Prevalence and Genetic Characterizations of Cryptosporidium spp. in Pre-Weaned and Post-Weaned Piglets in Heilongjiang Province, China

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Abstract

Background: Cryptosporidium spp. are common intestinal protozoa of humans and animals. There have been few studies conducted on the molecular characterizations of pig-derived Cryptosporidium isolates worldwide, especially in China. Thus, the aim of the present study was to understand the prevalence, distribution and genotypes of Cryptosporidium in pigs in Heilongjiang Province, China.

Methodology/Principal Findings: A total of 568 fecal samples from pre-weaned and post-weaned piglets were collected from eight pig farms from four areas of Heilongjiang Province. The average infection rate of Cryptosporidium was 1.6% (9/568) by microscopy. 113 samples were subjected to PCR amplification of the small subunit (SSU) rRNA gene of Cryptosporidium, with 55.8% (63/113) being positive for Cryptosporidium. Cryptosporidium suis (n = 31) and C. scrofarum (n = 32) were identified by DNA sequencing of the SSU rRNA gene. Three types of C. scrofarum were found at the SSU rRNA locus, with one novel type being detected. Using species/genotype-specific primers for pig-adapted Cryptosporidium spp., 22 and 23 respectively belonged to C. suis and C. scrofarum mono-infections, with 18 co-infections detected. The infection peaks for C. suis (60%, 24/40) and C. scrofarum (51.2%, 21/41) were respectively found in the piglets of 5 to 8 weeks and more than 8 weeks.

Conclusion/Significance: The detection of C. suis and C. scrofarum in pre-weaned and post-weaned piglets has public health implications, due to the fact that the two species are both zoonotic Cryptosporidium. The novel C. scrofarum type detected may be endemic to China.

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Introduction

Cryptosporidium spp. are common intestinal protozoa occurring in humans and many animal species, and do harm to the health of hosts. Animals and humans infected with Cryptosporidium are both potential sources of Cryptosporidium contamination in the environment. However, the contribution of each source is not clear, especially in developing countries. The distribution of Cryptosporidium spp. in humans and the human cryptosporidiosis burden of animal origin differ in different geographic regions. It has been proved that the transmission of C. parvum in humans is mostly anthroponotic in developing countries, with zoonotic infections play an important role in developed countries [1]. Cryptosporidium spp. are highly prevalent in livestock. Cattle have always been the subject of most of the studies in assessing the zoonotic potential of Cryptosporidium infections in animals. Years of epidemiological data have also documented the occurrence of natural infection of Cryptosporidium in other livestock. Cryptosporidium spp. have also been reported in pigs worldwide [2].

There is an extensive genetic variation within the genus Cryptosporidium, with 24 Cryptosporidium species having been recognized and more than 70 genotypes having been found [1,3,4]. To date, six Cryptosporidium species have been isolated from pigs, including C. suis, C. scrofarum (previously named as Cryptosporidium pig genotype II), C. parvum, C. muris, C. bovis (previously named as Cryptosporidium mouse genotype I) and C. andersoni [2] (Table 1). Meanwhile, experimental infection studies revealed the susceptibility of pigs to C. parvum, C. hominis and C. meleagridis [5–10]. However, there are differences in the population structure and the molecular characterizations of Cryptosporidium...
Cryptosporidium would help us to understand the transmission dynamics of cross-products of the SSU rRNA gene. Molecular epidemiological data study with those derived from humans available from GenBank. The present study focused on the investigation of Cryptosporidium in pigs by DNA sequencing of secondary PCR products of the SSU rRNA gene. Molecular epidemiological data would help us to understand the transmission dynamics of cross-Cryptosporidium species/genotypes between humans and pigs, and to assess the cryptosporidiosis burden attributable to zoonotic transmission by aligning the obtained sequences in the present study with those derived from humans available from GenBank.

### Materials and Methods

#### Ethics Statement

Before beginning work on this study, we contacted the farm owners and obtained their permission to have their animals involved. During sample collection, all animal work followed guidelines in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals, and was approved by the Animal Ethical Committee of Harbin Medical University.

#### Fecal Specimen Collection and Examination

In a one-year study from October 2011 to October 2012, 568 fecal samples were collected from eight intensive pig farms in four areas of Heilongjiang Province (Harbin, Daqing, Qiqihar and Mudanjiang). Farms were selected only according to each owner’s willingness and accessibility of animals for sampling. Approximately 20 g fresh fecal sample for each animal was collected immediately after being defecated on the ground of the pen by using a sterile disposal latex glove, and then placed in a disposable plastic bag individually. All the animals were healthy at the time of sampling, with their ages ranging from 15 to 105 days. Samples were transferred to the laboratory and stored in refrigerators at 4°C.

