

Seasonal monitoring of *Cryptosporidium* species and genetic diversity in neonatal dairy calves on two large-scale farms in Xinjiang, China

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Keywords: Cryptosporidium, Prevalence, Diarrhea, Neonatal dairy calf, Subtype, Season

Posted Date: August 4th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-52838/v1>

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Abstract

Background: Neonatal dairy calves infected with *Cryptosporidium* can possess a significant source of zoonotic infections and disease. To assess seasonal variations in the prevalence and genetic diversity of *Cryptosporidium* in neonatal dairy calves, 380 fecal samples from neonatal dairy calves on two large-scale farms in Xinjiang (Alaer and Wensu) were screened for the *Cryptosporidium* small subunit (SSU) rRNA gene.

Results: The overall prevalence of *Cryptosporidium* was 48.7% (185/380): 48.6% (108/222) in Alaer and 48.7% (77/158) in Wensu. *Cryptosporidium* was most frequent in summer (56.8%, 54/95), followed by spring (50.0%, 44/88), winter (46.8%, 44/94), and autumn (41.7%, 43/103) ($P > 0.05$). *Cryptosporidium* was significantly more prevalent in calves with diarrhea (72.4%, 113/156) than in those without (32.1%, 72/224) ($P < 0.01$). Based on a restriction fragment length polymorphism (RFLP) analysis, *C. parvum* (n = 173), *C. bovis* (n = 7), *C. ryanae* (n = 3), and co-infections of the three species (n = 2) were identified. Most (172/175) *C. parvum* samples were successfully sequenced at the 60-kDa glycoprotein gene (*gp60*), revealing two zoonotic subtypes: IIdA14G1 (n = 94) and IIdA15G1 (n = 7) in Alaer and IIdA15G1 (n = 71) in Wensu.

Conclusions: These results showed that neonatal dairy calves were commonly infected with *Cryptosporidium* throughout the year, and there was a significant association between the occurrence of diarrhea and *Cryptosporidium* infection. Presence of IIdA14G1 and IIdA15G1 indicated neonatal dairy calves may be a source of zoonotic *C. parvum* subtypes.

Background

Cryptosporidium is a ubiquitous pathogen that can cause severe diarrheal disease in infants and opportunistic infections in immunocompromised patients [1,2]. Cryptosporidiosis can be transmitted between hosts through the fecal–oral route, by direct contact with feces from infected hosts, or indirectly by the ingestion of contaminated food or water [3]. Among the valid *Cryptosporidium* species/genotypes so far discovered, *C. parvum*, *C. bovis*, *C. andersoni*, and *C. ryanae* are commonly responsible for infections in cattle [4,5]. Based on the age-related distributions of these four common species, *C. parvum* is recognized as the most important cause of neonatal enteritis in calves worldwide [6]. *C. andersoni* can cause gastritis, reduced milk yield, and poor weight gain in post-weaned calves and adult cattle, whereas *C. bovis* and *C. ryanae* usually infect post-weaned calves and yearlings, with no associated clinical disease [4,6].

The infection of pre-weaned calves with *C. parvum* has been linked to a number of outbreaks of human cryptosporidiosis, and evidence is increasing that contact with calves is the most likely source of *Cryptosporidium* infections [7-9]. Data on the global *C. parvum* subtypes in cattle indicate that although the IId subtypes are dominant in China, the IIda subtypes are more common in Europe, North America, and Australia. In China, cryptosporidiosis outbreaks were reported on two dairy farms in the Ningxia

Autonomous Region (referred to as Ningxia hereafter) and Jiangsu Province, caused by *C. parvum* subtypes IIdA15G1 and IIdA19G1, respectively [10,11]. The IId subtypes have been detected in humans and various animal species, indicating that neonatal calves may be a reservoir for the transmission of human *Cryptosporidium* infections [12].

A previous study suggested that the *C. parvum* IId subtypes were probably dispersed from Western Asia to other geographic regions with the introduction of cattle [13]. As the largest Chinese administrative division in China, Xinjiang Uygur Autonomous region (referred to as Xinjiang hereafter) is the necessary route for cattle trading and domestication between Central Asia and China, and has an increasing number of large-scale dairy farms. However, the seasonal prevalence of *C. parvum* infections in neonatal dairy calves in China is poorly understood, with pre-weaned calves only analyzed in Henan and Guangdong [14,15]. To address the knowledge gap in the seasonal variations in *C. parvum* prevalence, the incidence of associated diarrhea, and the genetic subtypes present in neonatal dairy calves in Xinjiang, we conducted a series of experiments to characterize the seasonal features of *Cryptosporidium* infection in neonatal dairy calves. The data generated extend our understanding of the causes of diarrhea in calves and the possible role of cattle in the transmission of *Cryptosporidium* to humans.

Methods

Study area and sample collection

Between September 2017 and September 2018, a total of 380 fresh fecal samples were collected from female neonatal dairy calves (mainly 7–20 days old) on two large-scale dairy farms in Alaer and Wensu in the Aksu area (N 41°09', E 80°19') of Xinjiang. According to the number of calves born per month, 50%–80% of the neonatal dairy calves were randomly sampled once a month for a year, and four seasons were divided according to the northern hemisphere. In Alaer, the calves were raised separately in a calf hutch with straw bedding that was not changed often, whereas in Wensu, the calves were raised separately in a calf hutch for a week, and then mixed breeding in an open outdoor stall. Fecal samples (approximately 30–50 g each) were collected directly from the rectum of each calf with disposable gloves. The fresh feces were placed into clean plastic bags marked with the date, calf age, and farm. All the samples were immediately transported to the laboratory and stored at 4 °C before genomic DNA extraction.

DNA extraction and PCR amplification

The samples were concentrated by centrifugation at 1,500 × g for 10 min, and the genomic DNA was extracted from approximately 200 mg of each sample with the E.Z.N.A.[®] Stool DNA Kit (Omega Biotek Inc., Norcross, GA, USA), according to the manufacturer's instructions. The extracted DNA samples were then frozen at -20 °C before their molecular analysis for *Cryptosporidium*.

