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Development of a highly sensitive method for the detection of Cryptosporidium parvum virus type 1 (CSpV1)

Ayman Ahmed Shehata[1,2], Hironori Bando[1], Yasuhiro Fukuda[1], Mohammad Hazzaz Bin Kabir[3,4], Fumi Murakoshi[5], Megumi Itoh[6], Atsushi Fujikura[7], Hiroaki Okawa[7], Takuto Endo[8], Akira Goto[9], Masayuki Kachi[10], Toshie Nakayama[11], Yuto Kano[12], Shoko Oishi[13], Konosuke Otomaru[13], Kei Kazama[14], Mohamed Ibrahim Essa[2] and Kentaro Kato[1,3,*]

[1]Laboratory of Sustainable Animal Environment, Graduate School of Agricultural Science, Tohoku University, 232-3 Yomogida, Naruko-onsen, Osaki, Miyagi 989-6711, Japan
[2]Department of Animal Medicine, Infectious Diseases, Faculty of Veterinary Medicine, Zagazig University, El-Shohada, Moawwad, Qesm Awel AZ Zagazig, 44511, Egypt
[3]National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan
[4]Department of Microbiology and Parasitology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207, Bangladesh
[5]Department of Infectious Diseases, Kyoto Prefectural University of Medicine, 465, Kajiicho, Kawaramachi-hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan
[6]Department of Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan
[7]Fukuoka Dairy Cattle Artificial Insemination Clinic, Fukuoka Prefecture Dairy Farming Cooperative, 1-13-4, Susenji, Nishiku, Fukuoka, Fukuoka 839-0832, Japan
[8]Kurume Dairy Cattle Artificial Insemination Clinic, Fukuoka Prefecture Dairy Farming Cooperative, 75-2, Airaku, Ohashimachi, Kurume, Fukuoka 839-0832, Japan
[9]Veterinary Medical Center, Obihiro University of Agriculture and Veterinary Medicine, 2-11 Inada-nishi, Obihiro, Hokkaido 080-8555, Japan
[10]Dairy Research Department, Gifu Prefectural Livestock Research Institute, 1975-615 Kubohara, Yamaoka-cho, Ena, Gifu 509-7601, Japan
[11]Miyazaki Agricultural mutual aid association, 17938-5 Nyuta Shintomi-cho, Miyazaki 889-1406, Japan
[12]Soo Agricultural mutual aid association, 2253 Osumicho Tsukino, Soo-shi, Kagoshima 899-8212, Japan
[13]Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065 Japan
[14]School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Chuou-ku, Sagamihara, Kanagawa 252-5201, Japan

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* Corresponding author: Kentaro Kato, D.V.M., Ph.D
Laboratory of Sustainable Animal Environment, Graduate School of Agricultural Science, Tohoku University, 232-3 Yomogida, Naruko-onsen, Osaki, Miyagi 989-6711, Japan
Phone: +81-229-84-7391, Fax: +81-229-84-7391, E-mail: kentaro.kato.c7@tohoku.ac.jp
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Abstract

***Cryptosporidium* is an apicomplexan zoonotic parasite that infects most mammals, including humans. Cryptosporidium parvum virus type 1 (CSpV1) is the first member within the Partitiviridae family recognized to infect protozoan hosts. *Cryptosporidium* tracking based on CSpV1 detection has been attempted; however, each study used different conditions for the PCR protocol, primers, and target viral sequences. Accordingly, the sensitivity of PCR-based CSpV1 detection remains unclear. In addition, oocyst purification from clinical samples can be problematic due to small number of oocysts, sample degradation and low yield efficiency of currently used purification methods. Here we show that the second half of the coding region of dsRNA2 can be detected from various types of clinical samples, without the need for oocyst purification, by using a semi-nested-PCR technique. Furthermore, we show that the short sequence targeted in this study has higher diversity than the *Cryptosporidium* GP60 gene. Taken together, our findings suggest that this method could be used as an important tracking marker for *Cryptosporidium* species.**

Key Words: Calves, *Cryptosporidium,* CSpV1, PCR

Introduction

Cryptosporidium is a zoonotic parasite that causes cryptosporidiosis. *Cryptosporidium* can infect a wide range of hosts including domestic animals and humans through ingestion of contaminated food or water[30]. The severe diarrhoea and related high mortality associated with the infection in young animals have a serious impact on animal husbandry[4]. *Cryptosporidium* infection occurs throughout the world but high prevalence rates are more common in poorly developed countries where prevalence was ranged from 20% to 60% in individual calves in Japan and may reach to 98% at herd level in Argentina[1,12,22]. Unfortunately, none of the drugs currently available to treat cryptosporidiosis are completely effective[29]. Therefore, the development of a quick and accurate detection method and genetic surveillance of *Cryptosporidium* are particularly important to predict and prevent the spread of infection.