Before microscopic examination, fecal samples, accounting for approximately 20% of the samples of each age group in each farm, were randomly selected and sieved, and then stored (approximate 10 g each) in 2.5% potassium dichromate at 4°C prior to being used in molecular identification. A total of 113 fecal samples were collected, with 16 being less than 5 weeks old, 43 being 5–8 weeks old and 54 being more than 8 weeks old. Meanwhile, all the 568 samples were processed for microscopic examination. Oocysts in the fecal samples from pre-weaned piglets (less than 5-week-old) were concentrated by formalin-ethyl acetate sedimentation method to remove the fats in the samples and were stained by modified fast-acid staining technique. Sugar floatation method was used to concentrate oocysts in fecal samples from post-weaned piglets (equal to or more than 5-week-old). All the processes were finished in the laboratory within 48 hours after collection.

#### DNA Extraction

Potassium dichromate was washed off with distilled water by centrifugation at 1500 g for 10 minutes four times at room temperature. DNA extraction was performed on 113 stored fecal samples. Genomic DNA of Cryptosporidium was extracted from 200 mg of each fecal sample using a commercially available kit (QIAamp DNA Mini Stool Kit, Qiagen, Hilden, Germany) in accordance with the manufacturer-recommended procedures.
Eluted DNA was kept frozen at −20°C in refrigerators until PCR amplification.

Genotyping of Cryptosporidium
An approximate 830bp fragment of the SSU rRNA gene was amplified from all DNA preparations by a nested PCR using genus-specific primers of Cryptosporidium as previously described [20]. All the secondary PCR products positive for Cryptosporidium were sequenced and identified to Cryptosporidium species/genotypes. For the assessment of mixed infection and age-specificity of C. suis and C. scrofarum in pigs, DNA preparations characterized as C. suis and C. scrofarum were respectively analyzed by a nested PCR protocol using pig-derived Cryptosporidium-specific primers to amplify a 443 bp fragment of the SSU rRNA gene from C. scrofarum and a 482 bp fragment of the SSU rRNA gene from C. suis, with the first set of genus-specific primers as described by Jiang et al and the second set of species/genotype-specific primers designed by Jenikova et al [21,22]. All the secondary PCR products were sequenced to confirm if mixed C. scrofarum and C. suis infections were detected.

DNA Sequence Analysis
All purified secondary PCR products were directly sequenced with secondary PCR primers on an ABI PRISM™ 3730 DNA Analyzer (Applied Biosystems, USA), using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA). Accuracy of the sequencing data was confirmed by sequencing in both directions and additional PCR products if required. The SSU rRNA gene sequences obtained in the present study were aligned with each other and reference sequences obtained from GenBank by using Clustal X 1.83. Sequences of the partial SSU rRNA gene from representative isolates obtained were deposited in the GenBank database under accession numbers: KC481228 to KC481231.

Results
Prevalence and Age Distribution of Cryptosporidium
568 fecal samples were microscopically screened for the presence of Cryptosporidium oocysts. Meanwhile, 113 randomly selected fecal samples were screened for the presence of Cryptosporidium by PCR amplification of the partial SSU rRNA gene. Cryptosporidium oocysts were found in all the eight pig farms selected in the present study. In total, the average infection rate of Cryptosporidium was 1.6% (9/568) by microscopy versus 53.8% (63/113) by PCR. The highest infection rates were both found in 5–8 week-old pigs, with the prevalence 2.8% (6/216) by microscopy versus 55.8% (63/113) by PCR, with the highest infection rate being found in 5–8 week-old pigs by either of the two identification methods (2.8% versus 83.7%) (Table 2). Many factors are generally considered to influence infection rates of Cryptosporidium in various hosts, including age and health status of the infected hosts, the size and structure of samples (experimental design), the variety of detection methods employed and so on.

