Cryptosporidiosis outbreak caused by Cryptosporidium parvum subtype IIdA20G1 in neonatal calves

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Abstract

Cryptosporidium parvum is a major zoonotic pathogen responsible for outbreaks of severe diarrhea in humans and calves. Almost all investigations of cryptosporidiosis outbreaks caused by C. parvum have focused on its IIa subtype family in industrialized nations. From December 2018 to April 2019, approximately 200 neonatal calves on a large cattle farm in Hebei Province, China presented watery diarrhea and over 40 died. To investigate the cause of the outbreak, 179 and 223 fecal specimens were collected during and after the diarrhea outbreak from the farm, including 40 and 56 from neonatal calves, respectively. Among them, 18 fecal specimens from ill calves during the peak of the outbreak were analyzed for four common enteric pathogens using enzymatic immunoassay (EIA), 75 additional specimens from neonatal calves were tested for rotavirus by EIA, and all specimens were analyzed for Cryptosporidium spp. using PCR and sequencing techniques. Of the initial 18 specimens from sick calves, ten were positive for C. parvum, five for rotavirus, and one for coronavirus. The overall prevalence of rotavirus in neonatal calves was 20.0% (15/75), with no significant differences during (21.6% or 8/37) and after (18.4% or 7/38) the outbreak. In contrast, the prevalence of C. parvum was significantly higher during the outbreak (60.0%, 24/40) than after the outbreak (30.4%, 17/56). C. parvum infection was associated with the occurrence of watery diarrhea in neonatal calves (odds ratio = 11.19), while no association was observed between C. bovis infection and diarrhea. All C. parvum isolates were identified as subtype IIdA20G1. Older animals were infected with C. bovis, C. ryanae, C. occultus, and C. andersoni. This is one of the few reports of outbreaks of severe diarrhea caused by C. parvum IId subtypes in calves. More attention should be directed toward preventing the dissemination of C. parvum in China.

Cryptosporidiosis outbreak caused by *Cryptosporidium parvum* subtype IIdA20G1inneonatal calves

Running title: Cryptosporidiosis outbreak in neonatal calves

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Summary

Cryptosporidium parvum is a major zoonotic pathogen responsible for outbreaks of severe diarrhea in humans and calves. Almost all investigations of cryptosporidiosis outbreaks caused by *C. parvum* have focused on its IIa subtype family in industrialized nations. From December 2018 to April 2019, approximately 200 neonatal calves on a large cattle farm in Hebei Province, China presented watery diarrhea and over 40 died. To investigate the cause of the outbreak, 179 and 223 fecal specimens were collected during and after the diarrhea outbreak from the farm, including 40 and 56 from neonatal calves, respectively. Among them, 18 fecal specimens from ill calves during the peak of the outbreak were analyzed for four common enteric pathogens using enzymatic immunoassay (EIA), 75 additional specimens from neonatal calves were tested for rotavirus by EIA, and all specimens were analyzed for *Cryptosporidium* spp. using PCR and sequencing techniques. Of the initial 18 specimens from sick calves, ten were positive for *C. parvum* , five for rotavirus, and one for coronavirus. The overall prevalence of rotavirus in neonatal calves was 20.0% (15/75), with no significant differences during (21.6% or 8/37) and after (18.4% or 7/38) the outbreak. In contrast, the prevalence of *C. parvum* was significantly higher during the outbreak (60.0%, 24/40) than after the outbreak (30.4%, 17/56). *C. parvum* infection was associated with the occurrence of watery diarrhea in neonatal calves (odds ratio = 11.19), while no association was observed between *C. bovis* infection and diarrhea. All *C. parvum* isolates were identified as subtype IIdA20G1. Older animals were infected with *C. bovis* , *C. ryanae* , *C. occultus* , and *C. andersoni* . This is one of the few reports of outbreaks of severe diarrhea caused by *C. parvum* IId subtypes in calves. More attention should be directed toward preventing the dissemination of *C. parvum* in China.

