post-Weaned and Adult Pigs in Shaanxi Province, Northwestern China

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Abstract: *Cryptosporidium* spp., ubiquitous enteric parasitic protozoa of vertebrates, recently emerged as an important cause of economic loss and zoonosis. The present study aimed to determine the distribution and species of *Cryptosporidium* in post-weaned and adult pigs in Shaanxi province, northwestern China. A total of 1,337 fresh fecal samples of post-weaned and adult pigs were collected by sterile disposable gloves from 8 areas of Shaanxi province. The samples were examined by Sheather's sugar flotation technique and microscopy at ×400 magnification for *Cryptosporidium* infection, and the species in positive samples was further identified by PCR amplification of the small subunit (SSU) rRNA gene. A total of 44 fecal samples were successfully amplified by the nested PCR of the partial SSU rRNA, with overall prevalence of 3.3%. The average prevalence of *Cryptosporidium* infection in each pig farms ranged from 0 to 14.4%. Species identification by sequencing of SSU rRNA gene revealed that 42 (3.1%) samples were *Cryptosporidium suis* and 2 (0.15%) were *Cryptosporidium scrofarum*. *C. suis* had the highest prevalence (7.5%) in growers and the lowest in breeding pigs (0.97%). *C. suis* was the predominant species in pre-weaned and adult pigs, while *C. scrofarum* infected pigs older than 3 months only. A season-related difference of *C. suis* was observed in this study, with the highest prevalence in autumn (5.5%) and the lowest (1.7%) in winter. The present study provided basic information for control of *Cryptosporidium* infection in pigs and assessment of zoonotic transmission of pigs in Shaanxi province, China.

Key words: *Cryptosporidium*, pig, SSU rRNA, Shaanxi province, China

Cryptosporidium, the worldwide distributed protozoans, have been recognized with 27 species and more than 70 genotypes that can infect humans and animals, including ruminants, amphibians, and birds, leading to serious enteritis even death in immunocompromised hosts and neonates [1-3]. Of susceptible animals, the pig is considered to be one of the main animal reservoir hosts of *Cryptosporidium* [4]. From pigs, 6 *Cryptosporidium* species have been isolated, namely, *C. suis*, *C. parvum*, *C. muris*, *C. andersoni*, *C. scrofarum* (formerly named as *Cryptosporidium* pig genotype II) [5], and *C. tyzzeri* (formerly named as *Cryptosporidium* mouse genotype I) [6]. Meanwhile, experimental infection studies demonstrated that pigs were also susceptible to infections with *C. hominis* and *C. meleagridis*, which suggested that pigs could be a source of infection for humans and other animals, posing an invisible threat to

human security [7-9].

Cryptosporidium infection in pigs occurs mainly through an oral-fecal route. Despite the fact that it does not always cause clinical signs, severe diarrhea, weight loss, and even death can appear in neonatal and immunodeficient pigs [10,11]. Considering that previous studies around the world have indicated pigs as the main host for *C. suis* and *C. scrofarum*, and that humans are infected with *C. suis* and *C. scrofarum*, these 2 pig-adapted *Cryptosporidium* species are potentially zoonotic [12,13]. The prevalence of *Cryptosporidium* infection in pigs have been reported in several provinces in China, with the highest in Anhui (56.8%) and lowest in Henan (8.2%) provinces [14,15]. The *Cryptosporidium* species have been identified in each research province, as mainly *C. suis* and *C. scrofarum*. Another species named *C. tyzzeri* was found only in eastern China [16]. However, it is unclear whether the pig naturally infected. Therefore, the present study aimed to determine the species and genotypes of *Cryptosporidium* in domestic pigs in Shaanxi province, northwestern China by DNA sequencing of the SSU rRNA gene, and to elucidate the public health significance of pigs in this province.

•Received 2 October 2014, revised 20 November 2014, accepted 27 November 2014.