It is known that pigs of all ages are affected by Cryptosporidium. However, a number of studies on the relationship between the prevalence of Cryptosporidium and the age of pigs demonstrate that Cryptosporidium infections are the most common in piglets more than one month but generally less than six months of age [23–26]. In fact, infections are detected less frequently in piglets younger than one month or in adults, and even in some studies, there is an absence of Cryptosporidium oocysts [24,27]. In the present study, Cryptosporidium were most frequently detected in 5–8 week-old piglets either by microscopy or by PCR (Table 2). The result was consistent with the previous reports that Cryptosporidium were mainly found in piglets within two months of weaning [2,19,22,28]. Maddox-Hyttel et al and Farzan et al suggested that Cryptosporidium is more likely to be detected in post-weaned pigs than any other age group, and they also attribute the result to a reduction in immunity as animals lose the maternally immunity while their own immunity still needs to develop [15,23]. A longitudinal study gave more exact data that piglets shed oocysts at the beginning of 45 days post weaning averagely [29]. However, two recent studies conducted in Vietnam have drawn the opposite conclusion that the prevalence of Cryptosporidium in pre-weaned pigs is significantly higher than that in post-weaned pigs [30,31]. In another study of a pig cryptosporidiosis survey in eastern China’s Shanghiai and Jiangsu, no age differences have been observed in the prevalence of Cryptosporidium [16]. To date, there two parasites appearing in all the age groups, C. suis (60%, 24/40) and C. scrofarum (31.2%, 21/41) had the highest percentage of positive samples in the pigs of 5 to 8 weeks and more than 8 weeks, respectively. C. scrofarum appeared to be more prevalent in older pigs than C. suis (Table 3).

Molecular Characterization of Cryptosporidium spp. at the SSU rRNA Locus
Sequence analysis of Cryptosporidium SSU rRNA gene revealed that all the 31 C. suis isolates were identical to each other (KC481228), having 100% similarity with the pig-derived sequences (JF710259, GQ227705 and GU254171-77 from China, AF108861 from Switzerland and EF489038 from Ireland). Three types were observed among 32 C. scrofarum isolates, with one to three nucleotide variations between one another. The most common type (KC481229) was found in 93.8% (30/32) of C. scrofarum samples and had 100% similarity with those isolates derived from a human (EU331243 from the Czech Republic), and pigs (HQ844734, GU254170, GQ227704 and GU254168 from China, DQ182600 from Denmark, GQ924105 from Brail, and JX424640 from Czech Republic). For the remaining two sequences, one (KC481231) had 100% similarity with a Chinese pig-derived sequence (HQ844730) and the other (KC481230) was never identical to any reported C. scrofarum.

Discussion
Pigs have been reported to be infected naturally with Cryptosporidium worldwide. The prevalence of pig cryptosporidiosis varies between different countries and between different areas within a country. In the present study, the average infection rate of Cryptosporidium in pigs was 1.6% (9/568) by microscopy versus 55.8% (63/113) by PCR, with the highest infection rate being found in 5–8 week-old pigs by either of the two identification methods (2.8% versus 83.7%) (Table 2). Many factors are generally considered to influence infection rates of Cryptosporidium in various hosts, including age and health status of the infected hosts, the size and structure of samples (experimental design), the variety of detection methods employed and so on.

A total of 63 fecal samples were successfully amplified at the SSU rRNA locus using the genus-specific nested PCR. DNA sequencing confirmed the presence of C. suis (n = 31) and C. scrofarum (n = 32) in piglets in the investigated areas. A combination of genus-specific and species/genotype-specific primers revealed 22 cases of C. suis mono-infection, 23 cases of C. scrofarum mono-infection and 18 cases of mixed infection of them both. Despite the
has been no definitive conclusion about age distribution of Cryptosporidium in pigs. It is difficult to explain why conventionally reared piglets less than one month of age have been reliably infected under experimental conditions in previous studies [8,9,32].

In the present study, PCR was 34.9 times as sensitive as microscopy for the diagnosis of pig cryptosporidiosis, with the prevalence 55.8% (63/113) versus 1.6% (9/568) for PCR versus microscopy respectively. Not surprisingly, molecular techniques have a greater power of Cryptosporidium diagnosis. PCR-based detection was demonstrated to be more sensitive than microscopy in the two studies of the Cryptosporidium prevalence in sheep; in Australia, the prevalence was 2.6% by microscopy compared to 26.3% by PCR, and in the United States, Cryptosporidium was identified in 20.6% by microscopy compared to 50.8% by PCR [33,34]. Although the prevalence of pig cryptosporidiosis conducted in Canada was 44.3% by microscopy versus 55.7% by PCR, there were statistically significant differences between the two testing methods [15]. The present higher ratio of PCR to microscopy might be attributable to the fact that all the animals were in a good health condition at the sampling and shed a lower number of oocysts in their feces. Thus, infection intensity of oocysts might be below the limit of detection of conventional morphological methods. Actually, pig fecal samples are generally reported to have lower intensity of oocysts than those from other animals based on recovery and enumeration of Cryptosporidium oocysts in their feces [16,25,31,35,36]. Therefore, PCR would be advised to be the preferred method for more accurate estimation of prevalence of Cryptosporidium in pigs. Besides the factors above, of course, differences in prevalence might be also related to differences in farm management systems. It has been reported that production in an intensive system might cause proliferation and maintenance of pathogens if techniques of handling are inadequate [37]. Therefore, measures should be taken to avoid the cross transmission of Cryptosporidium between different individuals within each farm.