To characterize *Cryptosporidium*, a nested PCR assay was performed based on the small subunit rRNA (SSU rRNA) gene with previously described primers and reaction conditions [16]. The *Cryptosporidium*

isolates were further identified with the restriction fragment length polymorphism (RFLP) method using the *SspI* and *MbolI* restriction enzymes (TaKaRa Shuzo Co. Ltd., Otsu, Japan) [17]. To determine the *C. parvum* subtypes, all *C. parvum*-positive samples were characterized to the subtype level based on a sequence analysis of an approximately 850-bp fragment of the 60-kD glycoprotein gene (*gp60*) [18]. To ensure the accuracy of amplification, positive controls (chicken-derived *C. bailey* DNA) and negative controls (containing no template DNA) were included in the PCR analysis of each sample.

All secondary PCR products were stained with GelRed (Biotium Inc., Hayward, CA, USA), separated with 1% agarose gel electrophoresis and visualized on a UV transilluminator. All secondary PCR amplification products of the expected size (~840 bp) were then bidirectionally sequenced on an ABI PRISM™ 3730 XL DNA Analyzer using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Molecular analysis

To identify the species of *Cryptosporidium* and the subtypes of *C. parvum*, the raw DNA sequences were edited with DNASTar Lasergene EditSeq version 7.1.0 (<http://www.dnastar.com/>) and aligned with reference sequences available from the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/>) using ClustalX version 2.1 (<http://www.clustal.org/>).

Statistical analysis

SPSS ver. 22.0 (IBM Corp., Armonk, NY, USA) was used for all statistical analyses. The infection rates and their 95% confidence intervals (CIs) were calculated with the Wald method. Differences in infection rates among seasons or symptoms were evaluated with the Chi-square test, and differences were considered significant at $P < 0.05$.

Results

Prevalence of *Cryptosporidium* spp.

The overall prevalence of *Cryptosporidium* in neonatal dairy calves was 48.7% (185/380, 95% CI: 43.5-53.8%), with 48.6% (108/222, 95% CI: 41.9-55.5 %) in Alaer and 48.7% (77/158, 95% CI: 40.6-56.8%) in Wensu. In Alaer, *Cryptosporidium* was detected most frequently in summer (53.7%, 29/54, 95% CI: 39.5-67.9%), followed by spring (44.9%, 22/49, 95% CI: 30.0-59.9%), winter (52.5%, 31/59, 95% CI: 39.0-66.1%), and autumn (43.3%, 26/60, 95% CI: 30.0-56.7%), while the prevalence of *Cryptosporidium* did not differ significantly among the seasons ($\chi^2 = 1.865$, $df = 3$, $P > 0.05$) (**Table 1**). In Wensu, the highest prevalence occurred in summer (61.0%, 25/41, 95% CI: 44.8-77.1%), then spring (56.4%, 22/39, 95% CI: 39.6-73.3%), autumn (39.5%, 17/43, 95% CI: 23.8-55.3%), and winter (37.1%, 13/35, 95% CI: 19.7-54.6%) ($\chi^2 = 6.718$, $df = 3$, $P > 0.05$) (**Table 1, Fig. 1**).

The prevalence of *Cryptosporidium* in neonatal dairy calves with and without diarrhea were 72.4% (113/156, 95% CI: 65.1-79.8%) and 32.1% (72/224, 95% CI: 25.8-38.5%), respectively, which differed significantly and there was a significant association between the occurrence of diarrhea and *Cryptosporidium* infection ($\chi^2 = 59.760$, $df = 1$, $P < 0.01$) (**Table 2**). In Alaer, the prevalence of *Cryptosporidium* with diarrhea in autumn (66.7%, 14/21, 95% CI: 44.1-89.2%) and summer (82.6%, 19/23, 95% CI: 65.0-100%) were significantly higher than those without diarrhea in autumn (30.8%, 12/39, 95% CI: 15.0-46.5%) ($\chi^2 = 7.163$, $df = 1$, $P < 0.01$) and summer (32.3%, 10/31, 95% CI: 14.2-50.3%) ($\chi^2 = 13.463$, $df = 1$, $P < 0.01$), respectively. However, the prevalence with diarrhea in winter 63.2% (12/19, 95% CI: 38.8-87.5%) and spring 58.8% (10/17, 95% CI: 32.5-85.2%) differed not significantly without diarrhea in winter (47.5%, 19/40, 95% CI: 30.8-64.2%) ($\chi^2 = 1.267$, $df = 1$, $P > 0.05$) and spring (37.5%, 12/32, 95% CI: 19.2-55.8%) ($\chi^2 = 2.040$, $df = 1$, $P > 0.05$), respectively. In Wensu, the prevalence of *Cryptosporidium* in neonatal dairy calves with diarrhea in the four seasons were 65.0% (13/20, 95% CI: 68.4-100%) in autumn, 75.0% (12/16, 95% CI: 50.7-99.3%) in winter, 85.7% (18/21, 95% CI: 44.1-89.2%) in spring, and 78.9% (15/19, 95% CI: 58.0-99.9%) in summer, which were significantly higher than those in calves without diarrhea in autumn (17.4%, 4/23, 95% CI: 0-35.1%) ($\chi^2 = 10.143$, $df = 1$, $P < 0.01$), winter (5.3%, 1/19, 95% CI: 0-17.9%) ($\chi^2 = 18.093$, $df = 1$, $P < 0.01$), spring (22.2%, 4/18, 95% CI: 2.4-44.2%) ($\chi^2 = 15.890$, $df = 1$, $P < 0.01$), and summer (45.5%, 10/22, 95% CI: 22.4-68.5%) ($\chi^2 = 4.806$, $df = 1$, $P < 0.05$), respectively (**Table 2, Fig. 2**).