Historically, microscopic techniques have been relied upon for the detection of *Cryptosporidium* oocysts, even though misdiagnosis is possible due to debris[5,9]. In addition, no major morphological differences among oocysts have been found between different species by using these techniques[8]. More recently, PCR-based techniques are being developed for detection, species identification, and phylogenetic analysis of *Cryptosporidium*[32,34,35]; however, problems remain. For example, although *Cryptosporidium* detection requires the purification of oocysts from faecal samples, the recovery rates of oocysts from frozen stock vary from 22% to 75%[3]. Accordingly, new techniques for *Cryptosporidium* detection and identification are needed.

Cryptosporidium parvum virus type 1 (CSpV1), which is a double-strand (ds) RNA virus belonging to the Cryspo-virus genus in the Partitiviridae family, was first detected in 1997 from C. *parvum*[13,14]. So far, there have been no reports demonstrating the extracellular transmission of the virus to *Cryptosporidium* because CSpV1 is vertically transmitted intracellularly during *Cryptosporidium* division[25]. In addition, it has been reported that *Cryptosporidium* oocysts contain a large number of viral RNAs relative to the number of oocyst genes[31]. Therefore, CSpV1-based techniques have been developed as an alternative approach to *Cryptosporidium* detection and identification[17,33]. In fact, the detection sensitivity of CSpV1-based techniques has been reported to be 2,000-fold higher than that of techniques designed to detect *Cryptosporidium* oocysts because of the stability and abundance of the viral RNA[15,31]. The virus possesses an encapsidated long dsRNA1 segment that encodes the RNA-dependent RNA

polymerase and a short dsRNA2 segment that encodes the capsid protein[24]. The complete sequences of the CSpV1 dsRNAs have been identified in *C. hominis*, *C. felis*, and *C. melegridis* and have been shown to be conserved[18]. Therefore, dsRNA2 has frequently been used for *Cryptosporidium* detection. However, different regions within or outside the coding region of the dsRNAs have been used as the targets for PCR-based detection[17-19], and the effects of differences in the PCR approaches used on the sensitivity of viral detection remain unknown.

Although CSpV1-based techniques show great potential for use in *Cryptosporidium* detection, the purification of *Cryptosporidium* oocysts remains an essential step for viral RNA extraction. Currently used methods for oocyst recovery appear to be unsuitable for samples with a small number of oocysts[7, 28]. Moreover, it has been reported that degradation of oocysts due to excystation or storage can lead to an extreme reduction in the efficiency of oocyst purification[11]. For these reasons, new methods that do not require *Cryptosporidium* oocyst purification are needed.

In the present study, we demonstrate that CSpV1 detection sensitivity is affected by the PCR target site within CSpV1 dsRNA2. In addition, we show that a partial dsRNA2 sequence of CSpV1 can be used to detect CSpV1 from clinical samples without the need for *Cryptosporidium* oocyst purification. Our data suggest that this method has potential as an alternative strategy for *Cryptosporidium* or CSpV1 detection and identification.

Materials and Methods

Clinical sample collection: A total of 62 diarrhoeic faecal samples were kindly provided by the veterinarians in various prefectures of Japan. Samples were from dairy and beef calves that ranged in age from 3 days to 50 days, acquired between October 2018 and March 2019, from eight different Japanese prefectures. In this study, three different breeds of calves were included: Holstein Friesians, Japanese Black, and cross-bred F1 hybrids. Samples were stored at -28 ˚C until use.

Table 1. different primer sequences used in this study.

Primer	Sequence
P1F	5'-ATTACAAGTTTTGAATCAATAGAG-3'
P2F	5'-CCTATGCACCATAGTGGAATTAC-3'
P3F	5'-CCTATCGCTGAGCATCTAACTAGATGG-3'
P4R	5'-ACTAACAGATTGCACTGCTTCCGGC-3'
P5R	5'-TCTGCGCTACACTCCGTCGTTACTAT-3'
P6R	5'-ATGGGAGCGATCTGCGCTACAC-3'

RNA extraction from faecal samples: RNA was extracted from all samples without oocyst purification by using 0.4–0.5 g of faeces according to the protocol of the Quick-RNA Faecal/Soil Microbe Microprep Kit (Zymo Research Corp, Irvine, CA, USA). RNA was stored at -80 ˚C until use. Some samples were examined microscopically for oocysts and were then subjected to at least three freezing-thawing cycles before RNA extraction.