DNA sequencing analysis of the partial SSU rRNA gene confirmed the presence of two Cryptosporidium spp. in 63 PCR-positive samples of Cryptosporidium in the present study, with 22 cases belonging to C. suis mono-infection, 23 cases belonging to C. scrofarum mono-infection and 18 cases belonging to mixed infection of them both. It has been reported previously that many hosts are susceptible to simultaneous infections with several Cryptosporidium spp., such as humans, cattle, pigs and so on [6,16,22,38–40]. In fact, mixed infections in farm animals might be more prevalent than expected before. The identification of Cryptosporidium spp. is commonly based on DNA sequencing alone or in combination with PCR-RFLP analysis of the SSU rRNA gene fragment. However, the ability of these methods to identify mixed infection is limited compared to the use of species/genotype-specific PCR tools. This is because genus-specific primers will preferentially amplify the predominant species/genotypes due to the inherent nature of PCR [39,41]. In the present study, the high percentage (28.6%, 18/63) of mixed infections may be due to the combined use of both the genus-specific and species/genotypes-specific primers for C. suis and C. scrofarum at the SSU rRNA locus. This may also explain why there are few reports of mixed infection cases in farm animals in previous studies as most previous studies did not use species/genotypes-specific primers.

The combination of genus-specific and species/genotypes-specific primers for C. suis and C. scrofarum not only enhanced our understanding of the population structure of Cryptosporidium in pigs but also helped us to clarify the age-related distribution of both parasites in pigs. In the present study, although C. suis and C. scrofarum were both found in all age groups, there appeared to be an age-related difference in the prevalence of pig cryptosporidiosis. The infection peaks for C. suis (60%, 24/40) and C. scrofarum (51.2%, 21/41) were respectively found in piglets that were 5 to 8 weeks old and more than 8 weeks old. Analysis of the current literature indicates that no clear conclusions can be drawn about the age distribution of C. suis and C. scrofarum in pigs. In general, C. suis seems to infect pigs of each age category, although the prevalence is lower in older pigs [3]. In a study of pig

### Table 2. Prevalence and age distribution of Cryptosporidium in pigs in Heilongjiang Province, China by microscopy and by PCR.

| Age (week) | By microscopy | By PCR |
|------------|---------------|--------|
|            | No. of examined | No. of positive (%) | No. of examined | No. of positive (%) |
| <5         | 81             | 0 (0.0)          | 16             | 4 (25.0)           |
| 5–8        | 216            | 6 (2.8)          | 43             | 36 (83.7)          |
| >8         | 271            | 3 (1.1)          | 54             | 23 (42.6)          |
| Total      | 568            | 9 (1.6)          | 113            | 63 (55.6)          |

### Table 3. Age-specific distribution of Cryptosporidium species in pigs in Heilongjiang Province, China.

| Age (week) | No. of positive | C. suis mono-infection | C. scrofarum mono-infection | Mixed infection |
|------------|-----------------|------------------------|----------------------------|-----------------|
| <5         | 4               | 2                      | 1                          | 1               |
| 5–8        | 36              | 14                     | 8                          | 10              |
| >8         | 23              | 6                      | 14                         | 7               |
| Total      | 63              | 22                     | 14                         | 18              |

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cryptosporidiosis in Czech Republic, C. suis was found to infect all 1–12-week-old pigs and showed the absence of age specificity of this species [22]. However, Kvač et al only identified C. suis in the pigs under 7 weeks of age [42]. It has been reported that C. suis preferentially infected sucking piglets [31,43]. Conversely, C. scrofarum appears to be specific for older pigs compared to C. suis [22,31,40,42–44], with a much lower prevalence in younger age categories, primarily in pre-weaned piglets [2,43]. C. scrofarum was found only in pigs at the age of 2–6 months [44], with more common in weaned piglets [2,28,31]. There are more accurate data reported that C. scrofarum appeared in pigs older than 6 weeks in one study [22]. In another study, C. scrofarum isolates were only identified in pigs from 7 weeks of age and was not identified in pre-weaners [42]. However, in the present study, two C. scrofarum isolates were identified in pre-weaned pigs, with one being mono infection and the other being mixed infection with C. suis.