Keywords: *Cryptosporidium parvum* ; Cryptosporidiosis; IIdA20G1; Outbreak; Cattle; Diarrhea

1. Introduction

Neonatal calf diarrhea caused by intestinal pathogens including bacteria, viruses and parasites is a leading cause of morbidity and mortality in pre-weaned calves (Gomez & Weese, 2017). Rotavirus, coronavirus, *Escherichia coli* , and *Cryptosporidium* spp. are considered the most important causes for calf diarrhea (Meganck, Hoflack, & Opsomer, 2014). Synergistic infections of these pathogens were often identified during outbreaks of diarrhea in calves (Foster & Smith, 2009; Gomez & Weese, 2017). Among them, *Cryptosporidium* spp. and rotavirus are the most frequently observed pathogens in fecal specimens from neonatal calves worldwide (Mohamed, Mansour, El-Araby, Mor, & Goyal, 2017; Thomson et al., 2017). In addition, moderate-to-severe diarrhea caused by *Cryptosporidium* spp. is of the leading causes for morbidity and mortality in young children in developing countries (Kotloff et al., 2013).

Cryptosporidium spp. are especially common in dairy calves. The cumulative infection rate of *Cryptosporidium* spp. in pre-weaned dairy calves in the United States is 100.0% (Santin, Trout, & Fayer, 2008; Xiao & Herd, 1994). In China, the average infection rate of *Cryptosporidium* spp. in dairy calves with diarrhea was 45.8%, much higher than that in cattle overall (14.5%) (Wang, Zhao, Gong, & Zhang, 2017). Numerous cryptosporidiosis outbreaks have been reported in neonatal calves in the United States, European countries, Australia, India, and China, causing serious harms to the dairy industry (Brar et al., 2017; Cui et al., 2014; Izzo et al., 2011; Li et al., 2019; Ouakli et al., 2018; Randhawa, Randhawa, Zahid, Singla, & Juyal, 2012).

Among the over 40 established *Cryptosporidium* species (Feng, Ryan, & Xiao, 2018), *C. parvum* , *C. bovis* , *C. ryanae* and *C. andersoni* are commonly found in cattle (Ryan, Fayer, & Xiao, 2014). Among the latter, *C. parvum* is the only major pathogenic and zoonotic species (Thomson et al., 2017). The distribution of *Cryptosporidium* spp. in cattle in China, however, is very different from other countries, with *C. bovis* being the most common species in pre-weaned calves rather than *C. parvum* elsewhere (Feng & Xiao, 2017). In addition, subtyping of *C. parvum* based on sequence analysis of the 60 kDa glycoprotein (*gp60*) gene

indicates that calves in China are exclusively infected with IId subtypes (Feng & Xiao, 2017). Elsewhere, IIa subtypes are mostly identified in calves, while IId subtypes are usually seen in lambs and goat kids (Xiao, 2010).

Thus far, almost all investigations of cryptosporidiosis outbreaks in humans and calves caused by *C. parvum* have focused on its IIa subtype family in industrialized nations. The clinical importance of the IId subtype family of *C. parvum* is less clear. Two recent outbreaks, however, have been reported in dairy calves in China, responsible for substantial mortality (Cui et al., 2014; Li et al., 2019). They were caused by IIdA15G1 and IIdA19G1 subtypes, two dominant *C. parvum* subtypes in calves and other animals in China (Feng & Xiao, 2017).

Here, we report the results of an investigation of a cryptosporidiosis outbreak in neonatal calves on a large cattle farm in Hebei Province, China caused by another IId subtype of *C. parvum* . Approximately 20% of neonatal calves on the farm died of watery diarrhea during the outbreak period.

2. Materials and Methods

2.1. Animals and Specimens

The investigated farm is located in Hebei Province in northern China. There were ˜2,000 cattle altogether, including about 350 calves on the farm. Among them, the majority of animals were of the Holstein breed, together with fewer than 200 cattle of the Simmental breed and 40 adult cattle of the Japanese Wagyu breed. There were no vaccinations against rotaviruses, coronaviruses, and *Escherichia coli* prior to the occurrence of the outbreak of diarrhea.

From December 2018 to April 2019, an outbreak of diarrhea was noticed in neonatal calves on the study farm (Fig. 1), with watery diarrhea occurring in all ˜200 neonatal calves born during the period. Forty-one calves died of diarrhea at 10 – 30 days of age despite antibiotic therapy. They experienced severe diarrhea before death, with the excretion of watery feces. Most of them also had other clinical signs such as poor appetite, weight loss and dehydration (Table 1). To identify the cause of the diarrhea, 402 fecal specimens were collected from the farm during the outbreak in January 2019 (n = 173) and after the outbreak in June 2019 (n = 229). Among the specimens collected during the outbreak, 40 were from neonatal calves (1 - 4 weeks in age) and 133 were from older cattle (> 4 weeks in age). Similarly, 56 and 173 specimens collected after the outbreak were from neonatal calves and older cattle, respectively. Altogether, 213 specimens were from calves under 24 weeks and 189 from older cattle. The specimens from calves included formed feces from animals with no diarrhea (n = 110), loose feces from animals with moderate diarrhea (n = 64), and liquid feces from animals with watery diarrhea (n = 39). The older animals were mostly healthy.