PCR protocol using RNA extracted from C. parvum HNJ-1 strain: *C. parvum* HNJ-1 is maintained in nude mice in the Department of Infectious Diseases, Kyoto Prefectural University of Medicine, Japan. Total RNA was extracted from *C. parvum* HNJ-1 oocysts. In this study, we used seven sets of primer pairs for CSpV1 detection. The CPVS_ORF_1F and CPVS_ORF_6R primers were previously used for CSpV1 detection[20]. The other four primers were designed for this study. All of the primers used in this study are listed in Table 1. First, cDNA was synthesized by using 1000 pg of total RNA extracted from *C. parvum* HNJ-1 with the CPVS_ORF_1F primer by using the Thermo Scientific Verso cDNA Synthesis Kit (Thermo Scientific, Waltham, Massachusetts, CA, USA) according to the manufacturer's instructions. The resulting cDNA was included the in initial and semi-nested

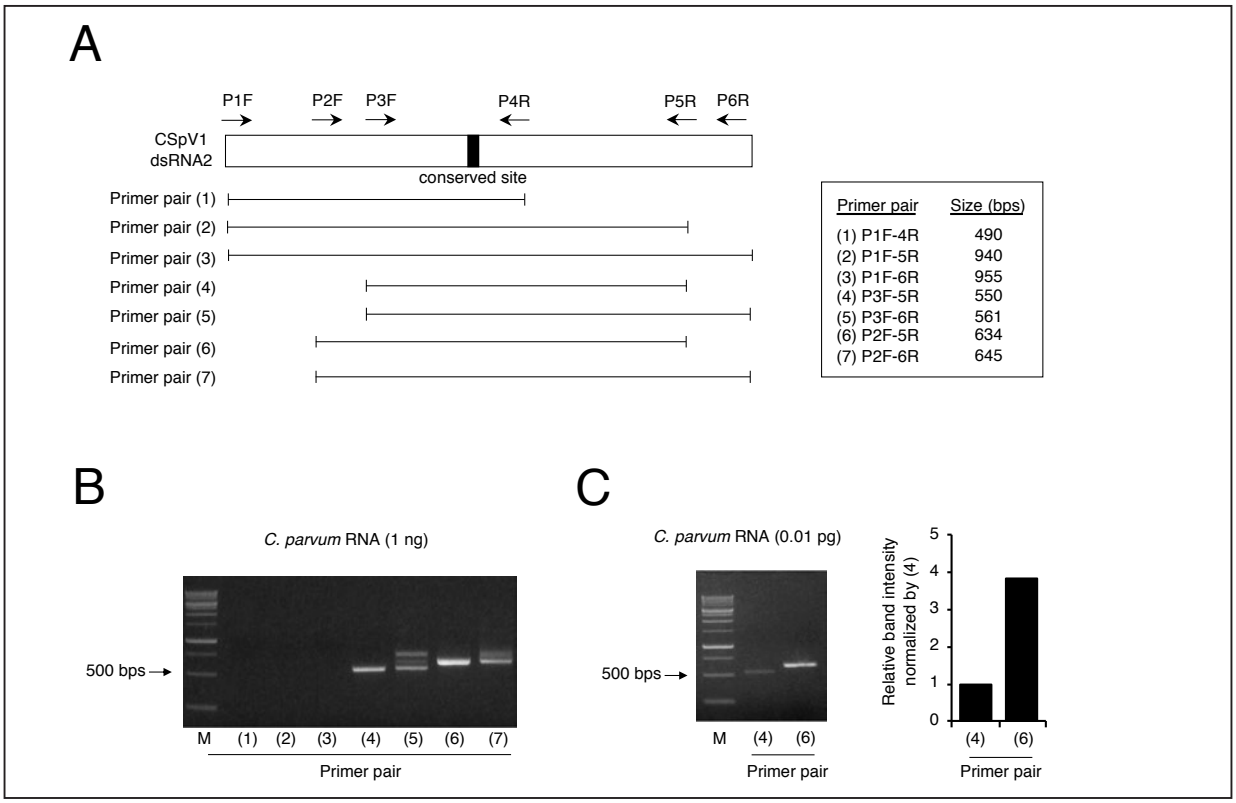


Fig. 1. PCR condition sare important for virus detection
(A) Schematic drawings of PCR analyses for CSpV1 dsRNA2 detection with different primer pairs and targeted sizes. **(B)** Detection of CSpV1 by PCR in 1 µg of RNA from *Cryptosporidium* oocysts was performed using the indicated primer pairs. Results shown in a 1.5% agarose gel. M: molecular weight marker (1-kb DNA ladder). **(C)** Detection of CSpV1 by PCR in 0.01 pg of RNA from *Cryptosporidium* oocysts was performed using the indicated primer pairs. Results shown in a 1.5% agarose gel. M: molecular weight marker (1-kb DNA ladder). The densitometry analysis of the amplified fragments was performed by using NIH Image J.

