NOTE *Parasitology*

Molecular characterization of *Cryptosporidium parvum* detected in Japanese black and Holstein calves in Iwate Prefecture and Tanegashima Island, Kagoshima Prefecture, Japan

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ABSTRACT. *Cryptosporidium* oocysts were found in 43 out of 77 calves from two farms in Iwate Prefecture and nine farms on Tanegashima Island, Kagoshima Prefecture, Japan. The DNA fragments of 18S ribosomal RNA (18S rRNA) gene were amplified by a nested PCR from 43 oocyst-positive as well as one oocyst-negative samples. All of them were precisely identified as *C. parvum* by analyzing the nucleotide sequences of the 18S rRNA gene. *C. parvum* oocyst-positive calves ranged in age from 6 to 13 days old and significantly have watery diarrhea ($P<0.05$). Sequences of the gene encoding the 60-kDa glycoprotein (*GP60*) in 43 *Cryptosporidium* oocyst-positive samples were identical to that of the zoonotic IIaA15G2R1 subtype. We therefore suggest that calves could be potential sources of *C. parvum* infections in humans.
KEY WORDS: calf, *Cryptosporidium parvum*, GP60, Japan, subtyping

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Cryptosporidium spp. are protozoan parasites belonging to the phylum Apicomplexa that parasitize the gastrointestinal tract of vertebrates [6, 8, 10]. Infections of *Cryptosporidium parvum*, *C. bovis*, *C. ryanae* and *C. andersoni* have been reported in cattle [5, 8, 9, 13]. The zoonotic subtype *C. parvum* [13] causes severe watery diarrhea in calves [3]. Although *C. parvum* has traditionally been diagnosed by the size and morphology of oocysts detected in fecal samples, the oocysts of *C. parvum* cannot be discriminated from those of *C. bovis* and *C. ryanae*, which are most likely asymptomatic [10]. It is therefore important to accurately distinguish these species from *C. parvum*.

The nucleotide sequences of the 18S ribosomal RNA (18S rRNA) gene have previously been employed for species determination of *Cryptosporidium* spp. [5]. Moreover, intraspecific subtyping of *C. parvum* has been conducted using the sequences of the gene encoding the 60-kDa glycoprotein (*GP60*) [1, 2, 7, 12, 13], because zoonotic subtypes can be identified by using the *GP60* gene. IIa, IId and IIl subtype families have been detected in calves worldwide, with the zoonotic subtype IIaA15G2R1 of the IIa subtype family most frequently reported [13]. Previous studies on *C. parvum GP60* sequences from Hokkaido [5], Gifu [1], Hyogo [9] and Okinawa [3] Prefectures, Japan, indicated that IIaA15G2R1 was the only subtype distributed in these regions. However, the number of samples and locations analyzed in those studies were insufficient to determine the genetic variation of *C. parvum* throughout Japan. Therefore, this study aimed to characterize the fecal conditions of *C. parvum* infections, as well as to perform molecular characterization of the species from Iwate Prefecture and Tanegashima Island, Kagoshima Prefecture, based on 18S rRNA and *GP60* genes, and to compare these results with those of previous studies.

Fecal samples of 46 Holstein calves (under 2 weeks old) were collected from two farms in Iwate Prefecture between March 2013 and September 2014. Fecal samples of 30 Japanese black calves (under 3 months old) were collected from eight farms, and that of one Holstein calf (20 days old) was also collected from a farm in Tanegashima Island, Kagoshima Prefecture between June 2013 and July 2014 (Table 1). The conditions of the fecal samples were recorded, and they were stored at 4°C in the laboratory. The fecal conditions were categorized into watery feces and other fecal conditions. Causation between fecal conditions and *C. parvum* infection was evaluated using Fisher's exact test. The centrifuge sucrose flotation method and microscopic examination were performed to detect *Cryptosporidium* oocysts. After three freeze-thaw cycles at −80°C for 15 min and then at 37°C for 15 min were performed, total DNA was extracted from fecal samples using a QIAamp® DNA Stool Mini Kit (Qiagen,

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Table 1. Profiles of diarrheal samples and the results of fecal and molecular analyses for *Cryptosporidium parvum*

