Genetic diversity of *Cryptosporidium parvum* in neonatal calves in Xinjiang, China

**CURRENT STATUS:** UNDER REVISION

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**DOI:**  
10.21203/rs.2.19253/v1

**SUBJECT AREAS**
**KEYWORDS**
*Cryptosporidium, Neonatal calves, Diarrhea, Subtype, Xinjiang*
Abstract

Background

The Cryptosporidium causes infection in wide spectrum of vertebrate hosts and is well known for leading to diarrhea and other gastrointestinal illness. To assess Cryptosporidium genetic diversity in neonatal calves and probe the cause for diarrhea of them, a total of 232 fecal samples from neonatal calves in 12 farms in Xinjiang were characterized for the presence of Cryptosporidium.

Results

The prevalence of Cryptosporidium was 38.4% (89/232), and three species were detected with SSU rRNA gene, including C. parvum (n = 88), C. ryanae (n = 9), and C. bovis (n = 1). Prevalence of C. parvum neonatal calves with diarrhea (52.6%, 51/97) was significantly higher than calves without diarrhea (28.1%, 38/135). All the C. parvum -positive samples were analyzed based on gp60 gene, IIdA15G1 (n = 35), IIdA20G1 (n = 21), IIdA14G1 (n = 17), and IIdA19G1 (n = 13) were successfully subtyped in this study.

Conclusions

These data indicated that C. parvum was a major contributor in diarrheal disease in neonatal calves, and C. parvum subtypes from neonatal calves in Xinjiang were high genetic diversity. Additionally, our findings implicating neonatal calves could be a potential source of human Cryptosporidium infection and provide further evidence for the uniqueness of C. parvum IId subtypes in cattle in China.

Background

Cryptosporidium is common causative pathogen caused gastroenteritis in humans and animals, especially responsible for significant morbidity and mortality in children, immuno-compromised individuals, and HIV patients [1]. Cryptosporidium is second only to Rotavirus as the cause pathogens of moderate-to-severe diarrhea in children younger than 2 years in the developing world [2]. Currently, 38 valid Cryptosporidium species and over 40 Cryptosporidium genotypes have been discovered, the most infections in human were Cryptosporidium hominis and C. parvum [3, 4]. Many studies have been drawn attention to cattle, with pre-weaned calves were considered the most
important reservoir for zoonotic infection [5]. Among of C. parvum, C. bovis, C. andersoni, and C. ryanae were common infection in cattle, only C. parvum being associated with clinical disease in neonatal calves [6].

To date, based on the 60 KDa glykoprotein (gp60, also known as gp40/15) gene, nearly twenty C. parvum subtype families have been identified by phylogenetic analysis [4, 7]. Compared with other countries, evidence is accumulating for the uniqueness of C. parvum subtypes distribution in China, especially in pre-weaned dairy calves. Early work pointed to IId subtypes is dominant in dairy calves in China, while pre-weaned dairy calves are most commonly infected with Ila subtypes in Europe, North America, and Australia [5, 7]. It was demonstrated by the study that C. parvum IId subtypes likely dispersed from Western Asia to other geographical regions by cattle introduction [8].

Previous study showed that C. parvum subtypes IIdA14G1 and IIdA15G1 in dairy calves in the Xinjiang Uyghur Autonomous Region (referred to as Xinjiang hereafter) were zoonotic subtypes [9]. In history, Xinjiang plays as a necessary part of trading route for cattle between Central Asia and China. To address the knowledge gap for C. parvum genetic diversity and pathogenicity in the region, this study was conducted to examine the occurrence of Cryptosporidium and determine C. parvum subtypes in neonatal calves, and to assess the zoonotic transmission risk of C. parvum.