Distribution of *Cryptosporidium* species

Three *Cryptosporidium* species, *C. parvum* (n = 173), *C. bovis* (n = 7), and *C. ryanae* (n = 3), and co-infections of *C. parvum*, *C. bovis*, and *C. ryanae* (n = 2) were detected with an RFLP analysis of the positive PCR products ($\chi^2 = 367.600$, $df = 2$, $P < 0.01$). *C. parvum* was clearly predominant in every season, whereas *C. bovis* and *C. ryanae* were found only occasionally, and co-infections were only seen in Alaer. Among the species detected, *C. parvum* (175/189) was the commonest species on the farms in Alaer (102/112) and Wensu (73/77) (**Table 1**).

In Alaer, the proportions of *Cryptosporidium* species identified in the four seasons were *C. parvum* 84.6% (22/26), *C. bovis* 7.7% (2/26), *C. ryanae* 3.8% (1/26), and co-infections 3.8% (1/26) in autumn; *C. parvum* 96.8% (30/31) and *C. ryanae* 3.2% (1/31) in winter; *C. parvum* 90.9% (20/22), *C. bovis* 4.5% (1/22), and co-infections 4.5% (1/22) in spring; and *C. parvum* 96.6% (28/29) and *C. bovis* 3.4% (1/29) in summer. In Wensu, the *Cryptosporidium* species distributions in the four seasons were *C. parvum* 88.2% (15/17) and *C. bovis* 11.8% (2/17) in autumn; *C. parvum* 92.3% (12/13) and *C. ryanae* 7.7% (1/13) in winter; *C. parvum* 100% (22/22) in spring; and *C. parvum* 96.0% (24/25) and *C. bovis* 4.0% (1/25) in summer (**Fig. 3**).

Subtypes of *C. parvum*

Among the 175 *C. parvum*-positive samples (102 from Alaer and 73 from Wensu), 173 were successfully sequenced and identified as two subtypes, IIdA14G1 (n = 94) and IIdA15G1 (n = 78) ($\chi^2 = 2.960$, $df = 1$, $P > 0.05$) (**Table 2**). In Alaer, the dominant subtype IIdA14G1 (n = 94, $\chi^2 = 149.881$, $df = 1$, $P < 0.01$) was

identified in every season, and the difference in the IIdA14G1 detection rates in dairy calves with diarrhea (51.5%, 52/101, 95% CI: 41.2-61.7%) and those without diarrhea (41.6%, 42/101, 95% CI: 31.5-51.7%) was not significant ($\chi^2 = 2.128$, $df = 1$, $P > 0.05$). Interestingly, IIdA15G1 ($n = 7$) was only seen in calves without diarrhea. The subtype distributions in the four seasons were IIdA14G1 95.7% (22/23, 95% CI: 85.1-100%) and IIdA15G1 4.3% (1/23, 95% CI: 0-14.9%) in autumn; IIdA14G1 89.7% (26/29, 95% CI: 76.9-100%) and IIdA15G1 10.3% (3/29, 95% CI: 0-23.2%) in winter; IIdA14G1 85.7% (18/21, 95% CI: 68.4-100%) and IIdA15G1 14.3% (3/21, 95% CI: 0-31.6%) in spring; and IIdA14G1 100% (28/28) in summer (**Fig. 4**). In Wensu, *C. parvum* was not detected in dairy calves without diarrhea in winter, and IIdA15G1 was the only subtype identified. The IIdA15G1 detection rate was significantly higher in calves with diarrhea (80.3%, 57/71, 95% CI: 70.3-90.2%) than in calves without diarrhea (19.7%, 14/71, 95% CI: 9.8-29.7%) ($\chi^2 = 52.085$, $df = 1$, $P < 0.01$) (**Table 2**).

Discussion

The overall prevalence of *Cryptosporidium* in neonatal dairy calves on the two farms was 48.7% (185/380), which was similar to that in a report from Heilongjiang in northeastern China (47.7%, 72/151) [19], and higher than those in Hebei and Tianjin (2.6%, 9/351) [20] in northern China; Ningxia (10.2%, 19/186 to 31.0%, 49/158) in three cases) [10,21,22], Shaanxi (24.7%, 46/186) [23], and Xinjiang (15.6%, 37/237) [24] in northwestern China; Henan (21.5%, 172/801) [14] and Hubei (15.8%, 42/265) [25] in central China; Sichuan (14.4%, 40/278) in southwestern China [26]; Shanghai (37.0%, 303/818) [27] and Jiangsu (22.7%, 139/612) [11] in eastern China; and Guangdong (24.0%, 93/388 and 6.4%, 19/297) in southern China [15,28].

In general, *Cryptosporidium* was significantly associated with calves younger than 2 weeks with diarrhea [29]. A previous study of a diarrhea outbreak among neonatal dairy calves in Jiangsu suggested that the prevalence of *Cryptosporidium* was significantly higher during the outbreak (80.3%) than after the outbreak (22.7%), and that the prevalence of *Cryptosporidium* in dairy calves less than 3 weeks old was much higher than in any other age group [11]. In a similar cryptosporidiosis outbreak in Ningxia, the highest prevalence of *Cryptosporidium* (83.3%, 40/48) was seen in 2–3-week-old calves with severe diarrhea [10]. In the present study, the neonatal dairy calves ranged in age from 1 week to 3 weeks, and this may have contributed to the higher prevalence of *Cryptosporidium* than has been seen in other studies in China. Another factor that may have influenced the prevalence of *Cryptosporidium* in this study is that the neonatal dairy calves were sampled continuously for a year, whereas dairy calves were sampled only once in most of the other studies.

Data on the seasonal variations in the infection rates of *Cryptosporidium* in pre-weaned dairy calves in Henan [14], Guangdong (without autumn) [15], and Western Australia [30] suggested that there is no significant seasonal difference in *Cryptosporidium* infections in pre-weaned dairy calves. The data in the present study were consistent with those studies. A previous study showed that the highest prevalence of *Cryptosporidium* in dairy calves was observed in summer in Henan, China (50.0%) [14], which was similar to our result, which showed a peak in occurrence in summer (56.8%, 54/95). In contrast, in New York

State, dairy cattle were significantly more likely to be infected by *Cryptosporidium* in winter than in summer [31]. Surprisingly, the farm in Alaer displayed its highest prevalence in December (71.4%) and lowest in late January (23.8%). Further research is required to clarify the cause.