To date, six Cryptosporidium species/genotypes have been identified molecularly in pig fecal samples, with C. suis and C. scrofarum being the most common (Table 1). The findings of only C. suis and C. scrofarum in piglets in the present study supported the conclusion above. The absence of C. parvum infection in the investigated areas was in agreement with previous observation that pigs are not a major source of C. parvum [1,42,43]. However, there appeared to be geographical differences in the preference of C. parvum in pigs. No C. parvum has been found in pigs in China. To date, C. parvum has been identified in pigs in European countries as well as Australia and Canada (Table 1). Also, it is interesting that C. parvum, which generally tends to be infective for juvenile animals, has been identified in mature sows and weaning piglets [25,40,45].

C. muris, C. tyzzeri and C. andersoni are minor Cryptosporidium species in pigs and were not been detected in the present study. To date, it is unclear whether the previous findings represented a natural infection of pigs although Chen and Huang suggested the possible transmission routes of C. tyzzeri between rodents and pigs [16]. It needs to be confirmed with more systematic studies of experimental infection of the three parasites, for we have no sufficient evidence to rule out the mechanical transmission of the oocysts in pig feces. C. hominis was previously reported in one pig fecal sample, however, it was considered to be a sequencing artifact [2,25].

In the present study, DNA sequence analysis of the SSU rRNA gene indicated that all the 31 C. suis isolates were identical to each other, having 100% homology with the pig-derived sequences from Switzerland, Ireland and China [2,25,47]. Among the 32 C. scrofarum isolates, three types were found based on the SSU rRNA gene sequences, including one novel type. In general, C. suis was more conserved than C. scrofarum. To date, 10 types have been observed worldwide from 67 SSU rRNA nucleotide sequences available in GenBank, which represented 895 C. suis isolates (including 31 in the present study). However, among 612 C. scrofarum isolates (including 32 in the present study), 16 types have been found in 46 SSU rRNA nucleotide sequences available in GenBank. By aligning obtained sequences with those from GenBank, it has been noticed that the SSU rRNA nucleotide sequence of the most prevalent type of C. scrofarum (95.75%, 30/32) (KC401229) had 100% homology with that from a diarrheal stool of 29-year-old immune-competent man (EU331243) [11]. Thus, the pigs infected with C. scrofarum may pose a threat to local inhabitants and may be of public health significance. C. suis, which is considered to have the potential to be zoonotic pathogens, has also been isolated in humans [12–14]. Due to the only sporadic humans cases infected with C. suis and C. scrofarum reported worldwide, we had no sufficient data available to assess the burden of human cryptosporidiosis caused by the two parasite attributable to zoonotic transmission.

C. suis and C. scrofarum appear to be adapted to pigs. Pig cryptosporidiosis might be paid less attention than it is supposed to due to the fact that the pigs infected with two parasites are generally reported to be asymptomatic [1,42,43]. Thus, pigs might have more opportunity to continually shed human-infective oocysts of C. suis and C. scrofarum into the environment through their feces. In fact, oocysts of C. suis and C. scrofarum have been found in water environment in some areas in China, including source water for drinking water plant, wastewater nearby pig farms as well as raw domestic wastewater in a wastewater treatment plant [40–50]. It is well-known that oocysts are extremely easily spread via water and it is difficult to remove or eliminate the parasites in water supply. Due to the lack of data of human cryptosporidiosis in the investigated area, even in China, the true prevalence of human cryptosporidiosis caused by C. suis and C. scrofarum, the transmission dynamic and the disease burden attributable to the two parasites of pig origin need to be assessed by more extensive molecular epidemiological surveys from humans and animals in the future. Unique SSU rRNA gene sequences of C. scrofarum in pigs in the investigated areas may reflect characteristic geographical distribution. The present data will help local authorities to develop protective strategies for the prevention and control of cryptosporidiosis in Heilongjiang Province. Considering the high prevalence of both parasites in pigs, further studies also need to be focused on the relationship between pig cryptosporidiosis and different farm breeding systems. It is important to develop better farm management systems to prevent the occurrence of cross transmission and re-infection of Cryptosporidium among the animals within each farm, and to reduce environmental contamination by reducing zoonotic agents from pig manure.

**Author Contributions**

Conceived and designed the experiments: AL HL. Performed the experiments: WZ FY AL. Analyzed the data: WZ FY RW. Contributed reagents/materials/analysis tools: LZ JC YS. Wrote the paper: AL WZ FY.