Results

**Prevalence of** Cryptosporidium spp.

A total of 232 fecal samples from neonatal calves were determined for Cryptosporidium based on SSU rRNA gene, with an overall prevalence of 38.4% (89/232). Cryptosporidium was detected in all twelve farms, with the highest prevalence in Tiemenguan (66.7%, 10/15), while the lowest prevalence in Hutubi2 (25.0%, 5/20) (Table 1). There was no significant difference in the prevalence of Cryptosporidium amongst farms (P > 0.05). The prevalence of neonatal calves with diarrhea or without diarrhea were 52.6% (51/97) and 28.1% (38/135), respectively (Table 2). There was a significant difference in the prevalence of Cryptosporidium between different symptoms (P < 0.05).
### Table 1
Occurrence of Cryptosporidium species and subtypes in neonatal calves in Xinjiang, China

| Farm    | No. samples | No. positive samples (%) | Cryptosporidium species and subtypes (no.) |
|---------|-------------|--------------------------|-------------------------------------------|
| Wushi1  | 11          | 7 (63.6)                 | C. parvum (7), IIdA20G1 (7)               |
| Wushi2  | 24          | 8 (33.3)                 | C. parvum (8)\(^a\), IIdA20G1 (7)        |
| Alaer   | 19          | 7 (36.8)                 | C. parvum (7), IIdA14G1 (6), IIdA15G1 (1) |
| Wensu1  | 17          | 7 (41.2)                 | C. parvum (7), IIdA15G1 (7)               |
| Wensu2  | 16          | 6 (37.5)                 | C. parvum (6), IIdA15G1 (6)               |
| Shihezi1| 23          | 6 (26.1)                 | C. parvum (6), IIdA14G1 (1), IIdA15G1 (5) |
| Shihezi2| 21          | 8 (38.1)                 | C. parvum (4), IIdA14G1 (3), IIdA15G1 (1); C. parvum + C. ryanae (4)\(^c\), IIdA14G1 (3), IIdA15G1 (1) |
| Shihezi3| 25          | 10 (40.0)                | C. parvum (6), IIdA14G1 (1), IIdA15G1 (5); C. parvum + C. ryanae (4)\(^c\), IIdA14G1 (3), IIdA15G1 (1) |
| Hutubi1 | 21          | 8 (38.1)                 | C. parvum (8), IIdA19G1 (8)               |
| Hutubi2 | 20          | 5 (25.0)                 | C. parvum (5), IIdA19G1 (5)               |
| Tiemenguan| 15         | 10 (66.7)                | C. parvum (9)\(^b\), IIdA15G1 (8); C. bovis + C. ryanae (1)\(^c\) |
| Kuitun  | 20          | 7 (35.0)                 | C. parvum (7), IIdA20G1 (7)               |
| **Total**| 232        | 89 (38.4)                | C. parvum (80)\(^b\), IIdA14G1 (11), IIdA15G1 (33), IIdA19G1 (13), IIdA20G1 (21); C. parvum + C. ryanae (8)\(^c\), IIdA14G1 (6), IIdA15G1 (2); C. bovis + C. ryanae (1)\(^c\) |

\(^a\) One isolate unsuccessfully subtyped.
\(^b\) Two isolates unsuccessfully subtyped.
\(^c\) Mixed infection.

### Table 2
Cryptosporidium species and subtypes according to clinical symptoms in present study

| Clinical symptom | Farm    | No. positive/No. examined (%) | Cryptosporidium species and subtypes (no.) |
|------------------|---------|------------------------------|-------------------------------------------|
| Diarrhea         | Wushi1  | 6/8 (75.0)                   | C. parvum (6), IIdA20G1 (6)               |
|                  | Wushi2  | 5/11 (45.5)                  | C. parvum (5)\(^a\), IIdA20G1 (4)        |
|                  | Alaer   | 3/7 (42.9)                   | C. parvum (3), IIdA14G1 (3)               |
|                  | Wensu1  | 4/11 (36.4)                  | C. parvum (4), IIdA15G1 (4)               |
|                  | Wensu2  | 6/11 (54.5)                  | C. parvum (6), IIdA15G1 (6)               |
|                  | Shihezi1| 5/9 (55.6)                   | C. parvum (5), IIdA15G1 (5)               |
|                  | Shihezi2| 3/4 (75.0)                   | C. parvum (2), IIdA14G1                   |
| Farm   | Samples | Isolates |
|--------|---------|----------|
| Shihezi3 | 4/7 (57.1) | C. parvum (4), IIdA14G1 (1), IIdA15G1 (3) |
| Hutubi1 | 2/4 (50.0) | C. parvum (2), IIdA19G1 (2) |
| Hutubi2 | 2/9 (22.2) | C. parvum (2), IIdA19G1 (2) |
| Tiemenguan | 7/9 (77.8) | C. parvum (7)\(^a\), IIdA14G1 (6) |
| Kuitun | 4/7 (57.1) | C. parvum (4), IIdA20G1 (4) |
| Subtotal 1 | 51/97 (52.6) | C. parvum (50)\(^b\), IIdA14G1 (6), IIdA15G1 (24), IIdA19G1 (4), IIdA20G1 (14); C. parvum + C. ryanae (1), IIdA14G1 (1) |

