Molecular Characterization of *Cryptosporidium* Isolates from Calves in Ishikari District, Hokkaido, Japan

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ABSTRACT.   Cattle are major hosts of *Cryptosporidium*. Cryptosporidiosis in neonatal calves is associated with retarded growth, weight loss and calf mortality, and zoonotic infections in humans. Fecal samples were collected from calves in Ishikari District, Hokkaido, Japan and examined by PCR and sequence analyses. Among the 107 fecal samples collected in May and June 2012, 25 (23%) were positive for *Cryptosporidium*, including 8 samples (7%) having *C. parvum*, 10 (9%) having *C. bovis* and 7 (7%) having *C. ryanae*. This is first time *C. ryanae* has been detected in Hokkaido. Furthermore, it is the first detection of *C. ryanae* from pre-weaned calves in Japan. Microscopic observation with the flotation method is powerful and traditional tool for screening for *Cryptosporidium* species, but it sometimes leads to low detection of *Cryptosporidium* with low oocyst shedding intensity. If calves with or without diarrhea are examined using the molecular diagnostic tools, *C. bovis* and *C. ryanae* might be detected in other areas of Japan including Hokkaido. Here, the zoonotic species, *C. parvum*, was also observed. Therefore, calves can be potential sources of cryptosporidial infections for humans and other animals. The detection of *C. parvum* was statistically correlated with diarrhea in calves.
KEY WORDS: cattle, *Cryptosporidium bovis*, *Cryptosporidium parvum*, *Cryptosporidium ryanae*, Hokkaido.

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Cattle are major hosts of *Cryptosporidium* spp. [23]. They are primarily infected with four *Cryptosporidium* species, namely *Cryptosporidium parvum*, *Cryptosporidium bovis*, *Cryptosoridium ryanae* and *Cryptosporidium andersoni*. *C. parvum* has been reported to be the zoonotic species and the major cause of diarrhea in calves [27, 32]. Oocyst shedding from cattle has been reported to be a contamination source in human cryptosporidiosis outbreaks [21]. Additionally, cryptosporidiosis is associated with neonatal diarrhea syndrome in calves, leading to direct and indirect economic losses [4]. Therefore, the studies on species distribution and transmission routes of *Cryptosporidium* spp. are important in public health and food production.

In Japan, there are very few studies on the epidemiology of *Cryptosporidium* in cattle. Additionally, most previous epidemiological studies of cryptosporidiosis in cattle were performed by only microscopic observations of oocyst shedding or microscopic observation used for initial screening of molecular epidemiology. However, these traditional screening methods might lead to low detection of *Cryptosporidium* species with low oocyst shedding intensity. There are dif-

fering results according to the type and age of host animals, area of study, detection methods and season, and because of this variation, more epidemiological studies are needed.

To assess the potential public health and production importance, *Cryptosporidium* infection in pre- and post-weaned calves in Ishikari District, Hokkaido, Japan was examined. Our study is an initial *Cryptosporidium* screening of Ishikari District. Specifically, we analyzed the relationships between the occurrence of diarrhea, genotype/subtype and age using molecular diagnostic tools.

MATERIALS AND METHODS

Collection of samples: Fecal samples were collected from 27 farms located in Ishikari District, Hokkaido, Japan. We collected samples from 86 dairy, 17 Japanese black and 4 crossbred calves (Bos taurus) that ranged in age from 2 to 120 days. A total of 107 samples were randomly collected at each farm (1–8 calves per farm) in May and June, 2012. Specimens were stored at 4°C prior to DNA extraction and microscopic examination.

Sample analysis: DNA was extracted using easy beads (AMR, Gifu, Japan) and QIAamp® DNAstool minikit (Qiagen, Hilden, Germany). For DNA extraction, 0.3–0.4 g of fecal specimen was used. *Cryptosporidium* spp. were detected and subtyped by nested polymerase chain reaction (PCR) amplification targeting a ~830 bp and ~850 bp fragments of the small subunit (SSU) rRNA and 60 kDa glycoprotein (GP60) genes, respectively, as described previously [8].

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Table 1.   Infection rates of *Cryptosporidium* in fecal samples from calves

	C. parvum	*C. bovis*	*C. ryanae*	sum
pre weaned 1–8 weeks (n=79)	8	5	4	17
post weaned 3–12 months (n=28)	0	5	3	8
sum	8	10	7	25

Table 2.   Correlation between diarrhea and detected genotype

	C. parvum	*C. bovis*	*C. ryanae*	ND
diarrhea	5	2	2	16
normal	3	8	5	64

ND: not detected.
C. parvum was statistically correlated with diarrhea (*P*<0.05).

