Distribution and Genetic Characterizations of Cryptosporidium spp. in Pre-Weaned Dairy Calves in Northeastern China’s Heilongjiang Province

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Abstract

Background: Cryptosporidium spp. are common parasites of humans and animals. Farm animals, especially pre-weaned calves, are considered to be one of main animal reservoir hosts of Cryptosporidium in the transmission of human cryptosporidiosis. The aim of this study was to determine the distribution and genotypes of Cryptosporidium spp. in pre-weaned calves using molecular tools and to assess zoonotic transmission and elucidate the public health significance in northeastern China.

Methodology/Principal Findings: A total of 151 fecal specimens from pre-weaned calves were collected in Heilongjiang Province and were screened for Cryptosporidium by PCR. The average prevalence of Cryptosporidium was 47.68% (72/151). Cryptosporidium spp. were characterized by DNA sequencing of the small subunit (SSU) rRNA gene and the 60-kDa glycoprotein (gp60) gene. Based on the SSU rRNA gene, five Cryptosporidium spp. were identified, including C. bovis (n = 34), C. andersoni (n = 26), C. ryanae (n = 5), C. meleagridis (n = 5) and C. parvum (n = 2). The SSU rRNA nucleotide sequences were identical to each other, respectively, within C. ryanae, C. parvum, C. meleagridis and C. andersoni. Four types of C. bovis were found in the SSU rRNA gene, with two novel types. The gp60 gene was successfully sequenced in one C. parvum isolate and three C. meleagridis isolates, with IldA19G1 for C. parvum and IleA22G2R1 for C. meleagridis.

Conclusion/Significance: Molecular analysis indicates that Cryptosporidium spp. are endemic in pre-weaned calves in Heilongjiang Province. The findings of C. parvum and C. meleagridis suggested the possibility of zoonotic transmission and public health significance. The transmission dynamics of C. parvum and C. meleagridis needed to be clarified by further molecular epidemiologic studies from humans and animals. Whether calves could act as the natural reservoirs of C. meleagridis needed to be confirmed by more systematic experimental infection studies.

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Introduction

Cryptosporidium spp. are the important intestinal pathogens with a wide host range, having the ability to infect humans and animals including mammals, birds, reptiles, amphibians and fish. Among the susceptible animals, cattle are considered to be one of main animal reservoir hosts of Cryptosporidium. Bovine cryptosporidiosis is of great concern due to the huge number of cattle and their economic importance. Cryptosporidium infections frequently result in morbidity, weight loss and delayed growth, and sometimes mortality of young animals. Contamination of cattle manure has led to several foodborne and waterborne outbreaks of human cryptosporidiosis [1,2].

Within the genus Cryptosporidium, extensive genetic variation has been reported. To date, at least 23 Cryptosporidium species and more than 70 genotypes have been recognized, with new genotypes being found by molecular bio-techniques [3,4]. PCR-based molecular analysis techniques have identified seven Cryptosporidium species in cattle, including C. parvum, C. bovis, C. andersoni, C. ryanae, C. felis, C. hominis, and C. suis, and two genotypes C. suis-like genotype and the Cryptosporidium pig genotype II [5]. The first four Cryptosporidium species listed above are responsible for most cases of bovine cryptosporidiosis with different clinical manifestations and there is an age-associated distribution in cattle. C. parvum is mostly found in pre-weaned calves with frequent diarrhea; C. andersoni is mostly identified in asymptomatic adults, but C. andersoni-infected...
cows have less milk production than uninfected ones. *C. bovis* and *C. ryanae* are frequently found in post-weaned calves and yearlings. Cattle infected with *C. bovis* and *C. ryanae* as well as other *Cryptosporidium* species/genotypes are generally considered to have no visible clinical signs, and no information of subclinical pathology is available. However, there is an exception in cattle in Sweden, with *C. ryanae* in one diarrheal case and *C. bovis* in four cases [6].