**No diarrhea**

| Farm   | Samples | Isolates |
|--------|---------|----------|
| Wushi1 | 1/3 (33.3) | C. parvum (1), IIdA20G1 (1) |
| Wushi2 | 3/13 (23.1) | C. parvum (3), IIdA20G1 (3) |
| Alaer | 4/12 (33.3) | C. parvum (4), IIdA14G1 (3), IIdA15G1 (1) |
| Wensu1 | 3/6 (50.0) | C. parvum (3), IIdA15G1 (3) |
| Wensu2 | 0/5 (0) | |
| Shihezi1 | 1/14 (7.1) | C. parvum (1), IIdA14G1 (1) |
| Shihezi2 | 5/17 (29.4) | C. parvum (2), IIdA14G1 (1), IIdA15G1 (1); C. parvum + C. ryanae (3), IIdA14G1 (2), IIdA15G1 (1) |
| Shihezi3 | 6/18 (33.3) | C. parvum (2), IIdA15G1 (2); C. parvum + C. ryanae (4), IIdA14G1 (3), IIdA15G1 (1) |
| Hutubi1 | 6/17 (35.3) | C. parvum (6), IIdA19G1 (6) |
| Hutubi2 | 3/11 (27.3) | C. parvum (3), IIdA19G1 (3) |
| Tiemenguan | 3/6 (50.0) | C. parvum (2), IIdA15G1 (2); C. bovis + C. ryanae (1) |
| Kuitun | 3/13 (23.1) | C. parvum (3), IIdA20G1 (3) |
| Subtotal 2 | 38/135 (28.1) | C. parvum (30), IIdA14G1 (5), IIdA15G1 (9), IIdA19G1 (9), IIdA20G1 (7); C. parvum + C. ryanae (7), IIdA14G1 (5), IIdA15G1 (2); C. bovis + C. ryanae (1) |

\(^a\) One isolate unsuccessfully subtyped.  
\(^b\) Two isolates unsuccessfully subtyped.

Based on SSU rRNA gene, RFLP analysis of the positive PCR products revealed three *Cryptosporidium* species, including *C. parvum* (n = 88), *C. ryanae* (n = 9), and *C. bovis* (n = 1) (Table 1). *C. parvum* was the dominant species detected in all twelve farms, which nine *C. ryanae* isolates were detected in Shihezi and Tiemenguan, and the sole *C. bovis* was detected in Tiemenguan. Two *Cryptosporidium* species were detected in diarrheal neonatal calves, with *C. parvum* (n = 51) and *C. ryanae* (n = 1)
Three Cryptosporidium species were detected in without diarrhea neonatal calves, with C. parvum (n = 37), C. ryanae (n = 8), and C. bovis (n = 1).

Nine samples from three farms were detected with mixed infection, one isolate co-infection of C. parvum and C. ryanae with diarrhea, while seven isolates co-infection of C. parvum and C. ryanae, one isolate co-infection of C. bovis and C. ryanae without diarrhea (Table 1, Table 2).