Whether *C. parvum* or *C. bovis* is the dominant *Cryptosporidium* species in pre-weaned dairy calves is controversial among publications that document *Cryptosporidium* prevalence among cattle in China. *Cryptosporidium bovis* was more common in central China (Henan and Hubei) [14,25], eastern China (Shanghai) [27], southwestern China (Sichuan) [26], and southern China (Guangdong) [15,28], whereas *C. parvum* is more common in northeastern China (Heilongjiang) [32], northern China (Beijing, Hebei, and Tianjin) [20,33], and most of northwestern China (Ningxia and Xinjiang) [10,22,24]. Dairy calves are most commonly infected with *C. parvum* within 1 month of birth, whereas *C. bovis* is mainly associated with dairy calves aged 2–3 months [6]. The differences in the distributions of *Cryptosporidium* species in pre-weaned dairy calves in China can be attributed to the ages of the calves sampled. In most studies, samples were collected from cattle < 3 months old, but the numbers of pre-weaned dairy calves sampled that were < 1 month are unclear.

Data on the seasonal distributions of *Cryptosporidium* species in pre-weaned dairy calves in Henan suggested that they varied as *C. parvum* was predominant in summer and *C. bovis* was predominant in autumn and winter [14]. However, the seasonal shift in pre-weaned dairy calves in New York showed that *C. bovis* was most common in summer and *C. parvum* was dominant in spring and winter [34]. However, in Guangdong, *C. parvum* was dominant in pre-weaned dairy calves in three seasons (not autumn) [15]. Our findings indicate that *C. parvum* was clearly predominant in every season, whereas *C. bovis* and *C. ryanae* were found only occasionally. This is similar to two studies of cryptosporidiosis outbreaks in Ningxia and Jiangsu, in which the occurrence of *C. parvum* was significantly higher than that of *C. bovis* or *C. ryanae* [10,11].

Subtyping *C. parvum* revealed two zoonotic subtypes, IIdA14G1 and IIdA15G1. Among the six IId family subtypes detected in dairy cattle in China (IIdA14G1, IIdA15G1, IIdA17G1, IIdA20G1, and IIdA21G1), IIdA14G1 has only been seen in Xinjiang [24]. IIdA15G1 was first detected in Ningxia and then in Xinjiang [24], Heilongjiang [32], Sichuan [26], and Beijing [33]. There have been several reports of human infections with IIdA14G1 and IIdA15G1 [35,36]. The presence of IIdA14G1 and IIdA15G1 indicates the genetic diversity of *C. parvum* in neonatal dairy calves in Xinjiang, and the data also suggest that the IId family of *C. parvum* subtypes derived from cattle is distributed uniquely in China. Therefore, neonatal dairy cattle infected with *C. parvum* may be a significant source of zoonotic infections and disease.

Conclusion

In this study, neonatal dairy calves on two farms in Xinjiang were commonly infected with *Cryptosporidium* throughout the year. The prevalence of *Cryptosporidium* was significantly higher in calves with diarrhea than in those without diarrhea. *C. parvum* was clearly predominant in every season. The detection of zoonotic subtypes IIdA14G1 and IIdA15G1 suggests that dairy cattle are a crucial

reservoir in the transmission human *Cryptosporidium* infections. These results extend our understanding of the epidemiology of cryptosporidiosis and the causes of diarrhea in dairy calves.

Abbreviations

SSU rRNA, small subunit rRNA; *gp60*, the 60 kDa glycoprotein gene; RFLP, restriction fragment length polymorphism; CI, confidence intervals

Declarations

Ethics approval and consent to participate

The research protocol was reviewed and approved by the Research Ethics Committee of Henan Agricultural University (Approval No: LVRIAEC 2017-019). The field studies did not involve endangered or protected species. Consent to participate was obtained from all the participating farm owners before sample collection and no specific permits were required for the field studies described. No neonatal dairy calves were injured and all efforts were made to minimize pain and suffering during the collection of fecal samples.

Consent for publication

Not applicable.

Availability of data and materials

All data used or analyzed during this study are available from the corresponding author on reasonable request.

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 (Grant No: 31702227); the Program for Young and Middle-aged Leading Science, Technology, and Innovation of Xinjiang Production & Construction Group (Grant No: 2018CB034); and the Key Technologies R & D Programme of Xinjiang Production & Construction Group (Grant No: 2020AB025).

Authors' contributions

MQ and LXZ conceived the study and participated in its design. YYW, KKZ, BJ, and ZLW collected fecal samples and performed the experiments. YZ, YCC and ZHC helped in discussion of data. CYX performed

the statistical analyses. YYW and KKZ interpreted the results and drafted the manuscript. All authors read and approved the final version of the manuscript.

Acknowledgements

We thank Janine Miller, PhD, from Liwen Bianji, Edanz Editing China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

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Tables

Table 1. Seasonal characterization of *Cryptosporidium* in neonatal dairy calves on two farms in Xinjiang, China