PCR reaction, which was performed by using the KOD FX Neo kit with the indicated primer pairs (**Fig. 1A**). Then, to evaluate the sensitivity of our protocol, the same procedure was repeated using four different concentrations (10, 1, 0.1, and 0.01 pg/ul) of template total RNA extracted from *C. parvum* HNJ-1 for cDNA synthesis and amplification of the various target fragments. The PCR protocol was as follows: 94 ˚C for 2 min, then 45 cycles of 98 ˚C for 10 s, 60 ˚C for 30 s and 68 ˚C for 1 min, followed by 68 ˚C for 7 min.

Semi-nested PCR protocol using RNA extracted from faecal samples: After optimization of our protocol using RNA from the *C. parvum* HNJ-1 strain, we examined the ability of this method to detect CSpV1 dsRNA2 from total RNA directly extracted from 62 *faecal* samples of calves in the field. Total RNA was extracted from these *faecal* samples as above. cDNA synthesis and the initial PCR, using primer pair 2, were carried out by using the protocol just described. Then, 2 µl of the first PCR product was used for the second PCR reaction, which was performed by using the KOD FX Neo kit (TOYOBO, Osaka, Japan) with the indicated primer pair 6. The PCR protocol was as follows: 94 ˚C for 2 min, then 45 cycles of 98 ˚C for 10 s, 60 ˚C for 30 s and 68 ˚C for 1 min, followed by 68 ˚C for 7 min.

Detection and visualization of CSpV1 and Cryptosporidium genetic diversity: PCR products were separated by electrophoresis through 1.5 % agarose gels and extracted by