Prevalence data have certificated the occurrence of cryptosporidiosis in cattle worldwide and the zoonotic *C. parvum* most commonly identified in pre-weaned calves. In parts of the USA, Belgium, Ireland, Germany, Malaysia, the UK, Sweden, Japan, Spain, Czech Republic and Iran, *C. parvum* is responsible for the majority of *Cryptosporidium* infections in pre-weaned calves and the minority in post-weaned calves and heifers [6,7–17]. However, there appeared to be geographical differences in the population and the prevalence of *Cryptosporidium* spp. in pre-weaned calves. In some countries or areas, *C. bovis* has been reported to be the most prevalent species in calves [5,6,18–20].

In China, molecular identification and genetic characterization of *Cryptosporidium* spp. in cattle have been limited to small numbers of *Cryptosporidium* isolates [5,19,21–23]. Based on previous evidence that pre-weaned calves are the most important source of zoonotic *Cryptosporidium* infection, the present study focused on the investigation of *Cryptosporidium* in pre-weaned calves by PCR and sequencing to better understand the prevalence of *Cryptosporidium* species in pre-weaned calves in Heilongjiang Province. The aim was to examine the distribution and species/genotypes of *Cryptosporidium* in pre-weaned calves at genotype and subtype levels in northeastern China and to assess zoonotic transmission and elucidate the public health significance.

**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 specimen collection, all animal work followed guidelines in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals, and approved by the Animal Ethical Committee of Harbin Medical University.

**Fecal Specimen Collection**

Between October 2009 and September 2011, a total of 151 fecal specimens (approximate 10 g each) were randomly collected from six dairy cattle farms in four areas of Heilongjiang Province (Harbin, Daqing, Mudanjiang and Qiqihar). Some were collected directly from the rectum of the animals, while the others were taken immediately from fresh feces deposited on the ground after animal defecation. Their ages ranged from 24 to 60 days. The number of collected specimens accounted for 10–15% of total pre-weaned calves in each farm. All the specimens were stored in 2.5% potassium dichromate at 4°C prior to being used in molecular biologic characterizations.

**DNA Extraction**

Potassium dichromate was washed off fecal specimens with distilled water by centrifugation at 1500 g for 10 minutes at room temperature four times. Genomic DNA was extracted from 200 mg of each specimen using a QIAamp DNA Mini Stool Kit (Qiagen, Hilden, Germany) according to the manufacturer-recommended procedures. Eluted DNA was stored at −20°C until further use in PCR analysis.

**Genotyping and Subtyping of Cryptosporidium**

All DNA preparations were screened for the presence of *Cryptosporidium* by nested PCR amplification of an approximate 830 bp fragment of the SSU rRNA gene as previously described [24]. DNA preparations characterized as *C. parvum* or *C. Meleagridis* were subsequently analyzed to determine subtypes using a nested PCR to amplify an approximate 830 bp fragment of the gp60 gene as described [25].

**DNA Sequence Analysis**

Purified PCR products were directly sequenced with secondary PCR primers on an ABI PRISM 3730 XL DNA Analyzer (i.e. superscript the TM) by Sinogeno-max Biotechnology Co. Ltd. (Beijing, China), using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequence accuracy was confirmed by two-directional sequencing and by sequencing a new PCR product if necessary. The identity of *Cryptosporidium* spp. and genotype was established by comparing the obtained sequences with reference sequences from GenBank by using Clustal x 1.83. The representative nucleotide sequences obtained in the present study were deposited in the GenBank database under the following accession numbers: JX416362 to JX416368 (SSU rRNA), and JX416369 and JX416370 (gp60).

**Results**

**Prevalence of Cryptosporidium**

A total of 151 fecal specimens were screened for the presence of *Cryptosporidium* using PCR amplification of the SSU rRNA gene. 72 specimens were positive for *Cryptosporidium*, thus, the average infection rate was 47.60% (72/151). All the six farms were positive for *Cryptosporidium*, with the infection rate ranging from 7.14% to 100% (Table 1).