Time	Alaer		Wensu	
	No. positive/ No. samples (%)	Species (n)	No. positive/No. samples (%)	Species (n)
Sep	8/20 (40.0) (16.0-64.0) ^a	<i>C. parvum</i> (6); <i>C. ryanae</i> (1); <i>C. bovis</i> (1)	8/15 (53.3) (24.8-81.9) ^a	<i>C. parvum</i> (8)
Oct	9/20 (45.0) (20.7-69.3) ^a	<i>C. parvum</i> (8); <i>C. bovis</i> (1)	6/16 (37.5) (10.7-64.4) ^a	<i>C. parvum</i> (4); <i>C. bovis</i> (2)
Nov	9/20 (45.0) (20.7-69.3) ^a	<i>C. parvum</i> (8); <i>C. parvum</i> + <i>C. ryanae</i> + <i>C. bovis</i> (1) ^b	3/12 (25.0) (0-53.7) ^a	<i>C. parvum</i> (3)
Autumn	26/60 (43.3) (30.0-56.7) ^a	<i>C. parvum</i> (22); <i>C. bovis</i> (2); <i>C. ryanae</i> (1) <i>C. parvum</i> + <i>C. ryanae</i> + <i>C. bovis</i> (1) ^b	17/43 (39.5) (23.8-55.3) ^a	<i>C. parvum</i> (15); <i>C. bovis</i> (2)
Dec	15/21 (71.4) (49.7-93.1) ^a	<i>C. parvum</i> (15)	2/11 (18.2) (0-45.5) ^a	<i>C. parvum</i> (2)
Jan	5/21 (23.8) (3.2-44.4) ^a	<i>C. parvum</i> (4); <i>C. ryanae</i> (1)	3/11 (27.3) (0-58.1) ^a	<i>C. parvum</i> (3)
Feb	11/17 (64.7) (39.1-90.4) ^a	<i>C. parvum</i> (11)	8/13 (61.5) (31.3-91.8) ^a	<i>C. parvum</i> (7); <i>C. ryanae</i> (1)
Winter	31/59 (52.5) (39.0-66.1) ^a	<i>C. parvum</i> (30); <i>C. ryanae</i> (1)	13/35 (37.1) (19.7-54.6) ^a	<i>C. parvum</i> (12); <i>C. ryanae</i> (1)
Mar	11/19 (57.9) (33.1-82.7) ^a	<i>C. parvum</i> (10); <i>C. parvum</i> + <i>C. ryanae</i> + <i>C. bovis</i> (1) ^b	9/15 (60.0) (31.9-88.1) ^a	<i>C. parvum</i> (9)
Apr	7/18 (38.9) (13.6-64.2) ^a	<i>C. parvum</i> (7)	7/13 (53.8) (22.9-84.8) ^a	<i>C. parvum</i> (7)
May	4/12 (33.3) (24.9-64.2) ^a	<i>C. parvum</i> (3); <i>C. bovis</i> (1)	6/11 (46.2) (20.6-88.5) ^a	<i>C. parvum</i> (6)
Spring	22/49 (44.9) (30.0-59.9) ^a	<i>C. parvum</i> (20); <i>C. bovis</i> (1) <i>C. parvum</i> + <i>C. ryanae</i> + <i>C. bovis</i> (1) ^b	22/39 (56.4) (39.6-73.3) ^a	<i>C. parvum</i> (22)
Jun	7/16 (43.8) (16.3-71.2) ^a	<i>C. parvum</i> (7)	9/15 (60.0) (31.9-88.1) ^a	<i>C. parvum</i> (8); <i>C. bovis</i> (1)
Jul	13/20 (65.0)	<i>C. parvum</i> (12); <i>C. bovis</i> (1)	8/12 (66.7)	<i>C. parvum</i> (8)

	(41.6-88.4) ^a		(35.8-97.5) ^a	
Aug	9/18 (50.0) (24.1-75.9) ^a	<i>C. parvum</i> (9)	8/14 (57.1) (27.7-86.6) ^a	<i>C. parvum</i> (8)
Summer	29/54 (53.7) (39.5-67.9) ^a	<i>C. parvum</i> (28); <i>C. bovis</i> (1)	25/41 (61.0) (44.8-77.1) ^a	<i>C. parvum</i> (24); <i>C. bovis</i> (1)
Total	108/222 (48.6) (41.9- 55.5) ^a	<i>C. parvum</i> (100); <i>C. bovis</i> (4); <i>C. ryanae</i> (2); <i>C. parvum</i> + <i>C. ryanae</i> + <i>C. bovis</i> (2) ^b	77/158 (48.7) (40.6- 56.8) ^a	<i>C. parvum</i> (73); <i>C. bovis</i> (3); <i>C. ryanae</i> (1)

^a 95% CI

^b Mixed infections

Table 2. Prevalence and distribution of *Cryptosporidium* species and *C. parvum* subtypes by symptoms

Symptom	Farm	Season	No. positive/ No. samples (%)	Species (n)	<i>C. parvum</i> subtype (n)
Diarrhea	Alaer	Autumn	14/21 (66.7) (44.1-89.2) ^a	<i>C. parvum</i> (13); <i>C. ryanae</i> (1)	IldA14G1 (13)
		Winter	12/19 (63.2) (38.8-87.5) ^a	<i>C. parvum</i> (11); <i>C. ryanae</i> (1)	IldA14G1 (11)
		Spring	10/17 (58.8) (32.5-85.2) ^a	<i>C. parvum</i> (9); <i>C. bovis</i> (1)	IldA14G1 (9)
		Summer	19/23 (82.6) (65.0-1) ^a	<i>C. parvum</i> (19)	IldA14G1 (19)
		Subtotal 1	55/80 (68.8) (58.0-79.5) ^a	<i>C. parvum</i> (52); <i>C. ryanae</i> (2); <i>C. bovis</i> (1)	IldA14G1 (52)
	Wensu	Autumn	13/20 (65.0) (41.6-88.4) ^a	<i>C. parvum</i> (13)	IldA15G1 (13)
		Winter	12/16 (75.0) (50.7-99.3) ^a	<i>C. parvum</i> (12)	IldA15G1 (12)
		Spring	18/21 (85.7) (68.4-1) ^a	<i>C. parvum</i> (18)	IldA15G1 (18)
		Summer	15/19 (78.9) (58.0-99.9) ^a	<i>C. parvum</i> (15) ^c	IldA15G1 (14)
		Subtotal 2	58/76 (76.3) (66.1-86.5) ^a	<i>C. parvum</i> (58) ^c	IldA15G1 (57)
Total 1		113/156 (72.4) (65.1-79.8) ^a	<i>C. parvum</i> (110) ^c ; <i>C. ryanae</i> (2); <i>C. bovis</i> (1)	IldA14G1 (52); IldA15G1 (57)	
Without diarrhea	Alaer	Autumn	12/39 (30.8) (15.0-46.5) ^a	<i>C. parvum</i> (9); <i>C. bovis</i> (2); co-infection (1) ^b	IldA14G1 (9); IldA15G1 (1)
		Winter	19/40 (47.5) (30.8-64.2) ^a	<i>C. parvum</i> (19) ^c ;	IldA14G1 (15); IldA15G1 (3)
		Spring	12/32 (37.5) (19.2-55.8) ^a	<i>C. parvum</i> (11); co- infection (1) ^b	IldA14G1 (9); IldA15G1 (3)
		Summer	10/31 (32.3) (14.2-50.3) ^a	<i>C. parvum</i> (9); <i>C. bovis</i> (1)	IldA14G1 (9)
		Subtotal 3	53/142 (37.3) (29.0-45.6) ^a	<i>C. parvum</i> (48) ^c ; <i>C. bovis</i> (3); co-infection (2) ^b	IldA14G1 (42); IldA15G1 (7)
	Wensu	Autumn	4/23 (17.4) (0- 35.1) ^a	<i>C. parvum</i> (2); <i>C. bovis</i> (2)	IldA15G1 (2)