Location	Farm	Breed of calf	Age (days)	Number of diarrhea samples	Oocyst positive	18S rRNA		GP60	
						Positive	Species identification	Positive	Subtype
Iwate	A	Holstein	6–15	45	32	33	*C. parvum*	32	IIaA15G2R1
	B	Holstein	8	1	1	1	*C. parvum*	1	IIaA15G2R1
Subtotal	2			46	33 (72%)	34 (74%)		33 (72%)	
Kagoshima (Tanegashima)	C	Japanese black	6–9	5	4	4	*C. parvum*	4	IIaA15G2R1
	D	Japanese black	8	1	0	0	*C. parvum*	0	IIaA15G2R1
	E	Japanese black	8	1	1	1	*C. parvum*	1	IIaA15G2R1
	F	Holstein	20	1	0	0	*C. parvum*	0	IIaA15G2R1
	G	Japanese black	10–75	5	1	1	*C. parvum*	1	IIaA15G2R1
	H	Japanese black	7–49	5	2	2	*C. parvum*	2	IIaA15G2R1
	I	Japanese black	26–76	5	0	0	*C. parvum*	0	IIaA15G2R1
	J	Japanese black	4–35	3	0	0	*C. parvum*	0	IIaA15G2R1
	K	Japanese black	6–33	5	2	2	*C. parvum*	2	IIaA15G2R1
Subtotal	9			31	10 (32%)	10 (32%)		10 (32%)	
Total	11			77	43 (56%)	44 (57%)		43 (56%)	

Hilden, Germany) according to the manufacturer's instructions. 18S rRNA and *GP60* genes were amplified by nested PCR, as described previously [3, 14]. Second PCR amplicons were sequenced in both directions with the secondary PCR primers, using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) and the 3500 Genetic Analyzer (Applied Biosystems).

Small oocysts were detected in 33 out of the 46 calves from Iwate Prefecture and 10 out of the 31 calves from Tanegashima Island. The age of calves discharging oocysts ranged from 6 to 13 days old. 18S rRNA gene fragments were amplified in 34 and 10 samples from Iwate Prefecture and Tanegashima Island, respectively. The nucleotide sequences (786 bp) of these amplicons were identical to that of *C. parvum* deposited in GenBank (accession no. AB746195). The fecal conditions of *C. parvum*-positive samples are summarized in Table 2. The presence of watery feces was causally related with *C. parvum* infection ($P<0.05$). *GP60* was amplified in 33 and 10 samples from Iwate Prefecture and Tanegashima Island, respectively. The nucleotide sequences (925 bp) of these amplicons were identical to that of the IIaA15G2R1 subtype (AB237131). The representative nucleotide sequences of both genes were deposited in GenBank under accession nos. LC012015–LC012018. In one oocyst-negative fecal sample by the sucrose floatation method and microscopic examination, we were unable to obtain *GP60* amplicons, but successfully amplified the 18S rRNA gene. Because the multi-locus 18S rRNA gene is more efficiently amplified by PCR than the single-locus *GP60* [11], these results suggest that the 18S rRNA nested PCR and sequencing are a superior method capable of accurately detecting *C. parvum.*

Cryptosporidium parvum infection is frequently seen in calves under 2 weeks old [4], so the infected calves in our study (6 to 13 days old) were consistent with the previous report. However, the positive rates of the two locations could not be compared, because the calf ages differed between them. Calves infected with *C. parvum* were previously shown to have watery diarrhea [3]. Watery diarrhea causes severe dehydration in neonatal calves and decreases growth rates of the calves. Therefore, *C. parvum* may lead to a significant economic loss in the cattle industry.

Table 2. Causation between *Cryptosporidium parvum* oocyst-positive samples and watery feces

	C. parvum positive	*C. parvum* negative
Watery feces	27[a)]	14
Other fecal conditions[b)]	16	20
Total	43	34

a) *C. parvum* and watery feces were causally related. ($P<0.05$), b) Loose feces, mucous feces, muddy feces and formed feces are included.

No other subtypes, except for zoonotic IIaA15G2R1 [13], have been reported from calves in Japan [1, 5, 9]. This was also the only subtype detected in the present study. IIaA15G2R1 has previously been identified in a human *Cryptosporidium* infection in Japan [1]. These results indicate that there is a risk of *C. parvum* transmission from calves to humans. Since the subtyping analysis on the basis of *C. parvum GP60* amplicons from different areas is as yet insufficient, further work should analyze *C. parvum* from different areas within Japan to investigate the genetic diversity of the species.

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