**Distribution of C. parvum subtype**

As 88 C. parvum-positive samples selected for subtyped, 86 (97.8%, 86/88) samples were successfully sequenced based on gp60 gene. Four subtypes were identified, IIdA15G1 was the dominant subtype (40.7%, 35/86), followed by IIdA20G1 (24.4%, 21/86), IIdA14G1 (19.8%, 17/86), and IIdA19G1 (15.1%, 13/86) (Table 1). The predominant subtype IIdA15G1 was detected from seven farms (three from Shihezi, two from Wensu, one from Alaer, and one from Tiemenguan). As for the remaining three subtypes, IIdA20G1 was identified in three farms (two from Wushi and one from Kuitun), IIdA14G1 was detected in four farms (three from Shihezi and one from Alaer), while IIdA19G1 was only detected in two farms from Hutubi (Table 1).

The subtype IIdA15G1 was the dominant subtype in neonatal calves with diarrheal (49.0%, 24/49), followed by IIdA20G1 (28.6%, 14/49), IIdA14G1 (14.3%, 7/49), and IIdA19G1 (8.2%, 4/49). Similarly, subtype IIdA15G1 was also the dominant subtype in neonatal calves without diarrhea (29.7%, 11/37), followed by IIdA14G1 (27.0%, 10/37), IIdA19G1 (24.3%, 9/37), and IIdA20G1 (18.9%, 7/37).

**Discussion**

The prevalence of Cryptosporidium in pre-weaned calves was 3.4–96.6% worldwide [6]. In this study, Cryptosporidium was detected in all 12 dairy cattle farms, and the overall prevalence was 38.4% (89/232). Compared to subsequent studies conducted in pre-weaned dairy calves of China, of which calves ages strictly less than 2 months, the prevalence of Cryptosporidium was similar to several reports in Shanghai (37.0%, 303/818) [10], Heilongjiang Province (33.2%, 86/259) [11], Ningxia Autonomous Region (referred to as Ningxia hereafter) (31.0%, 49/158) [12]. The prevalence higher than reports in Shaanxi (24.7%, 46/186) [13], Guangdong (24.0%, 93/388) [14], Henan (21.5%, 172/801) [15], Hubei (15.8%, 42/265) [16], Xinjiang (15.6%, 37/237) [9], Sichuan (14.4%, 40/278)
[17], Ningxia (14.0%, 122/871 and 10.2%, 19/186) [18, 19], Guangdong (6.4%, 19/297) [20], Hebei and Tianjin (2.6%, 9/351) Provinces [21], respectively. However, the prevalence was lower than one case in Heilongjiang Province (47.7%, 72/151) [22].

Epidemiology of bovine Cryptosporidium in China both suggests C. bovis is the predominant species in pre-weaned dairy calves of China [5]. Such as Shanghai [10], Guangdong [14, 20], Henan [15], Shaanxi [13], Hubei [16], and Sichuan Provinces [17]. However, evidence is accumulating that C. parvum is the dominant species in pre-weaned dairy calves from Ningxia [12, 18], Xinjiang [9], Beijing [23], Hebei and Tianjin [21], and Heilongjiang [11]. In this study, C. parvum was also the dominant species while C. bovis was only detected in one sample here. In addition, C. ryanae and C. andersoni can be occasionally isolated from pre-weaned dairy calves in China [5]. C. parvum mainly infections dairy calves within one month, while C. bovis and C. ryanae were more commonly detected in dairy calves 2–3 months old [6, 24]. So the differences of infection species between different locations may attribute to the age of dairy calves sampled.

Four species of Cryptosporidium are commonly found in cattle: C. parvum, C. bovis, C. ryanae and C. andersoni, and more than 90% of the infection cases in pre-weaned dairy calves are attributed to C. parvum, which has been reported as a major cause of calf enteritis [4]. In China, severe diarrhea was observed in pre-weaned calves on a dairy farm in the Ningxia in 2013, and C. parvum was the major cause for the outbreak [12]. In another report, severe diarrhea was observed in neonatal dairy calves on a large dairy farm in Jiangsu Province (East China), and approximately 360 calves died due to watery diarrhea despite antibiotic therapy [25]. Additionally, in a longitudinal study of the USA, a group of calves (n = 30) from birth to 24 months showed that the highest prevalence of Cryptosporidium infection was at 2 weeks of age and C. parvum constituted 97% of infections in pre-weaned calves [26]. The C. parvum infection rate of neonatal calves with diarrhea was significantly higher than calves without diarrhea in this study, which further suggests that C. parvum was associated with clinical disease in neonatal calves.