	Winter	1/19 (5.3) (0-17.9) ^a	<i>C. ryanae</i> (1)	-
	Spring	4/18 (22.2) (2.4-44.2) ^a	<i>C. parvum</i> (4)	IldA15G1 (4)
	Summer	10/22 (45.5) (22.4-68.5) ^a	<i>C. parvum</i> (9) ^c ; <i>C. bovis</i> (1)	IldA15G1 (8)
	Subtotal 4	19/82 (23.2) (13.4-32.9) ^a	<i>C. parvum</i> (15) ^c ; <i>C. bovis</i> (3); <i>C. ryanae</i> (1)	IldA15G1 (14)
	Total 2	72/224 (32.1) (25.8-38.5) ^a	<i>C. parvum</i> (63) ^d ; <i>C. bovis</i> (6); co-infection (2) ^b ; <i>C. ryanae</i> (1)	IldA14G1 (40); IldA15G1 (21)

^a 95% CI

^b one unsuccessfully subtyped

^c infected with *C. parvum* + *C. ryanae* + *C. bovis*

^d two unsuccessfully subtyped

Figures

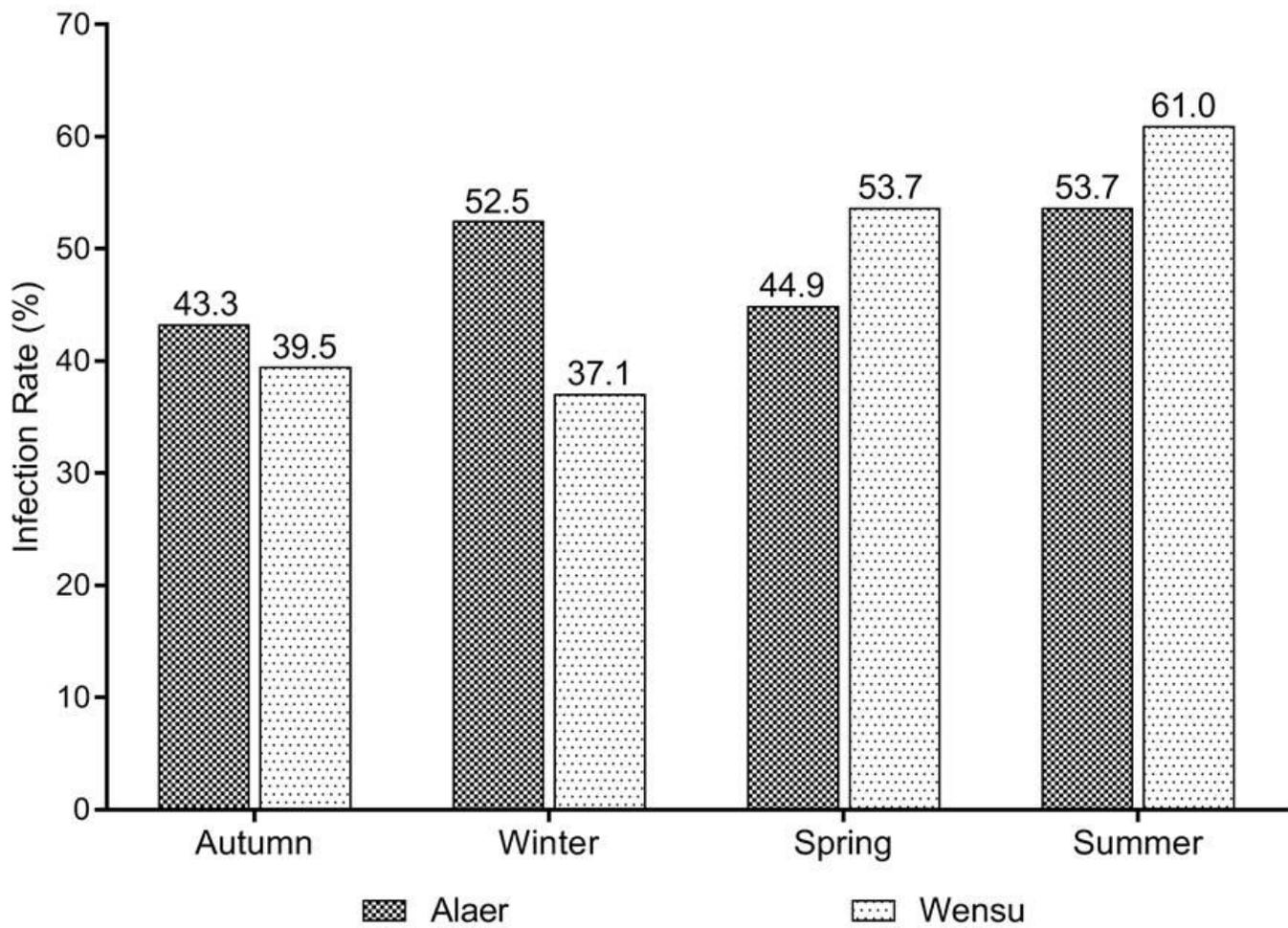


Figure 1

Seasonal variation in prevalence of Cryptosporidium in neonatal dairy calves by farms