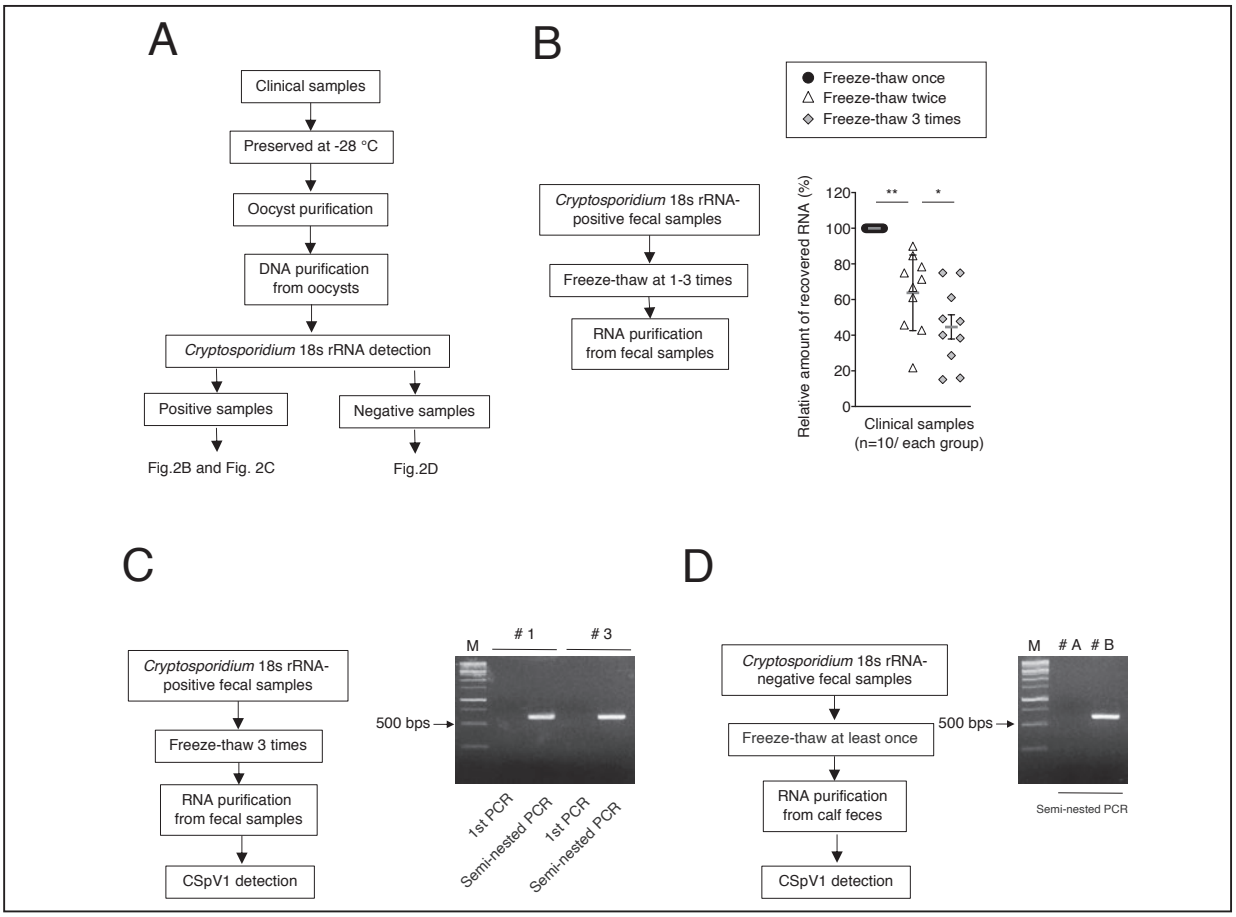


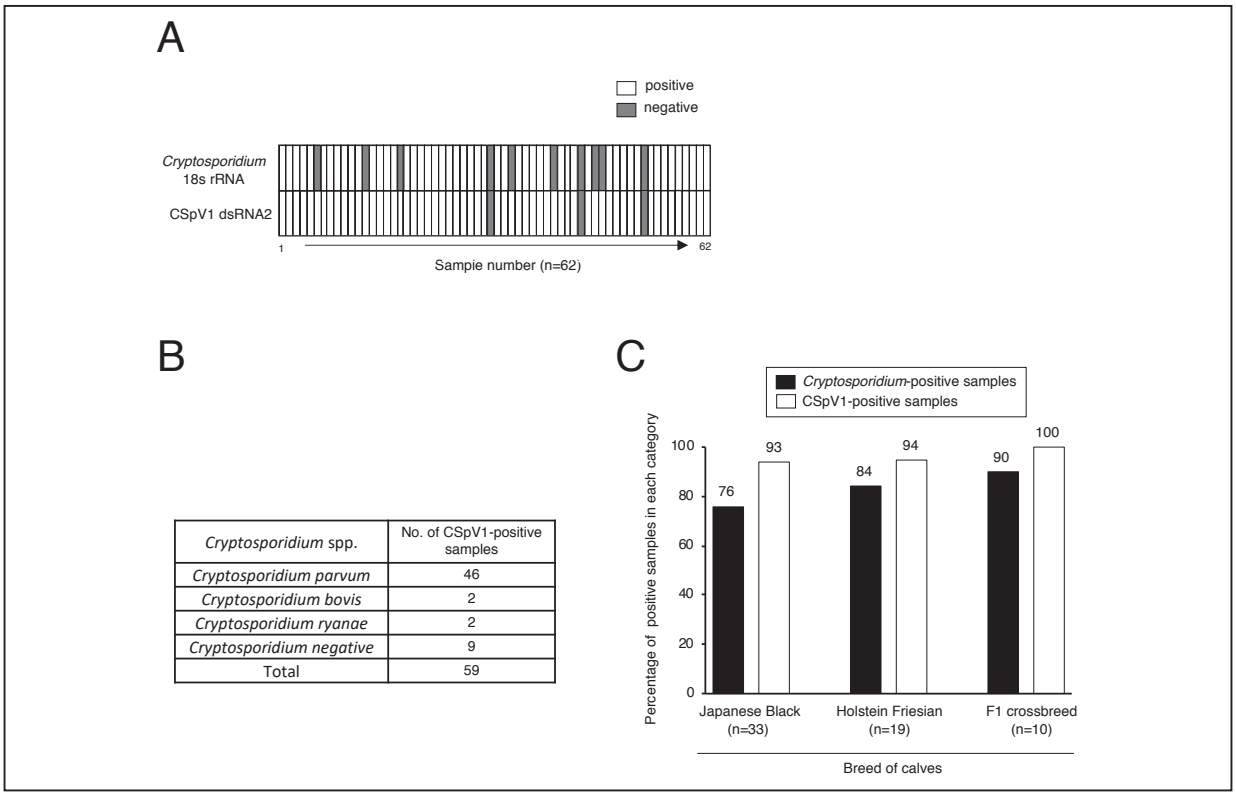
Fig. 2. CSpV1 can be detected in damaged clinical samples.
(A) Scheme of the experimental strategy used to evaluate PCR sensitivity and specificity using clinical samples. **(B)** Effect of frequent freeze-thawing on RNA extraction. Each point represents the mean of one sample (n=10). *$p < 0.05$, ** $p<0.01$; (Student's t-test). **(C)** Detection of CSpV1 in *Cryptosporidium*-positive clinical samples by using primary PCR alone or semi-nested PCR. Results shown in a 1.5% agarose gel. M: molecular weight marker (1-kb DNA ladder) **(D)** Detection of CSpV1 in *Cryptosporidium*-negative clinical samples by using semi-nested PCR. Results shown in a 1.5% agarose gel. M: molecular weight marker (1-kb DNA ladder).