Nearly twenty C. parvum subtype families have been identified based on gp60 sequencing analysis [7]. In a series of subtyping studies published, there is a high prevalence of I1a subtype family in both
humans and cattle in Europe, North America and Australia [4]. However, Ild subtypes were major subtype identified from C. parvum isolates in dairy cattle of China, and the distribution of C. parvum subtype families in dairy cattle seems to be distinct in different areas [5].

To date, nearly five hundred C. parvum-positive isolates from cattle in China were identified (Table 3) and a total of seven Ild subtypes were detected, with IldA14G1, IldA15G1, IldA19G1, and IldA20G1 zoonotic. Subtype IldA15G1 was mostly found in Ningxia, Xinjiang, Heilongjiang, Sichuan, Beijing, and Gansu Provinces [9, 11, 12, 17-19, 23]. Subtype IldA19G1 was mostly found in Jiangsu, Henan, Shanghai, Xinjiang, Guangdong, Hebei, Tianjin, Beijing, and Heilongjiang Provinces [10, 14, 15, 21-23, 25]. Subtype IldA20G1 was found in Heilongjiang Province [11]. The rest of subtypes, IldA14G1, IldA17G1, IldA18G1, IldA21G1 was only detected in Xinjiang [9], Beijing [23], Tibet [27], and Shandong (data unpublished), respectively.

Table 3
Geographical distribution of C. parvum Ild subtype family from cattle in China

| Subtype   | Animals | No. of positive (no.) | Province (no.) | Reference                        |
|-----------|---------|-----------------------|----------------|----------------------------------|
| IldA14G1  | Dairy cattle | 21                    | Xinjiang (21) | [21], this study                  |
| IldA15G1  | Dairy cattle | 165                   | Ningxia (85)\(^a\), Xinjiang (46), Heilongjiang (24), Sichuan (7), Gansu (1), Beijing (1), Shandong (1)\(^b\) | [11, 12, 17-19, 21, 23], this study |
|           | Yak     | 3                     | Gansu (2), Qinghai (1) | [27]                      |
| IldA17G1  | Dairy cattle | 1                     | Beijing (1)   | [23]                             |
| IldA18G1  | Yak     | 1                     | Qinghai (1)   | [27]                             |
| IldA19G1  | Dairy cattle | 250                   | Jiangsu (77)\(^a\), Henan (67), Shanghai (66), Xinjiang (13), Guangdong (10), Hebei (5), Tianjin (5), Shandong (5)\(^b\), Beijing (1), Heilongjiang (1) | [10, 14, 15, 21-23, 25], this study |
|           | Yak     | 1                     | Tibet (1)     | [27]                             |
| IldA20G1  | Dairy cattle | 69                    | Heilongjiang (48), Xinjiang (21) | [11], this study             |
| IldA21G1  | Dairy cattle | 4                     | Shandong (4)\(^b\) |                                  |

\(^a\) With farm cryptosporidiosis outbreak

\(^b\) Data unpublished

Generally, one or two subtypes were detected from every place in the above studies, while four Ild subtypes were successfully subtyped (IldA14G1, IldA15G1, IldA19G1, and IldA20G1), and all subtypes zoonotic in this study. The data indicate the subtypes of C. parvum in dairy cattle of Xinjiang appear to high genetic diversity, which are more heterogeneous than other research areas in China. We
speculate that more subtypes may be detected in Xinjiang, so more systematic epidemiological studies focus on other species animals are needed to further clarify the genetic diversity and zoonotic transmission risk of Cryptosporidium in this region.

Conclusions
Four IId subtypes (IIdA14G1, IIdA15G1, IIdA19G1, and IIdA20G1) were successfully subtyped, and the data indicate that C. parvum subtypes from neonatal calves in Xinjiang are high genetic diversity. The significant statistical differences between calves with diarrhea or not indicate C. parvum is a major contributor in diarrheal disease in neonatal calves. Our findings implicating neonatal calves could be a potential source of human Cryptosporidium infection and provide further evidence for the uniqueness of C. parvum IId subtypes in dairy cattle in China.