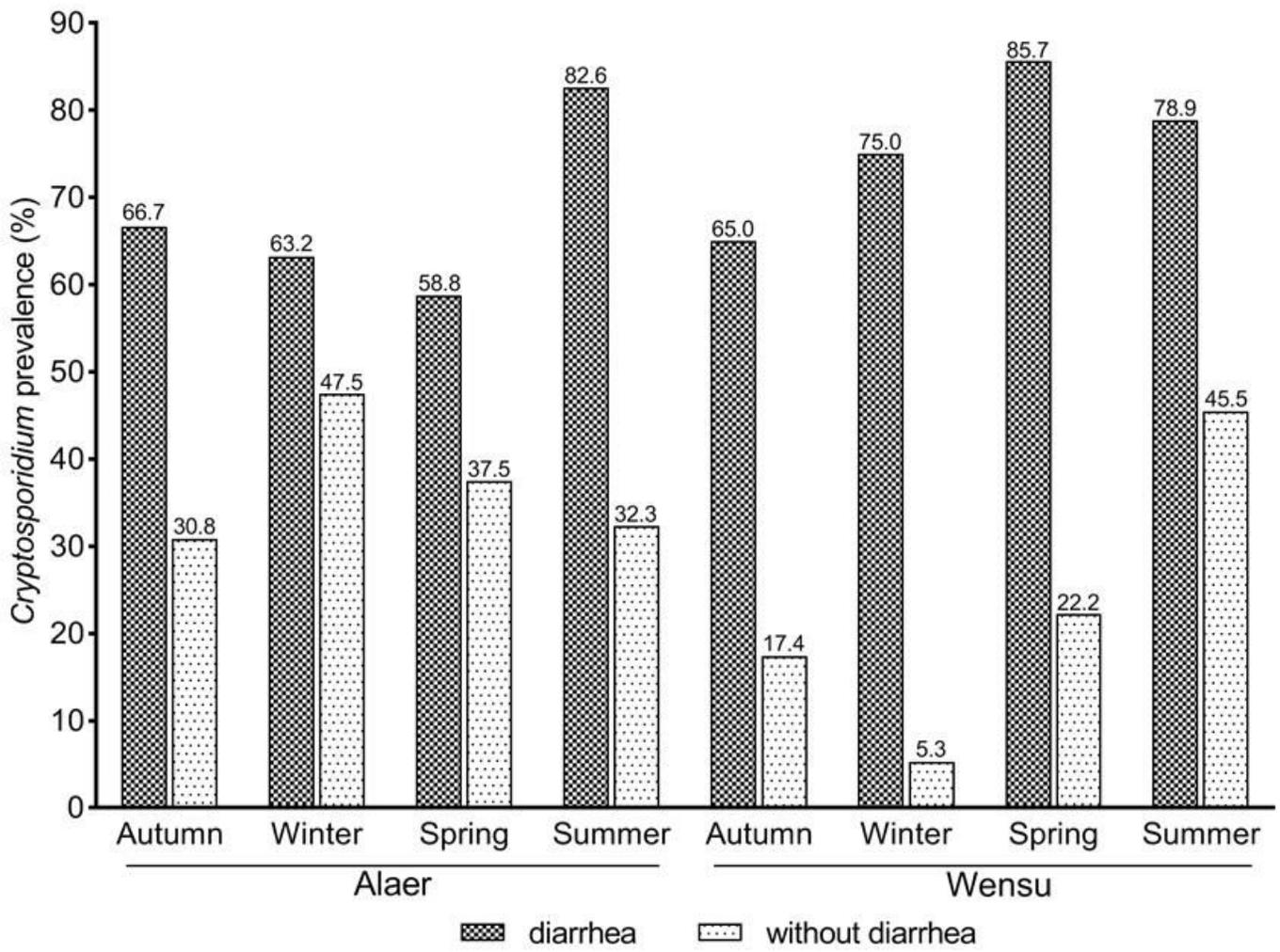


Figure 2

Seasonal variation in prevalence of *Cryptosporidium* in neonatal dairy calves by diarrhea status