GP60 gene was used to subtype *C. parvum*. For each sample, PCR detection was repeated independently at least two times. All secondary PCR products determined to be *Cryptosporidium* positive were sequenced in both directions using an ABI 3130 Genetic Analyzer (Applied Biosystems Japan, Tokyo, Japan) with the secondary primers and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Japan). The sequences obtained were aligned using ClustalX 2 [15], edited manually in BioEdit 7.0.5.3 [9] and compared to *C. parvum*, *C. bovis* and *C. ryanae* reference sequences in GenBank. Unique 18S rRNA and GP60 sequences acquired in this study were deposited in the GenBank database under accession numbers; AB746195– AB746198.

The sugar flotation method and microscopic examination were also performed for *Cryptosporidium* positive fecal samples as described elsewhere. In brief, 1 g of feces was suspended in sucrose (1.2 g/m*l*), and oocysts were floated to slide grass.

Statistical analyses: Data were compared using a Fisher's exact test with the use of R 2.13.0 software. Differences at *P*<0.05 were considered significant.

RESULTS

Genotype and analysis: Of the 107 fecal samples, 25 samples (23%) were positive for *Cryptosporidium* infection based on PCR-sequence analysis. Three *Cryptosporidium* species (*C. parvum*, *C. bovis and C. ryanae*) were detected; 8 (7%), 10 (9%) and 7 (7%) samples had *C. parvum*, *C. bovis and C. ryanae*, respectively. The percentage of each genotype of *Cryptosporidium* represented among the 25 *Cryptosporidium* positive specimens in the two age categories (pre-weaned; 1–8 weeks and post-weaned; 3–12 months) (Table 1). The infection rate of pre-weaned calves was 21.5%; 10%, 6% and 5% of them had *C. parvum*, *C. bovis* and *C. ryanae,* respectively. The infection rate of post-weaned calves was 29%; 0%, 18% and 11% of the calves were infected with *C. parvum*, *C. bovis* and *C. ryanae*, respectively. Fragments of the small subunit (SSU) rRNA nucleotide sequences acquired in this study were deposited in the GenBank database under accession numbers; AB746195- AB746197. For *C. parvum* (AB746195), nucleotide sequences of the SSU rRNA gene derived from our fecal samples were identical

to those of HQ009805 from dairy cattle from China. *C. bovis* (AB746197) was identical to JX515546 from dairy calves, China, and *C. ryanae* (AB746196) was identical to HQ179574 from dairy calves, China. Therefore, we conclude that *C. parvum*, *C. bovis* and *C. ryanae* detected here were not geographically unique. The sugar flotation method and microscopic examination were also performed for all 25 *Cryptosporidium* PCR-positive specimens. All *C. bovis* and *C. ryanae* PCR-positive specimens were negative by sugar flotation method. The *C. parvum* PCR-positive specimens were positive by sugar flotation method, except for only one specimen.

Correlation between diarrhea and detected genotype: We detected *C. parvum*, *C. bovis* and *C. ryanae* were detected in calves, however, only *C. parvum* was statistically correlated with diarrhea (*P*<0.05) (Table 2). Among diarrheal *Cryptosporidium* positive samples, *C. parvum* was identified in 5 cases, but some calves with diarrhea were not infected *C. parvum*.

Relationship between Cryptosporidium infection and animal age: The percentages of each *Cryptosporidium* genotype represented among the 25 *Cryptosporidium*-positive specimens in the 2 age categories are presented in Table 1. There were no significant correlations between *Cryptosporidium* infection and animal age. In post-weaned calves, the majority of infections were *C. bovis* and *C. ryanae*. *C. bovis* and *C. ryanae* were found in pre-weaned and post-weaned calves. The percentages of *C. parvum*, *C. bovis* and *C. ryanae* detected from pre-weaned calves were 47%, 29% and 24%, respectively, whereas those from post-weaned calves were 0%, 63% and 38%, respectively.

Subtyping of C. parvum by GP60 gene: Sequencing of GP60 gene PCR products was successful in all *C. parvum* isolates. DNA sequencing indicated that all GP60 nucleotide sequences were identical to each other and had a 100% similarity with *C. parvum* subtype IIaA15G2R1. GP60 nucleotide sequences acquired in this study were deposited in the GenBank database under accession numbers; AB746198.

DISCUSSION

Genotype analysis: Of the 107 fecal samples, 25 samples (23%) were positive for *Cryptosporidium* infection based on

PCR-sequence analyses. In Kenya, 20% of dairy cattle were PCR-positive for *Cryptosporidium* [12]. In China, 18.82% positive samples were identified from dairy cattle [3]. In India, 17.65–86.67% of calves were *Cryptosporidium* positive [29]. Again for calves, 25%, 21.5% and 10.7% were *Cryptosporidium* positive in Romania, China and Brazil, respectively [10, 18, 30]. Therefore, our results were consistent with those epidemiologic data in the world. Furthermore, in this study, we collected 1–8 samples per farm. Those from more than half of farms were *Cryptosporidium* positive. *Cryptosporidium* positive farms were widely distributed in Ishikari District.