Methods
**Study area and sample collection**
From April 2017 to April 2018, a total of 232 individual fresh fecal sample (approximately 20 g to 40 g each) were collected from 12 dairy cattle farms from the cities of Wushi, Alaer, Wensu, Shihezi, Hutubi, Tiemenguan, and Kuitun in Xinjiang, northwest China. Neonatal calves, all aged within one month (0–30 days), were separately kept in calf hutch or raised in neonatal calves cowshed. All fecal samples were collected directly from the rectum of each by sterile disposable latex glove. The status of diarrhea was observed according to clinical symptom, and neonatal calves were divided into two groups for diarrhea or without diarrhea. The samples were transported to the laboratory and stored at 4 °C for use in subsequent molecular analysis.

**DNA extraction and PCR amplification**
The genomic DNA was extracted from approximately 200 mg of each sample using the E.Z.N.A.R® Stool DNA (Omega Biotek Inc., Norcross, GA, USA). The DNA was stored at −20 °C prior to being used in PCR amplification.

The small subunit rRNA (SSU rRNA) gene was used to identify the Cryptosporidium species with primers and under the reaction conditions described previously [28]. To determine the Cryptosporidium species, positive samples were further analyzed by restriction fragment length polymorphism (RFLP) method using the SspI and MboII restriction enzymes (Fermentas, Shenzhen,
China) [24]. C. parvum-positive samples, confirmed by DNA sequencing of PCR products, were subtyped based on the sequence analysis of the 60 kDa glycoprotein (gp 60) gene [29]. Two replicates were used in the PCR analysis for each sample, and positive controls (chicken-derived C. bailey DNA) and negative controls (containing no template DNA) were set during the amplification.

**Sequence analysis**
The raw sequences obtained here were edited with DNAstar Lasergene Editseq version 7.1.0 (http://www.dnastar.com/) and aligned with reference sequences downloaded from GenBank (http://www.ncbi.nlm.nih.gov/) by Clustal X version 2.1 (http://www.clustal.org/).

**Statistical analysis**
The infection rates of Cryptosporidium were calculated and compared by the Chi-square test.

Differences were considered significant at $P < 0.05$.

**List Of Abbreviations**
HIV, human immunodeficiency virus; gp60, 60 KDa glycoprotein; SSU rRNA, Small subunit rRNA; RFLP, restriction fragment length polymorphism.

**Declarations**

**Ethics approval and consent to participate**
The experiment protocol was performed strictly according to the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, China. In accordance with the field study guidelines of local legislation, written consent to participate was obtained from all participating farm owners in our study. In addition, the research protocol was reviewed and approved by the Research Ethics Committee of Henan Agricultural University (approval number IRC-HENAU-20160225).

No neonatal calves were injured during the fecal samples collection.

**Consent for publication**
Not applicable.

**Availability of data and materials**
Not applicable.

**Competing interests**
The authors declare that they have no competing interests.
**Funding**

This work was supported in part by the National Natural Science Foundation of China (31702227); the Program for Young and Middle-aged Leading Science, Technology, and Innovation of Xinjiang Production & Construction Group (2018CB034); and the President Foundation of Tarim University (TDZKCX201701). The sponsors had no role in study design, in the collection, analysis, or interpretation of data, in the writing of the report, or in the decision to submit the article for publication.

**Authors’ contributions**

Design of study and experiments: LXZ and MQ. Sample collection: KKZ, ZLW, and YZ. Analysis of results and data interpretation: KKZ, YYW, ZHC, DFL, and YCC. Manuscript drafting: KKZ, YYW, and BJ. All authors read and approved the final version of the manuscript.

**Acknowledgements**

We thank Traci Raley, MS, ELS, from Liwen Bianji, Edanz Editing China (www.liwenbianji.cn/ac) for editing a draft of this manuscript.

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