**Distribution and Percentage of Cryptosporidium spp.**

DNA sequencing of the SSU rRNA gene PCR products confirmed the presence of 5 *Cryptosporidium* spp., including 2.78% (2/72) for *C. parvum* on one farm, 47.22% (34/72) for *C. bovis* on six farms, 36.11% (26/72) for *C. andersoni* on four farms, 6.94% (5/72) for *C. ryanae* on three farms, and 6.94% (5/72) for *C. meleagridis* on one farm (Table 2). At least two *Cryptosporidium* species were detected on five farms with the exception of Farm 4 (Table 1). *C. bovis* was the most prevalent and the most widespread in the investigated areas.

**Molecular Characterizations of Cryptosporidium spp. at the SSU rRNA Locus**

Two SSU rRNA gene sequences of *C. parvum* were identical to each other and showed 100% similarity with the previous cattle-derived sequences (HQ009805, AB513857–AB513881, AB441687, EF611871 and AF093493). The human-derived sequences (EU331237–EU331241, DQ525304 and DQ656352). All the five *C. meleagridis* isolates had 100% homogeneity with each other at the SSU rRNA locus and were identical to the sequence (HM485432) derived from a British human. 100% homology was observed within the SSU rRNA gene sequences of the five *C. ryanae* isolates, which was identical to those derived from cattle (HQ2822137, EU410344, AY587166, AB513679, HQ009807 and HQ179574).

*C. bovis* showed intra-species variations at the SSU rRNA locus in the present study. Sequence analysis of *Cryptosporidium* SSU rRNA gene of 34 *C. bovis* isolates revealed the presence of four distinct types, respectively named as Type I, Type II, Type III and...
Type IV for a convenient description (Table 3). Type I and Type II had 100% similarity with the cattle-derived isolates HQ179575 and HQ179573, respectively, and Type I was in a high percentage of 91.18% (31/34) of C. parvum-positive specimens. Type III and Type IV have never been described previously, with one or two base variations compared to Type I and Type II, respectively (Table 3).

Subtypes of C. parvum and C. meleagridis at the gp60 Locus

At the gp60 locus, only one out of the two C. parvum isolates produced the expected PCR product and was successfully sequenced. It was identified as the subtype IIdA19G1, which showed 100% homology with the cattle-derived IIdA19G1 isolates (HQ099809 and JF961561) and the human-derived IIdA19G1 isolate (DQ280496), and showed 99% homology with the cattle-derived IIdA19G1 isolate (EF073048) with three base differences. Four out of five C. meleagridis isolates produced the expected PCR products, but only three of them were successfully sequenced. All the three C. meleagridis isolates belonged to the subtype IIIeA22G2R1, which was not identical to any known C. meleagridis subtypes.

Discussion

The prevalence of bovine cryptosporidiosis varies between and within countries in the world. Although hosts of all ages are affected, the prevalence of Cryptosporidium declined with the increasing ages of animals. In the present study, the prevalence of Cryptosporidium in pre-weaned calves was much higher (47.7%) than a previous study in Henan Province (21.5%), China [5]. It might be attributable to different detection methods, with PCR in this study and Sheather’s sugar flotation technique in Henan [5]. Not surprisingly, molecular methods have the advantage of increased sensitivity of detection. In addition, the differences in prevalence might partially be related to the experimental design, specimen size and host health status at the time of sampling. On the other hand, geographical and seasonal differences can also affect the prevalence of Cryptosporidium.