Here, *Cryptosporidium* species were detected; 8 (7%), 10 (9%) and 7 (7%) samples had *C. parvum*, *C. bovis* and *C. ryanae*, respectively. In Hyogo Prefecture, 93% of calves were reported to pass *C. parvum* oocysts [28]. Oocysts of this species have previously been isolated from cattle in many areas in Japan [1, 2, 13, 22, 33]. Here, the zoonotic species, *C. parvum*, was observed. Therefore, calves can be potential sources of cryptosporidial infections for humans and animals in Hokkaido.

C. bovis has been identified previously as *Cryptosporidium* genotype bovine B [7], and *C. ryanae* has been identified previously as the *Cryptosporidium* deer-like genotype [6]. These species have been detected all over the world [8]. It is very difficult to distinguish *C. bovis* and *C. ryanae* from *C. parvum* morphologically, and molecular analysis is needed for the differentiation of three intestinal *Cryptosporidium* spp. in cattle. For this reason, in Japan, these species have been described in only three studies. In Japan, *C. ryanae* was first detected from adult dairy cattle in Miyagi Prefecture [2], and *C. bovis* was first detected from pre-weaned calves with diarrhea in Hokkaido [13]. These studies showed only a single case infected with these species. Subsequently, 15 samples of *C. bovis*, 6 of *C. ryanae* and 2 of mixed infection were detected from post-weaned and adult beef cattle in Miyagi Prefecture [19]. This is the first description of *C. ryanae* in Hokkaido. Furthermore, this is first detection of *C. ryanae* from pre-weaned calves in Japan. All *C. bovis* and *C. ryanae* PCR-positive specimens were negative by sugar flotation method. Therefore, the low detection frequency of *C. bovis* and *C. ryanae* in Japan might have been the result of the wide use of microscopy in the previous studies. *C. bovis* and *C. ryanae* in younger calves might be concealed by an overwhelming *C. parvum* infection [8]. Using molecular diagnostic tools and examination of normal and calves with diarrhea, *C. bovis* and *C. ryanae* might be detected in other areas of Japan including Hokkaido.

C. andersoni is also a major species detected in cattle, especially post-weaned or adults [24]. There are several studies on *C. andersoni* detected in cattle in Japan [2, 11, 14, 16, 17, 20, 26]. Here, we did not detect this species, perhaps because we only sampled from calves.

Correlation between diarrhea and detected genotype: *C. parvum*, *C. bovis* and *C. ryanae* were detected along with the occurrence of diarrhea, but only *C. parvum* was statistically correlated with diarrhea ($P<0.05$) (Table 2). Not all diarrheal symptoms were caused by *Cryptosporidium*, but cattle with diarrhea are associated with *C. parvum*. There were some diarrhea samples that were *C. bovis* or *C. ryanae* positive. In a previous study, none of the calves infected with *C. ryanae* and *C. bovis* had any signs of disease [6, 7]. Therefore, there is a possibility that other pathogens or other environmental factors were related to diarrhea calves not infected *C. parvum*. However, our data are not sufficient to address this question. It is noteworthy that one calf which was *C. parvum*-positive by PCR did not have diarrhea and was negative by sugar flotation method. Although these results may come from recovering calves, these calves still discharge viable oocysts during their recovery. Therefore, asymptomatic infection of *C. parvum* is probably results in transmission to other calves.

Relationship between Cryptosporidium infection and animal age: In pre-weaned calves, 47% versus 29% and 24% *Cryptosporidium*-positive calves were infected with *C. parvum* versus *C. bovis* and *C. ryanae*. In United States, 85% versus 9% and 5% of *Cryptosporidium*-positive dairy calves, which were 2 months of age and younger, were infected with *C. parvum* versus *C. bovis* and *C. ryanae* [25]. When the percentage of each species/genotypes of *Cryptosporidium* is represented, *C. parvum* constituted 97% of the species in pre-weaned calves [24]. Therefore, percentages of *C. bovis* and *C. ryanae* positive pre-weaned calves were higher than those of other countries. Our results are consistent with the report that *C. parvum* was most prevalent in pre-weaned calves [5].

Subtyping of C. parvum by GP60 gene: DNA sequencing showed that all *C. parvum* positive specimens had the *C. parvum* subtype IIaA15G2R1. This is the common subtype found in humans and cattle in many countries [24]. It is noteworthy that the obtained sequence was identical to that of *C. parvum* calf genotype reported from Kobe, Hokkaido and Gifu in Japan [1, 31].

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