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From May 2011 to May 2014, a total of 1,337 fresh fecal samples of post-weaned and adult pigs (Tables 1, 2) were collected from 9 intensive farms located in 8 counties/districts in Shaanxi province by sterile disposable gloves. Samples were firstly examined by Sheather's sugar flotation technique and microscopy at $\times 400$ magnification. The microscopically positive samples were stored in 2.5% potassium dichromate. All microscopy-positive samples were then used to identify species/genotypes using the nested PCR and sequencing. Genomic DNA was extracted using the E.Z.N.A.[®] Stool DNA Kit (OMEGA Biotek Inc., Doraville, Georgia, USA) according to the manufacturer's instructions, and stored at -20°C until further processed.

A 2-step nested PCR amplification of the small subunit (SSU) rRNA was used to determine species/genotypes of *Cryptosporidium*. The procedure of Xiao et al. [17] was followed except Taq polymerase-KOD FX Neo (Toyobo, Japan). Negative (without DNA template) and positive controls were included in each amplification running. Each secondary amplification was detected by 1.5% (w/v) agarose gel stained with ethidium bromide, and the positive amplicons were directly sequenced with secondary PCR primers on an ABI 3370 DNA sequencer at Sangon Company (Shanghai, China). Precise of the data was ensured by sequencing in both directions and hand assembly using DNASTar software as a sequence editor [18]. Sequences from all samples (n=44) in this study were deposited in GenBank database under accession nos. KJ790201 to KJ790244.

Assembled sequences were identified into species/genotype in GenBank[™] database using the BLAST (<http://www.ncbi.nlm.nih.gov>) and phylogenetic analyses. The phylogenetic tree was reconstructed using the Neighbor-joining (NJ) method implemented in Mega 4.0 [19] and the Kimura 2-parameter model.

Table 1. Information of representative *Cryptosporidium* isolates in the present study

Sample code	Location	Age group	GenBank accession no.
CMX1	Meixian	Grower	KJ790236
CTC2	Tongchuan	Grower	KJ790244
CAK2	Ankang	Fattener	KJ790235
CWQ4	Wuquan	Grower	KJ790239
CWQ5	Wuquan	Post-weaner	KJ790234
CYL1	Yangling	Grower	KJ790242
CYL4	Yangling	Breeding pig	KJ790201
CWG6	Wugong	Post-weaner	KJ790243
CWG7	Wugong	Post-weaner	KJ790237
CWG14	Wugong	Post-weaner	KJ790202

The consensus tree was obtained after bootstrap analysis, with 1,000 replications. The phylogenetic analysis based on SSU rRNA gene utilized *C. suis* (GenBank accession no. JQ936485) and *C. scrofarum* (no. JX424840) as ingroups, and *Plasmodium ovale* (KF018658) as the outgroup. The prevalence differences of *Cryptosporidium* oocysts among the factors of origins, cultivation modes, seasons, and ages were evaluated using Regression Analysis in Statistical Product and Service Solutions (SPSS) with 95% confidence intervals (CI). The probability level (P) of < 0.05 was regarded as statistically significant.

Of 1,337 fresh fecal samples, 44 were successfully detected by both microscopy (Fig. 1) and nested PCR, with an overall prevalence of 3.3%. The average prevalence of *Cryptosporidium* infection in each pig farm ranged from 0 to 14.4%. The farm in Wugong county using Fermentation Bed breeding conditions had the highest prevalence of 14.4% (27/187), whereas the average prevalence of the others under conventional breeding techniques was 1.5% (17/1,150). No infection was found in pigs from farms in Ankang and Xianyang counties. Statistical analysis showed the prevalence between different pig farms fluctuated greatly ($P < 0.05$). For different age groups of pigs, the prevalence were 1.3%, 1.4%, 7.5%, and 2.8% for breeding pigs (> 6 months), fatteners (3-6 months), growers (1-2 months), and

Table 2. Prevalence and risk factors associated with *Cryptosporidium* infection in pigs in Shaanxi province, China