Sequence analysis of Cryptosporidium at the SSU rRNA locus identified five Cryptosporidium species in 72 specimens, with 2.78% prevalence for C. parvum, 47.22% for C. bovis, 36.11% for C. andersoni, 6.94% for C. ryanae, and 6.94% prevalence for C. meleagridis. C. bovis and C. andersoni were the two most common species in calves in the investigated areas instead of C. parvum. Previously, age-specific preferences for some Cryptosporidium species have been observed in cattle. Most Cryptosporidium infections in pre-weaned calves are caused by C. parvum, whereas Cryptosporidium infections in post-weaned calves are mostly caused by C. bovis and C. ryanae. Two studies in the USA indicated that C. parvum is responsible for approximately 85–97% of the Cryptosporidium infections in pre-weaned calves [26,27]. However, recently, there are three studies reporting C. bovis as the most prevalent species of Cryptosporidium in pre-weaned calves, which respectively occurred in Sweden (74%, 54/73) and in Henan (37.8%, 65/172) and Shanghai (80%, 4/5) of China [5,6,19]. In India, C. bovis was also observed as the most common species in both pre- and post-weaned calves [19]. The true reason for the high occurrence of C. bovis in pre-weaned calves remains unclear. But early in 2007, Feng et al suggested that the low infection rates of C. bovis and C. ryanae in calves may be because C. parvum may outcompete C. bovis and C. ryanae [19]. Even in concurrent infection cases with mixed Cryptosporidium species/genotypes, unless genotype-specific PCR tool are used, only the predominant species/genotypes will be amplified due to the inherent nature of exponential amplification by PCR [19,28,29].

The age pattern of cattle of C. ryanae is very similar to that of C. bovis. Most Cryptosporidium infections in post-weaned calves are...
attributable to Cryptosporidium spp. However, C. ryanae has been found in a variety of birds and farm animals, especially pre-weaned calves [6,9,12–15,31,32]. Subtyping results have shown that C. meleagridis in calves needs to be determined with more systematic experimental infection studies. The transmission dynamics of C. meleagridis in calves needs to be elucidated further by characterizing additional Cryptosporidium isolates from humans and animals from the same geographic areas at genotype and subtype levels.

By DNA sequence analysis within the partial SSU rRNA gene, intra-species variations were noticed in Cryptosporidium spp. Four types were obtained from 34 C. bovis sequences at nine nucleotide sites. Two of them (Type I and Type II) appear to be worldwide with no apparent differences in geographical distribution, while the remaining two (Type III and Type IV) have never been reported previously and might be with characteristic geographical distributions. The findings might represent the genetic characterizations of Cryptosporidium spp. in calves in the investigated areas, although the meaning of the sequence polymorphism remains unclear. More data from large-scale investigations regarding the new types of Cryptosporidium spp. will contribute to the study of geographical distributions of Cryptosporidium spp.

Genetic characterizations of two C. parvum isolates in the present study were observed, respectively by analyzing the SSU rRNA gene and the gp60 gene. Within the SSU rRNA gene, both of them showed 100% homology with each other and were identical to those reported in cattle from Egypt, Iran, India, the USA and Henan of China [5,12,38–40] and in humans from the Czech Republic, Spain and Iran [41–43]. Sequence analysis of the gp60 gene showed that the C. parvum isolate belonged to the subtype IIaA19G1. The same sequences had been obtained from 67 calves from Henan of China, one calf from Hungary and three HIV-seropositive patients from Portugal [5,44,45]. However, the calf-derived IIaA19G1 isolate (EF073048) has three base differences compared to the isolates above mentioned. Due to the lack of epidemiologic data from animals and humans in the investigated areas, we could not draw the definite conclusion about the source and endemic characteristic of C. parvum in pre-calves. The finding of the same sequence of IIaA19G1 identified in Portuguese cryptosporidiosis patients suggested the possible zoonotic transmission of the subtype IIaA19G1 and public health significance [45]. To characterize the transmission dynamics and zoonotic potential of C. parvum, numerous studies should focus on subtyping C. parvum in farm animals, especially pre-calves. Currently, sequence analysis of the gp60 gene revealed the presence of three subtype families of C. parvum in calves worldwide, including IIa, IIId and III [3,5]. Subtyping results have shown that calves are commonly infected with the IIa subtype family, and the subtype, IIaA15G2R1, is the most prevalent in most areas studied [3,46]. IIId and III subtype families are occasionally found in calves. So far, some IIId subtypes have been found in dairy calves in Belgium, Germany, Hungary, Portugal, Malaysia, Spain, Sweden, Egypt, Henan of China, and Belgrade, Serbia and Montenegro [5,6,9,15,32,38,44,45,47,48]. III subtype owns the least share among the three subtype families of C. parvum. Only 11 isolates presenting five III subtypes have been identified in calves, with IIIA16R2 (two isolates) and IIIA18R2 (two isolates) in Slovenia, IIIA24R2 (one isolate) in the Netherlands [49,50], IIIA16R2 (four

