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

Kuankuan Zhang

Tarim University

Yayun Wu

Henan Agricultural University

Zilin Wei

Tarim University

Ying Zhang

Tarim University

Bo Jing

Tarim University

Zhaohui Cui

Henan Agricultural University

Dongfang Li

Henan Agricultural University

Yuancai Chen

Henan Agricultural University

Meng Qi (✉ qimengdz@163.com)

Tarim University

Longxian Zhang

Henan Agricultural University

Research article

Keywords: Cryptosporidium, Neonatal calves, Diarrhea, Subtype, Xinjiang

Posted Date: December 19th, 2019

DOI: <https://doi.org/10.21203/rs.2.19253/v1>

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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 IId 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 (%)	<i>Cryptosporidium</i> species and subtypes (no.)
Wushi1	11	7 (63.6)	<i>C. parvum</i> (7), IIdA20G1 (7)
Wushi2	24	8 (33.3)	<i>C. parvum</i> (8) ^a , IIdA20G1 (7)
Alaer	19	7 (36.8)	<i>C. parvum</i> (7), IIdA14G1 (6), IIdA15G1 (1)
Wensu1	17	7 (41.2)	<i>C. parvum</i> (7), IIdA15G1 (7)
Wensu2	16	6 (37.5)	<i>C. parvum</i> (6), IIdA15G1 (6)
Shihezi1	23	6 (26.1)	<i>C. parvum</i> (6), IIdA14G1 (1), IIdA15G1 (5)
Shihezi2	21	8 (38.1)	<i>C. parvum</i> (4), IIdA14G1 (3), IIdA15G1 (1); <i>C. parvum</i> + <i>C. ryanae</i> (4) ^c , IIdA14G1 (3), IIdA15G1 (1)
Shihezi3	25	10 (40.0)	<i>C. parvum</i> (6), IIdA14G1 (1), IIdA15G1 (5); <i>C. parvum</i> + <i>C. ryanae</i> (4) ^c , IIdA14G1 (3), IIdA15G1 (1)
Hutubi1	21	8 (38.1)	<i>C. parvum</i> (8), IIdA19G1 (8)
Hutubi2	20	5 (25.0)	<i>C. parvum</i> (5), IIdA19G1 (5)
Tiemenguan	15	10 (66.7)	<i>C. parvum</i> (9) ^a , IIdA15G1 (8); <i>C. bovis</i> + <i>C. ryanae</i> (1) ^c
Kuitun	20	7 (35.0)	<i>C. parvum</i> (7), IIdA20G1 (7)
Total	232	89 (38.4)	<i>C. parvum</i> (80) ^b , IIdA14G1 (11), IIdA15G1 (33), IIdA19G1 (13), IIdA20G1 (21); <i>C. parvum</i> + <i>C. ryanae</i> (8) ^c , IIdA14G1 (6), IIdA15G1 (2); <i>C. bovis</i> + <i>C. ryanae</i> (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), IldA20G1 (6)
	Wushi2	5/11 (45.5)	C. parvum (5) ^a , IldA20G1 (4)
	Alaer	3/7 (42.9)	C. parvum (3), IldA14G1 (3)
	Wensu1	4/11 (36.4)	C. parvum (4), IldA15G1 (4)
	Wensu2	6/11 (54.5)	C. parvum (6), IldA15G1 (6)
	Shihezi1	5/9 (55.6)	C. parvum (5), IldA15G1 (5)
	Shihezi2	3/4 (75.0)	C. parvum (2), IldA14G1 (2); C. parvum + C. ryanae (1), IldA14G1 (1)
	Shihezi3	4/7 (57.1)	C. parvum (4), IldA14G1 (1), IldA15G1 (3)
	Hutubi1	2/4 (50.0)	C. parvum (2), IldA19G1 (2)
	Hutubi2	2/9 (22.2)	C. parvum (2), IldA19G1 (2)
	Tiemenguan	7/9 (77.8)	C. parvum (7) ^a , IldA15G1 (6)
	Kuitun	4/7 (57.1)	C. parvum (4), IldA20G1 (4)
	Subtotal 1	51/97 (52.6)	C. parvum (50) ^b , IldA14G1 (6), IldA15G1 (24), IldA19G1 (4), IldA20G1 (14); C. parvum + C. ryanae (1), IldA14G1 (1)
No diarrhea	Wushi1	1/3 (33.3)	C. parvum (1), IldA20G1 (1)
	Wushi2	3/13 (23.1)	C. parvum (3), IldA20G1 (3)
	Alaer	4/12 (33.3)	C. parvum (4), IldA14G1 (3), IldA15G1 (1)
	Wensu1	3/6 (50.0)	C. parvum (3), IldA15G1 (3)
	Wensu2	0/5 (0)	
	Shihezi1	1/14 (7.1)	C. parvum (1), IldA14G1 (1)