using the NucleoSpin Gel and PCR Clean-up kit (MACHEREY-NAGEL GmbH & Co, Düren, Germany) according to the manufacturer's instructions. An automated sequencer (Applied Biosystems 3130xl Genetic Analyzer; Applied Biosystems, Tokyo, Japan) was used for analysing sequences. The resulting sequences were read on 4Peaks Genetic Analyzer software and aligned using Clustal W on the MEGA7 program. The aligned dsRNA2 sequences in which all gaps were eliminated were applied to the popART software equipped with the integer neighbor-joining (IntNJ) method to visualize genetic diversity by nucleotide substitutions[2]. Similarly, GP60 gene sequences (published data) for the same twenty-five samples were used to construct haplotype networks to be compared with the dsRNA2-based tree.

Results

The PCR target region of CSpV1 has an effect on virus detection sensitivity

To test the PCR amplification efficiency in relation to the PCR conditions, we used 1 ng of RNA extracted from purified *Cryptosporidium parvum* oocysts and different primer pairs targeting dsRNA2 (**Fig. 1A**). We found that



Cryptosporidium spp.	No. of CSpV1-positive samples
Cryptosporidium parvum	46
Cryptosporidium bovis	2
Cryptosporidium ryanae	2
Cryptosporidium negative	9
Total	59

Fig. 3. The CSpV1-based dsRNA2 detection method is applicable to field samples.
(A) CSpV1 and *Cryptosporidium* detection in 62 clinical samples; correlation between the results based on *Cryptosporidium* 18S rRNA and the results based on CSpV1 dsRNA2. **(B)** CSpV1 detection in three *Cryptosporidium* species. **(C)** The infection rates for CSpV1 and *Cryptosporidium* in different breeds of host animals was analysed.

CSpV1 was only detected by primer pairs targeting the second half of dsRNA2 (i.e., primer pairs 4, 5, 6, or 7; **Fig. 1B**). The specificity of each primer pair was confirmed through amplicon sequencing. Additionally, whereas primer pairs 5 and 7 showed non-specific amplification, specific amplicons were clearly produced by primer pairs 4 and 6 (**Fig. 1B**). Therefore, we next tested the limit of detection of CSpV1 by using low RNA concentrations from *C. parvum* oocysts and primer pair 4 or 6 (**Fig. 1C**). We found that CSpV1 was strongly detected by primer pair 6 compared with primer pair 4 (**Fig. 1C**). Taken together, these data indicate that both the viral target sequence and the primer pair combination are important for CSpV1 detection.

Establishment of a direct method for the detection of CSpV1 from faecal samples

Cryptosporidium oocyst purification has been essential for sensitive molecular or serological CSpV1 detection[17, 20], even though the current purification procedures have limitations and drawbacks. Therefore, we assessed whether a PCR-based CSpV1 detection method using primer pair 6 could be used to assess faecal samples without having to purify the *Cryptosporidium* oocysts (**Fig. 2A, 2B, 2C, and 2D**). Storage conditions reportedly affect the extraction efficiency of RNA[11]. We confirmed that the extraction efficiency of RNA from faecal samples was markedly reduced by repeat freeze-thawing (**Fig. 2B**). We tried to detect CSpV1 by using RNA extracted from damaged faecal samples (i.e., samples that had been freeze-thawed three times). However, we failed to detect CSpV1 from these samples even though *Cryptosporidium* infection was confirmed by using a nested-PCR