Fecal specimens were collected directly from the rectum of each animal using disposable gloves into 50-mL centrifuge tubes. Approximately 1.0 g of each fecal specimen was transferred into a 1.5-mL tube and kept frozen at -80 , while the remaining fecal material was stored in 2.5% potassium dichromate at 4 .

2.2. Detection of Common Enteric Pathogens by Enzymatic Immunoassay (EIA)

To identify the possible etiological agent of calf diarrhea, 18 specimens from ill calves collected during the peak of the diarrhea outbreak were examined for four common enteric pathogens. For this, 200 mg frozen fecal material was tested for rotavirus, coronavirus, *Escherichia coli* , and *Cryptosporidium* spp. using the Pathasure Enteritis EIA kit (Biovet, Saint-Hyacinthe, Canada). In addition, rotavirus in another 75 fecal specimens collected during (n = 37) and after (n = 38) the diarrhea outbreak from 1 to 4-week-old calves were tested using the ProSpecT Rotavirus Kit (Thermo-Oxoid, Basingstoke, UK).

2.3. *Cryptosporidium* Genotyping and Subtyping

Fecal specimens preserved in potassium dichromate were washed three times with distilled water by centrifugation at 2500×g for 5 min. Genomic DNA was extracted from 0.2 g washed fecal material using the FastDNA SPIN Kit for Soil (MP Biomedicals, CA, USA). *Cryptosporidium* species were detected using polymerase chain reaction (PCR) and sequence analysis of the SSU rRNA gene. To identify the *C. parvum*

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subtype involved, a ˜850 bp fragment of the *gp60* gene was amplified using nested PCR (Feng, Li, Duan, & Xiao, 2009). Reagent-grade water was used as the negative control, whereas *C.serpentis* DNA was used as the positive control for the SSU rRNA PCR and *C. hominis* DNA as the positive control for the*gp60* PCR. All positive PCR products of the SSU rRNA and*gp60* genes were sequenced bi-directionally on an ABI 3730 Genetic Analyzer (Applied Biosystems, CA, USA). Nucleotide sequences generated were aligned together with reference sequences using ClustalX 2.1 (*http://www.clustal.org/*) for the identification of *Cryptosporidium* species and *C. parvum*subtypes.

2.4. Statistical Analysis

Data in this study were analyzed statistically using the software packages R version 3.6.2 (*https://www.r-project.org/*) and RStudio version 1.1.463 (*https://rstudio.com/*). The χ^2 test was used to compare differences in*Cryptosporidium* or rotavirus infection rates by age group or diarrhea status. Logistic regression was used to assess the association between putative risk factors and the occurrence of diarrhea, using data from 213 pre-weaned and post-weaned calves during and after the diarrhea outbreak. Initially, the strength of association was assessed using univariate analysis. Variables with P [?] 0.2 were thereafter used to build a multivariate model, with $P < 0.05$ being considered significant in the final model. Odds ratios (OR) with 95% confidence intervals (CI) were used to identify risk factors for diarrhea occurrence.

3. Results

3.1. Occurrence of Enteric Pathogens inIll Calves during the Outbreak

In the initial EIA analysis of major enteric pathogens in the 18 specimens from ill calves, 10 were positive for *Cryptosporidium*spp., five for rotavirus, one for coronavirus, and none for enteropathogenic *E. coli* . All five rotavirus-positive specimens were also positive for *Cryptosporidium* spp. (Table 2). These 18 specimens were further analyzed for *Cryptosporidium* spp. using the SSU rRNA-based PCR, leading to the identification of *C. parvum* in 10 of them, including 9 of the 10 EIA-positive specimens. All the *C. parvum* -positive specimens were successfully subtyped as IIdA20G1 by sequence analysis of the *gp60* gene.