Factor	Category	No. examined	No. positive (%)	<i>C. suis</i> (%)	<i>C. scrofarum</i> (%)
Season					
	Spring	148	3 (2.0)	3 (2.0)	0
	Summer	404	9 (2.2)	9 (2.2)	0
	Autumn	438	26 (5.9)	24 (5.5)	2 (0.46)
	Winter	347	6 (1.7)	6 (1.7)	0
Age					
	3 weeks-1 month	252	7 (2.8)	7 (2.8)	0
	1-3 months	358	27 (7.5)	27 (7.5)	0
	3-6 months	417	6 (1.4)	5 (1.2)	1 (0.24)
	> 6 months	310	4 (1.3)	3 (0.97)	1 (0.32)
Location					
	Ankang	76	0	0	0
	Wugong	187	27 (14.4)	26 (13.9)	1 (0.53)
	Wuquan	185	5 (2.7)	4 (2.2)	1 (0.54)
	Yangling	448	5 (1.1)	5 (1.1)	0
	Xianyang	92	0	0	0
	Meixian	121	3 (2.5)	3 (2.5)	0
	Tongchuan	124	3 (2.4)	3 (2.4)	0
	Xi'an	104	1 (0.96)	1 (0.96)	0
	Total	1,337	44 (3.3)	42 (3.1)	2 (0.15)

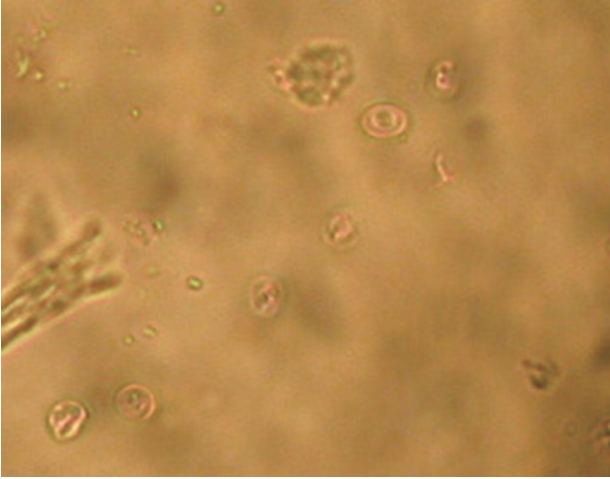


Fig. 1. *Cryptosporidium* oocysts detected in fecal samples of pigs. Unstained.

post-weaners (3 weeks-1 month), respectively. The difference of *Cryptosporidium* infection rates between different age categories was statistically significant ($P < 0.05$). The prevalence of *Cryptosporidium* infection in 4 seasons was significantly different ($P < 0.05$), with the highest in autumn (5.9%) and the lowest in winter (1.7%) (Table 2).

The sequence analysis and phylogenetic analysis (Fig. 2) based on SSU rRNA gene locus revealed the presence of 2 *Cryptosporidium* species, namely *C. suis* (42/44) and *C. scrofarum* (2/44). *C. suis* was found in all *Cryptosporidium*-positive areas, all seasons, and age groups, but *C. scrofarum* was detected only in pigs older than 3 months in autumn in Wugong and Wuquan counties. *C. suis* had the highest prevalence (7.5%) in growers and the lowest in breeding pigs (0.97%). A season-related difference of *C. suis* was observed in this study, with the highest in autumn (5.5%) and the lowest (1.7%) in winter. Pairwise comparison of obtained sequences indicated that no variation was observed within isolates of *C. scrofarum*, but 2 sequence types of *C. suis* isolates were detected. For sequences of *C. suis* isolates, only 1 Ins (G/-) was found in isolate CWQ4 from Wuquan.