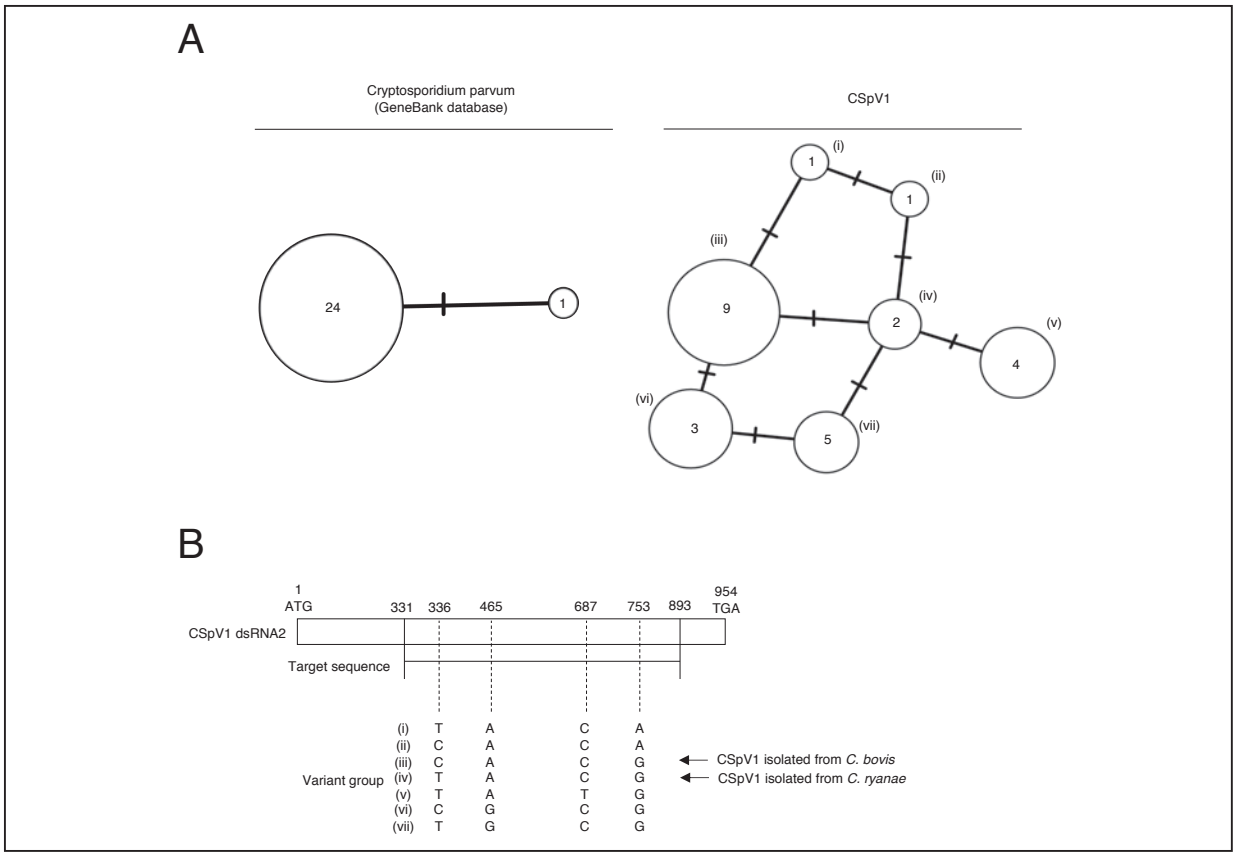


Fig. 4. Partial sequence analysis of dsRNA2 shows high genetic diversity compared with GP60 gene sequences. **(A)** Genetic diversities based on *Cryptosporidium* GP60 and CSpV1 dsRNA2 nucleotide substitutions as visualized by using popART software equipped with the Integer neighbor-joining (IntNJ) method. Numbers inside circles indicate the total number of samples within the group. The number of mutation sites between groups is indicated on each line. Variant groups of CSpV1 dsRNA2 sequences are shown (i–vii). **(B)** Nucleotide substitutions at different sites within the dsRNA2 sequence resulting in variant alignment groups based on virus sequence.

method targeting *Cryptosporidium* 18S rRNA (**Fig. 2C**). Then, we examined whether a semi-nested PCR method, using primer pair 2 for the first PCR and primer pair 6 for the second PCR, could be used to test faecal samples without purification of the *Cryptosporidium* oocysts. By using this approach, we were able to confirm the presence of CSpV1 in the damaged faecal samples (**Fig. 2C**), thereby demonstrating that the semi-nested PCR method could directly detect CSpV1 in damaged faecal samples. To test the sensitivity of the semi-nested PCR method, we attempted to detect CSpV1 from faecal samples that were freeze-thawed at least once and were negative for the parasite by a nested-PCR method targeting *Cryptosporidium* 18s rRNA(**Fig. 2D**). Some of the samples were CSpV1-negative in this assay(**Fig. 2D**), suggesting that our method is highly specific. Other samples were CSpV1-positive (**Fig. 2D**), indicating that our method is more sensitive than the existing method. Taken together, our findings demonstrate that this direct CSpV1 detection method is effective for use with faecal samples.