3.2. Occurrence of Rotavirus inNeonatal (1-4-Week-Old) Calves

During the outbreak, 8/37 (21.6%) specimens from neonatal calves were positive for rotavirus. In contrast, 7/38 (18.4%) specimens from neonatal calves were positive after the outbreak (Fig. 2). Altogether, 15 of the 75 fecal specimens from neonatal calves of 1 - 4 weeks were positive for rotavirus by EIA. Among them, the infection rate was 21.1% (8/38) in calves with watery diarrhea, 0% (0/6) in calves with moderate diarrhea, and 22.6% (7/31) in calves without diarrhea ($\chi^2 = 1.66$, $df = 2$, $P = 0.437$).

3.3. Occurrence of *C. parvum* in Neonatal (1-4-Week-Old) Calves

During the outbreak, 27 of 40 (67.5%) fecal specimens collected from neonatal calves were positive for *Cryptosporidium* spp. by PCR, including *C. parvum* (n = 24) and *C. bovis* (n = 3). After the outbreak, 35 of 56 (62.5%) fecal specimens collected from neonatal calves were positive for *Cryptosporidium* spp., including *C. parvum* (n = 17), *C. bovis* (n = 17) and *C. ryanae* (n = 1) (Fig. 2). The infection rate of *C. parvum* during the outbreak (60.0%, 24/40) was significantly higher ($\chi^2 = 8.38$, $df = 1$, $P = 0.004$) than after the outbreak (30.4%, 17/56) (Fig. 2). Animals older than 4 weeks had no *C. parvum*infection during and after the outbreak. Instead, they were mostly infected with *C. bovis* (n = 56) and *C. ryanae* (n = 39), together with some occurrence of *C. occultus* (n = 6) and *C. andersoni* (n = 5) (Table 3). Therefore, during the outbreak, *C. parvum* was the dominant species in neonatal calves, while after the outbreak, *C. bovis* and *C. parvum* occurred at the same frequency (Table 3).

Altogether, 39/41 specimens positive for *C. parvum* were successfully subtyped at the *gp60* locus. The sequences obtained were all identical to the reference sequence KU248815 of the IIdA20G1 subtype from GenBank.

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In neonatal (1-4-week-old) calves, *Cryptosporidium* infection rates were 66.7% (26/39), 69.2% (9/13) and 61.4% (27/44) in animals with watery diarrhea, moderate diarrhea and no diarrhea, respectively. The differences among groups were not significant in χ^2 analysis ($\chi^2 = 0.40$, $df = 2$, $P = 0.820$) (Tables 4). By *Cryptosporidium* species, *C. parvum* infection rates were 61.5% (24/39), 38.5% (5/13), and 27.3% (12/44) in animals with watery diarrhea, moderate diarrhea and no diarrhea, respectively. The difference in *C. parvum* infection rates between animals with watery diarrhea and those with no diarrhea was significant ($\chi^2 = 9.88$, $df = 1$, $P = 0.002$) (Tables 4). In contrast, the infection rate of *C. bovis* in animals with no diarrhea (31.8%, 14/44) was significantly higher than those with watery diarrhea (5.1%, 2/39) ($\chi^2 = 9.46$, $df = 1$, $P = 0.002$). Infection rates of *C. ryanae* were similar among the three groups ($\chi^2 = 1.19$, $df = 2$, $P = 0.550$) (Tables 4).

No *C. parvum* infection nor watery diarrhea was observed in calves older than 4 weeks. In this age group, the infection rates of *Cryptosporidium* spp. were 58.8% (30/51) and 60.6% (40/66) in animals with moderate diarrhea and those without diarrhea, respectively ($\chi^2 = 0.04$, $df = 1$, $P = 0.845$) (Tables 4). By *Cryptosporidium* species, the infection rates of *C. bovis* ($\chi^2 = 0.078$, $df = 1$, $P = 0.780$) or *C. ryanae* ($\chi^2 = 0.005$, $df = 1$, $P = 0.941$) were not significantly different between the two groups (Tables 4).