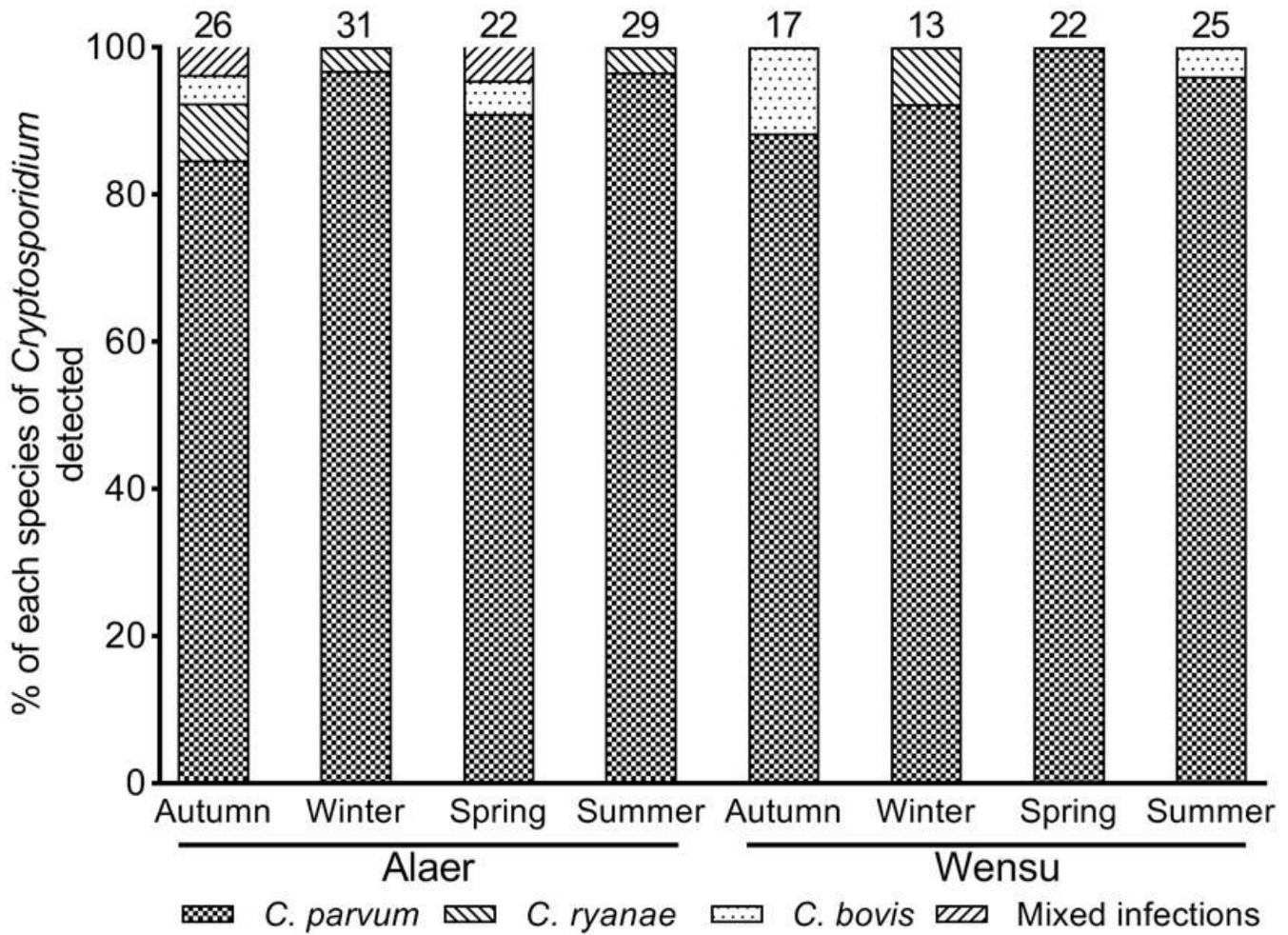


Figure 3

Seasonal distribution of *Cryptosporidium* spp. in neonatal dairy calves

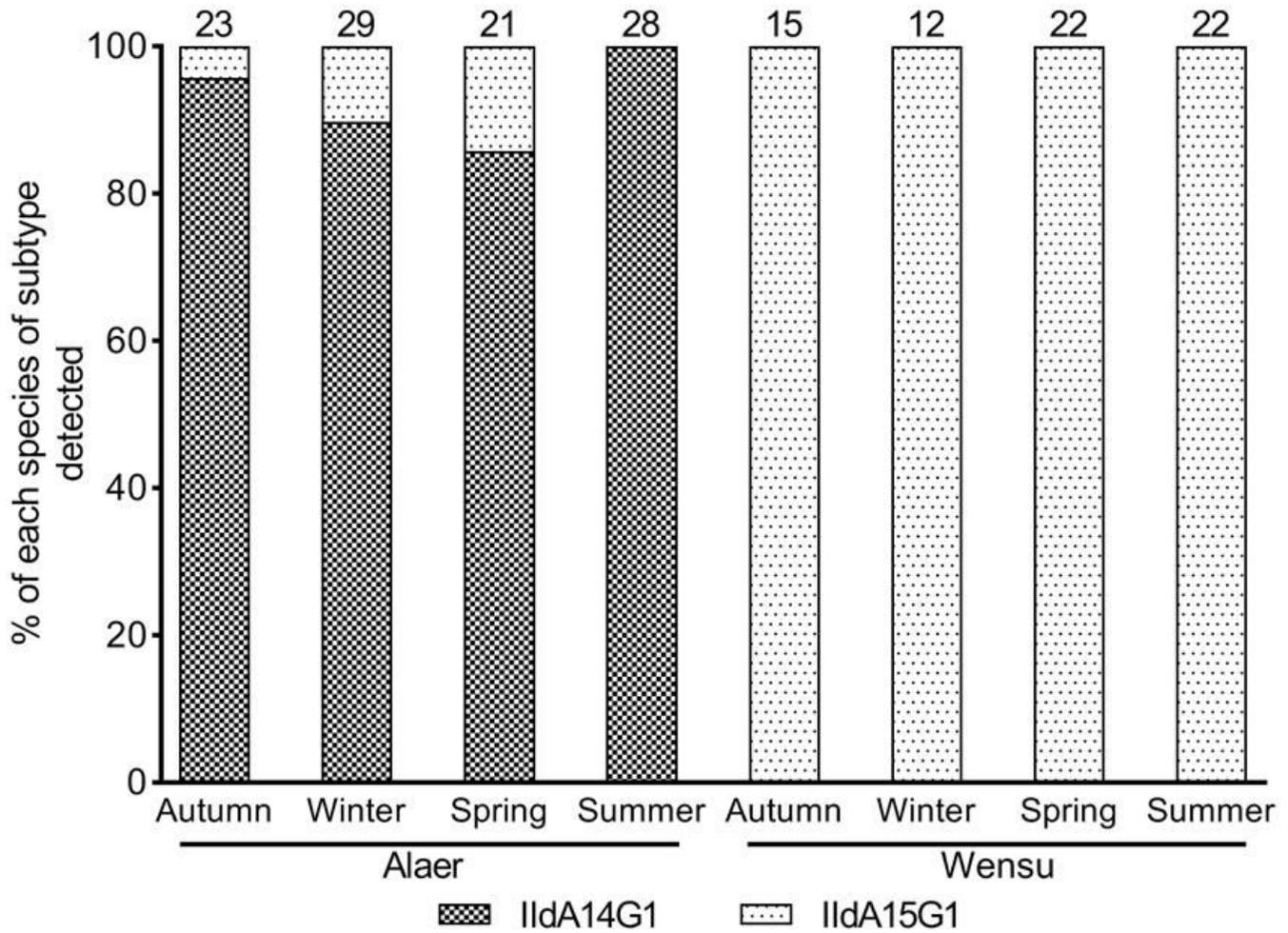


Figure 4

Seasonal distribution of *C. parvum* subtype in neonatal dairy calves

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