The present study indicated *Cryptosporidium* infection in pigs in Shaanxi province, with an overall infection rate of 3.3%, showing a lower infection rate than that in Henan (8.2%) and Anhui (56.8%) provinces, China [20,21]. Compared with the prevalence of *Cryptosporidium* infection in pigs in other countries, it was relatively lower than that in western Australia (6.0%), northeastern Spain (22.5%), and Denmark (16%, 31%, and 100% for sows, piglets, and weaners) [22-26], but

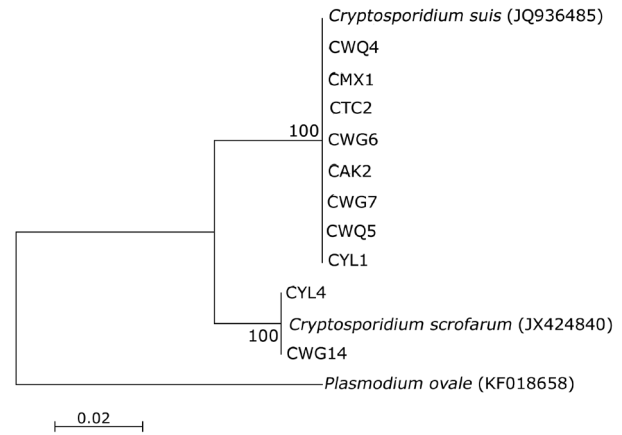


Fig. 2. Phylogenetic tree of representative samples reconstructed using the Neighbor-joining (NJ) method and the Kimura 2-parameter model. The consensus tree was obtained after bootstrap analysis, with 1,000 replications.

higher than that in Germany with the prevalence of 1.4% [27]. Many factors have been considered to influence the prevalence of pig cryptosporidiosis among different areas and countries, including pig health status, age categories, breeding mode, and management style [28,29].

Cryptosporidium species have been molecularly genotyped in pigs in many countries, including China [20,22,23,30]. *C. suis*, *C. scrofarum*, *C. muris*, *C. tyzzeri*, *C. parvum*, and *C. andersoni* were successfully isolated and identified [25,31-35]. However, DNA sequencing of the SSU rRNA gene indicated only 2 *Cryptosporidium* species in this study, namely *C. suis* and *C. scrofarum*, and *C. suis* was the predominant species in pre-weaned and adult pigs. These findings further confirmed that *C. suis* and *C. scrofarum* were the most common species in pigs [12,13,30]. Although the 2 main species of human cryptosporidiosis, namely *C. parvum* and *C. hominis*, were not detected, both *C. suis* and *C. scrofarum* are also important zoonotic pathogens [13,36,37]. In fact, the oocysts of 2 species have been found in water environment in some areas of China, including the source of drinking water companies, tap water as well as the wastewater nearby pig farms [38-40]. Therefore, pigs in Shaanxi province would pose an invisible threat to human security because of their asymptomatic infection and close contact with humans and water due to their huge numbers and economic importance. To further evaluate the public concern of pigs in this province, extensive molecular epidemiological surveys for better understanding of the transmission dynamics should be studied in the future.

Interestingly, season-related and age-related differences in

Cryptosporidium infection were observed in this study, with the highest prevalence in autumn (5.9%) and the lowest (1.7%) in winter. *C. suis* predominantly infected younger piglets less than 3 months, while *C. scrofarum* infected only pigs older than 3 months. Such season-specific and age-specific susceptibility were consistent with findings of some previous studies [41,42]. These results provided basic data for control of cryptosporidiosis in pigs in different seasons and age categories.

In conclusion, *Cryptosporidium* infection was observed in pigs in Shaanxi province, with the highest prevalence in autumn and the lowest in winter. *C. suis* and *C. scrofarum* were identified by sequencing of the SSU rRNA gene, with *C. suis* as the predominant species. The present study provided basic information for control of *Cryptosporidium* infection in pigs and assessment of zoonotic transmission source of pigs in Shaanxi province, China.

ACKNOWLEDGMENTS

This work was supported, in part, by the National Natural Science Foundation of China (grant no. 31101805), the Program for New Century Excellent Talents in University (NCET-13-0489), the Fund for Basic Scientific Research (ZD2012010), and the Open Funds of the State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (SKLVEB2013KFKT007), China.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests in this paper.

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