3.5. Association of *C. parvum* Infection with Diarrhea Occurrence as Indicated by Univariate and Multivariate Analyses

In the univariate analysis of data, *C. parvum* -positive calves had a significantly higher occurrence of diarrhea (odds ratio = 3.20, $P = 0.002$, Table 5), especially watery diarrhea (odds ratio = 14.78, $P = 0.000$). There was no correlation between *Cryptosporidium* infection and occurrence of moderate diarrhea. In addition, neonatal calves were more likely to have watery diarrhea (odds ratio = 34.12, $P = 0.001$, Table 5) and moderate diarrhea (odds ratio = 3.09, $P = 0.000$, Table 5) than older animals. Further analysis using the multivariate model showed that *C. parvum* infection was the only species associated with the occurrence of diarrhea (odds ratio = 2.98, $P = 0.016$, Table 5), especially watery diarrhea (odds ratio = 11.19, $P = 0.000$, Table 5). In addition, age was an important risk factor associated with the occurrence of watery diarrhea (odds ratio = 40.00, $P = 0.001$, Table 5) and moderate diarrhea (odds ratio = 2.91, $P = 0.001$, Table 5) in these animals.

4. Discussion

Cryptosporidiosis was identified as the cause for outbreak of severe diarrhea in neonatal calves in this study. The initial EIA analysis of 18 fecal specimens from ill calves at the peak of the outbreak showed that *C. parvum* and rotavirus were possible etiological agents rather than *E. coli* and coronavirus. The role of rotavirus was excluded in comparative detection of the pathogen in specimens from neonatal calves during and after the outbreak. There was no significant difference in infection rates of rotavirus during and after the outbreak, and rotavirus infection was not significantly associated with the occurrence of moderate or watery diarrhea in these animals. In contrast, the infection rate of *C. parvum* in neonatal calves was higher during the outbreak than after it, and the infection was significantly associated with the occurrence of watery diarrhea in these animals. In addition, all *C. parvum* infections were observed in calves of 1-4 weeks in concurrence with watery diarrhea, reinforcing the role of *C. parvum* in the occurrence of the outbreak.

The present study constitutes one of several lines of evidence for the pathogenicity of the IId subtype family of *C. parvum* . The pathogenicity of IIa subtypes of *C. parvum* has been well established, as cryptosporidiosis outbreaks have been reported in neonatal calves in many industrialized countries where they are prevalent (Holzhausen, Lendner, Gohring, Steinhofel, & Daugschies, 2019; Izzo et al., 2011; Niine, Dorbek-Kolin, Lassen, & Orro, 2018). The pathogenicity of IId subtypes of *C. parvum* , however, has only been indicated in molecular epidemiological investigations of *Cryptosporidium* spp. in China, which have shown a dominance of the IId subtype family and association of it with the occurrence of diarrhea in pre-weaned calves (Cai et al., 2017; Wu et al., 2020). Recently, the IId subtype family was implicated as the cause for two outbreaks of cryptosporidiosis in calves in China, including one by the IIdA15G1 subtype in Ningxia, leading to the death of 356 calves (Cui et al., 2014), and one by the subtype IIdA19G1 in Jiangsu, leading to the death

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of 360 calves (Li et al., 2019). In the present study, the IIdA20G1 subtype was identified as the cause of cryptosporidiosis outbreak in calves in China.

IIdA20G1 appears to be an emerging *C. parvum* subtype. In China, it has been recently reported in cattle in Heilongjiang, Hebei and Xinjiang and deer in Hubei (Tao et al., 2018; Wu et al., 2020; Xie et al., 2019; Zhang et al., 2020). Elsewhere, it has been reported in livestock and humans in Egypt, Kuwait, Qatar, Jordan, Turkey, Iran, Italy and Romania (Amer et al., 2010; Amer, Zidan, Adamu, et al., 2013; Amer, Zidan, Feng, et al., 2013; Boughattas et al., 2017; Diaz et al., 2018; Helmy, Krucken, Nockler, von Samson-Himmelstjerna, & Zessin, 2013; Hijjawi, Mukbel, Yang, & Ryan, 2016; Ibrahim, Abdel-Ghany, Abdel-Latef, Abdel-Aziz, & Aboelhadid, 2016; Imre et al., 2013; Kiani et al., 2017; Mahfouz, Mira, & Amer, 2014; Majeed et al., 2018; Naguib et al., 2018; Taylan-Ozkan et al., 2016). Most of these countries are located in the Middle East. Nevertheless, this is the first report of cryptosporidiosis outbreak caused by *C. parvum*IIdA20G1 subtype.