^a One isolate unsuccessfully subtyped.^b Two isolates unsuccessfully subtyped.

Clinical symptom	Farm	No. positive/No. examined (%)	Cryptosporidium species and subtypes (no.)
	Shihezi2	5/17 (29.4)	C. parvum (2), IldA14G1 (1), IldA15G1 (1); C. parvum + C. ryanae (3), IldA14G1 (2), IldA15G1 (1)
	Shihezi3	6/18 (33.3)	C. parvum (2), IldA15G1 (2); C. parvum + C. ryanae (4), IldA14G1 (3), IldA15G1 (1)
	Hutubi1	6/17 (35.3)	C. parvum (6), IldA19G1 (6)
	Hutubi2	3/11 (27.3)	C. parvum (3), IldA19G1 (3)
	Tiemenguan	3/6 (50.0)	C. parvum (2), IldA15G1 (2); C. bovis + C. ryanae (1)
	Kuitun	3/13 (23.1)	C. parvum (3), IldA20G1 (3)
	Subtotal 2	38/135 (28.1)	C. parvum (30), IldA14G1 (5), IldA15G1 (9), IldA19G1 (9), IldA20G1 (7); C. parvum + C. ryanae (7), IldA14G1 (5), IldA15G1 (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) (Table 2). 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, IldA15G1 was the dominant subtype (40.7%, 35/86), followed by IldA20G1 (24.4%, 21/86), IldA14G1 (19.8%, 17/86), and IldA19G1 (15.1%, 13/86) (Table 1). The predominant subtype IldA15G1 was detected from seven farms (three from Shihezi, two from Wensu, one from Alaer, and one from Tiemenguan). As for the remaining three subtypes, IldA20G1 was identified in three farms (two e from Wushi and one from Kuitun), IldA14G1 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 Ila subtype family in both humans and cattle in Europe, North America and Australia [4]. However, IId 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 IId subtypes were detected, with IIdA14G1, IIdA15G1, IIdA19G1, and IIdA20G1 zoonotic. Subtype IIdA15G1 was mostly found in Ningxia, Xinjiang, Heilongjiang, Sichuan, Beijing, and Gansu Provinces [9, 11, 12, 17–19, 23]. Subtype IIdA19G1 was mostly found in Jiangsu, Henan, Shanghai, Xinjiang, Guangdong, Hebei, Tianjin, Beijing, and Heilongjiang Provinces [10, 14, 15, 21–23, 25]. Subtype IIdA20G1 was found in Heilongjiang Province [11]. The rest of subtypes, IIdA14G1, IIdA17G1, IIdA18G1, IIdA21G1 was only detected in Xinjiang [9], Beijing [23], Tibet [27], and Shandong (data unpublished), respectively.

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

Subtype	Animals	No. of positive (no.)	Province (no.)	Reference
IIdA14G1	Dairy cattle	21	Xinjiang (21)	[21], this study
IIdA15G1	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]
IIdA17G1	Dairy cattle	1	Beijing (1)	[23]
IIdA18G1	Yak	1	Qinghai (1)	[27]
IIdA19G1	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]
IIdA20G1	Dairy cattle	69	Heilongjiang (48), Xinjiang (21)	[11], this study
IIdA21G1	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 IId subtypes were successfully subtyped (IIdA14G1, IIdA15G1, IIdA19G1, and IIdA20G1), 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 Ild subtypes (IldA14G1, IldA15G1, IldA19G1, and IldA20G1) 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* Ild 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 *Ssp*I and *Mbo*II 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.

Authors' information

¹College of Animal Science, Tarim University, Alar 843300, China

²College of Animal Science and Veterinary Medicine, Henan Agricultural University, Zhengzhou 450002, China

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