### Table 3. Intra-species variations in the SSU rRNA nucleotide sequences of C. bovis.

| Type | No. of isolates (%) | Nucleotide at position* | Accession no. in GenBank |
|------|---------------------|-------------------------|-------------------------|
| Ref sequence | A A A C T A T C A G | | HQ179575 |
| Type 1 | 31 (91.18) | A A A C T A T C A G | JX416363 |
| Type 2 | 1 (2.94) | A A G T C G C T G A | JX416364 |
| Type 3 | 1 (2.94) | G T A C T A T C A G | JX416365 |
| Type 4 | 1 (2.94) | A A G T C G C T G G | JX416366 |

*Denotes the nucleotide at position within the SSU rRNA gene.

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Montenegro [48]. C. parvum many of the common IIa subtypes of cryptosporidiosis. It has been confirmed by numerous studies that generally considered to be potentially responsible for zoonotic lands were also reported to be infected with three IId subtypes infected patients in Lisbon, and two of which (IIdA17G1 and IIdA19G1, IIdA21G1 and IIdA22G1) identified from HIV-infected patients in Lisbon, and two of which (IIdA17G1 and IIdA21G1) were found, respectively in calves and in lambs in the same areas [45]. Human cryptosporidiosis cases in the Netherlands were also reported to be infected with three IId subtypes (IIdA15G1, IIdA16G1, IIdA18G1) of C. parvum, which were not found in calves in this study [50]. In a study of genetic classification of Cryptosporidium isolates from humans and calves in Slovenia, one III subtype (IIIA16R2) has been found in both humans and calves [49]. The finding indicated that III subtype families might have the potential of zoonotic transmission.

In the present study, five SSU rRNA gene sequences of C. meleagridis were identical to each other and had 100% similarity with the sequence (HM485432) derived from a human case [56]. C. meleagridis was found to be as prevalent as C. parvum in some countries and areas [46]. It is identified frequently in children and immunocompromised adults from developing and industrialized countries [57]. DNA sequencing of the gp60 gene showed that three C. meleagridis isolates sequenced successfully all belonged to the subtype IIIa22G2R1, and all of them were identical to each other and have never been described previously. Sequence analysis of the gp60 gene has been used widely in subtyping and tracking the transmission of some Cryptosporidium spp (C. meleagridis, C. fayeri, C. cuniculus) and genotypes besides C. parvum and C. hominis [3,58–60]. To date, a total of six different subtype families (IIa-III) have been identified worldwide [3,58,61]. We are unable to determine the true source of infection and transmission dynamics of C. meleagridis in calves due to the lack of C. meleagridis subtype data from humans and animals in the investigated areas. Even in China, there are no subtype descriptions of C. meleagridis although a few studies molecularly identified 22 C. meleagridis isolates (seven from wastewater, one from source water, three from chickens, two from quails, three from birds and six from children) [62–67].

In conclusion, the present findings, including population, distribution and genetic characterizations of Cryptosporidium at genotype and subtype levels in pre-weaned calves, might represent the endemcity of bovine cryptosporidiosis in Heilongjiang Province, China. The reasons behind geographic differences in the distribution of Cryptosporidium species/genotypes and subtypes between and within countries remain unclear. Whether the finding of C. meleagridis in pre-weaned calves represented a natural infection needs to be confirmed with more systematic characterization of cryptosporidiosis in calves and by experimental infection studies. The transmission dynamics and the risky extent of zoonotic transmission of C. parvum and C. meleagridis need to be elucidated with sufficient molecular epidemiologic data from humans, animals and water source in the future.

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

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