As with the *C. parvum* IIa subtype family, young age is associated with diarrhea caused by the IIdA20G1 subtype. In present study, *C. parvum* all appeared in neonatal calves under four weeks of age. This is likely due to the immature immune system in these animals, which also makes them prone to the occurrence of clinical signs after *C. parvum* infection (Santin et al., 2008). In the present study, neonatal calves had high occurrence of *C. bovis* after the outbreak. This, however, was not associated with the occurrence of diarrhea. Previously, in a few reports, *C. bovis*and *C. ryanae* had been associated with the occurrence of moderate diarrhea (Lee et al., 2016; Li et al., 2019; Silverlas & Blanco-Penedo, 2013; Silverlas, Bosaeus-Reineck, Naslund, & Bjorkman, 2013).

The role of rotavirus in causing the diarrhea outbreak appears to be minor. Although rotavirus was commonly found in neonatal calves in the present study, there was no significant difference in its infection rates during and after the outbreak. In statistical analyses, its occurrence was not correlated with the occurrence of diarrhea. Nevertheless, rotavirus is a well-known cause of diarrhea in 1-2-week-old calves (Cho, Han, Wang, Cooper, & Yoon, 2013). It has been frequently identified in co-infections with *C. parvum* , contributing to the occurrence of diarrhea in infected calves (Brar et al., 2017; Izzo et al., 2011). Thus, the concurrence of rotavirus in neonatal calves could have worsen the deleterious impact of *C. parvum* infection in the investigated outbreak of diarrhea.

5. Conclusion

This represents the first report of an outbreak of bovine cryptosporidiosis caused by the *C. parvum* subtype IIdA20G1, which appears to be an emerging pathogen in China and the Middle East. Significant morbidity and mortality have been associated with this outbreak, possibly as a result of concurrence of rotavirus on the farm. Control measures should be implemented to effectively reduce the transmission of *C. parvum* , which still occurs on only a portion of the dairy farms in China. Further studies are required to examine the clinical significance of rotavirus and *C. parvum* coinfections in neonatal calves.

Acknowledgements

Conflict of Interest Statement

The authors declare that they have no competing interests.

Ethics Statement

Fecal specimens from cattle in this study were collected with the permission of the farm managers as part of the technical assistance in the investigation of an outbreak of diarrhea. The animals were handled in accordance with the Animal Ethics Procedures and Guidelines of the People's Republic of China. The research protocol was reviewed and approved by the Ethics Committee of the South China Agricultural University.

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Legends to Figures

Fig. 1 Calves with diarrhea on a cattle farm in Hebei, China

Fig. 2 Infection rates of rotavirus and *Cryptosporidium* spp. in 1-4-week-old calves during and after the outbreak of watery diarrhea in Hebei, China

Table 1. Number of births and deaths of calves during a diarrhea outbreak provided by the farm owner

Time	Number of newborn calves	No. of dead calves	No. of deaths due to diarrhea
Sept. 2018	23	2	2
Oct. 2018	19	6	2
Nov. 2018	43	11	3
Dec. 2018*	27	12	9
Jan. 2019*[a]	71	7	7
Feb. 2019*	45	7	6
Mar. 2019*	41	13	13
Apr. 2019*	16	11	6
May 2019	20	2	2
Jun. 2019[b]	36	6	3
Jul. 2019	45	2	1
Total	386	79	54

*The time period during which the outbreak of diarrhea was noticed.

[a]Sampling time during the diarrhea outbreak.

[b]Sampling time after the diarrhea outbreak.

Table 2. Detection of common enteric pathogens in fecal specimens from 18 seriously ill calves during a diarrhea outbreak on a cattle farm in Hebei, China using an enzymatic immunoassay (EIA) test and PCR

Specimen	Breed	Age (weeks)	EIA test	EIA test	EIA test	EIA test	*Cryptospor*
			Rotavirus	**Coronavirus**	Escherichia coli	Cryptosporidium	**Positivity**
12359	Holstein	1	-	-	-	+	+
12406	Holstein	1	-	-	-	-	+
12394	Holstein	2	+	-	-	+	+
12407	Holstein	2	-	-	-	+	+
12401	Holstein	2	-	-	-	-	-
12403	Holstein	2	-	-	-	-	-
12404	Holstein	2	-	-	-	-	-
12385	Holstein	1	-	+	-	-	-
12386	Holstein	1	-	-	-	-	-
12532	Simmental	1	-	-	-	-	-
12533	Holstein	1	-	-	-	+	+
12537	Holstein	1	-	-	-	+	-
12539	Simmental	1	-	-	-	-	+
12531	Simmental	1	+	-	-	+	+
12534	Simmental	1	+	-	-	+	+
12536	Simmental	1	+	-	-	+	+
12540	Simmental	1	+	-	-	+	+

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Specimen	Breed	Age (weeks)	EIA test	EIA test	EIA test	EIA test	*Cryptospor*
12541	Holstein	1	-	-	-	-	-

+, positive; -, negative.

Table 3. Prevalence of *Cryptosporidium* infection in cattle during and after a diarrhea outbreak on a farm in Hebei, China

Time	Age group	No. positive/No. sampled (%)	*Cryptosporidium* spp.	*C*
			C. parvum	C.
During the outbreak	Neonatal calves (1 – 4 weeks)	27/40 (67.5)	24	3
	Older cattle* (> 4 weeks)	78/133 (58.6)	0	39
After the outbreak	Neonatal calves (1 – 4 weeks)	35/56 (62.5)	17	17
	Older cattle* (> 4 weeks)	28/173 (16.2)	0	17
Total	**-**	**168/402 (41.8)**	**41**	**76**

*Including calves of 5 – 24 weeks, heifers (7 – 12 months) and adults (> 12 months)

Table 4. Associations between *Cryptosporidium* or rotavirus infection and occurrence of moderate or watery diarrhea in neonatal and older calves during and after a diarrhea outbreak on a farm in Hebei, China, as indicated by results of χ^2 analysis

Factors	Factors	No diarrhea	No diarrhea
		n	χ^2
Neonatal calves (1 – 4 weeks)	**Neonatal calves (1 – 4 weeks)**	**Neonatal calves (1 – 4 weeks)**	**Neonatal ca**
	Sample size	44	-
	Cryptosporidium spp.	27	Reference
	C. parvum	12	Reference
	C. bovis	14	Reference
	C. ryanae	1	Reference
	Rotavirus	7	Reference
Older cattle (5 – 24 weeks) Sample size	**Older cattle (5 – 24 weeks)**	**Older cattle (5 – 24 weeks)**	**Older cattle**
	Sample size	66	-
	Cryptosporidium spp.	40	Reference
	C. bovis	21	Reference
	C. ryanae	19	Reference

Table 5. Association between *Cryptosporidium* infection and occurrence of diarrhea in neonatal and older calves during and after a diarrhea outbreak on a farm in Hebei, China, as indicated by results of univariate and multivariate analyses

Diarrhea status	Factor	Univariate model	Univariate model	Univariate model	Mu
		OR	**95% CI**	P	**OR**
Watery diarrhea	Cryptosporidium	1.28	0.63 - 2.74	0.505	NA
	C. parvum	14.78	6.66 - 34.33	0.000	11.
	C. bovis	0.12	0.02 - 0.4	0.004	0.29
	No *Cryptosporidium*	1	Reference		1

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Diarrhea status	Factor	Univariate model	Univariate model	Univariate model	Mu
	Age				
	Neonatal (1 - 4 weeks)	34.12	$4.32 - 269.51$	0.001	40.0
	Other ages (5 - 24 weeks)	1	Reference		1
Moderate diarrhea	Cryptosporidium	0.94	0.52 - 1.73	0.839	NA
	C. parvum	0.27	0.09 - 0.66	0.009	0.82
	C. bovis	1.28	0.66 - 2.44	0.461	NA
	C. ryanae	1.97	$0.93 - 4.15$	0.074	1.0
	No *Cryptosporidium*	1	Reference		1
	Age				
	Neonatal (1 - 4 weeks)	3.09	$1.93 - 5.16$	0.000	2.9
	Other ages (5 - 24 weeks)	1	Reference		1
Any diarrhea	Cryptosporidium	1.1	0.63 - 1.91	0.741	NA
	C. parvum	3.2	1.56 - 6.91	0.002	2.98
	C. bovis	0.55	0.29 - 1.02	0.060	0.72
	C. ryanae	0.77	0.36 - 1.59	0.477	NA
	No *Cryptosporidium*	1	Reference		1
	Age				
	Neonatal (1 - 4 weeks)	0.74	0.50 - 1.09	0.125	1.04
	Other ages (5 - 24 weeks)	1	Reference		1