Direct methods to detect CSpV1 in clinical samples

Our current study has shown that the semi-nested PCR method using primer pair 6 is highly sensitive for the detection of CSpV1 from faecal samples without the need for purification of *Cryptosporidium* oocysts (**Fig. 3A**). We, therefore, next investigated whether this method could be used to detect CSpV1 in 62 clinical samples

that had undergone freeze-thawing at least once; these samples were collected in Japan between October 2018 and March 2019 (**Fig. 3A**). Although a previous study reported that 50 out of these 62 samples were *Cryptosporidium*-positive (personal communication), we found that 59 of the same 62 samples were CSpV1-positive (**Fig. 3A**). Importantly, we found that the CSpV1-positive samples matched completely with the *Cryptosporidium* 18S rRNA-positive samples (**Fig. 3A**). Thus, we detected an additional 9 CSpV1-positive samples among a total of 12 *Cryptosporidium* 18S rRNA-negative samples (**Fig. 3A**). These results demonstrate that our direct viral detection method is highly sensitive even for clinical samples.

CSpV1 is present in C. bovis and C. ryanae

To date, CSpV1 has been reported in only four *Cryptosporidium* species: *C. parvum, C. felis, C. hominis, and C. meleagridis*[17, 18]. Our results suggest that CSpV1 is also present in *C. bovis* and *C. ryanae* (**Fig. 3B**). This is the first report to detect CSpV1 both in *C. bovis* and *C. ryanae*, suggesting that our method can detect CSpV1 regardless of the *Cryptosporidium* species (**Fig. 3B**). Moreover, we found a high number of CSpV1-positive samples in all breeds of beef and dairy calves(**Fig. 3C**), suggesting that our method can detect CSpV1 regardless of the cattle breed. These results demonstrate that our direct viral detection method can be used regardless of parasite species or host breed.

CSpV1 partial sequences show a high level of genetic polymorphism compared with Cryptosporidium GP60

Although the GP60 gene is frequently used for subtyping *Cryptosporidium parvum*, the *C. parvum* subtype IIaA15G2R1 is the most common subtype found in cattle in many countries[26] and is the only subtype of *C. parvum* that has been detected in Japan[1]. In a previous study, the *Cryptosporidium* infection rate was about 80% where *C. parvum* isolates represented 92% of the total positive samples with IIaA15G2R1 as the most common subtype (personal communication). In contrast, previous reports have shown variation in the sequence of CSpV1 dsRNA2[17, 33]. In one study, although 64 of 80 samples were found to be *C. parvum*-positive, GP60 gene sequences were obtained from only 41 samples (personal communication), and only 25 samples were found to express both the GP60 gene and partial CSpV1 dsRNA2 sequences. Therefore, to assess whether our viral target sequence could serve as an alternative marker for *C. parvum*, we attempted a genetic polymorphism analysis using the 25 sequences of the GP60 gene or partial CSpV1 dsRNA2 from these samples (**Fig. 4A**). We found that 24 of the 25 GP60 gene sequences comprised one group, whereas the other group contained only one sequence. In contrast, we obtained seven CSpV1 dsRNA2 variant groups based on the virus sequences: 2 groups contained one sequence, whereas the other 5 groups contained 2, 3, 4, 5, and 9 sequences, respectively. These results suggest that CSpV1 dsRNA2 sequences have greater genetic diversity than the GP60 gene sequence, even though the viral target sequence is shorter (**Fig. 4A**). Four nucleotide substitutions were found within the dsRNA2 target sequences; as a result, distinct nucleotide alignments were obtained and the samples could be clustered into 7 groups (**Fig. 4B**). Specifically, these 4 nucleotide substitutions at base pair positions 336, 465, 687, or 753 of the dsRNA2 target sequence are responsible for genetic diversity of the virus (**Fig. 4B**). In addition, virus sequences recovered from *C. bovis*-infected samples were 100% identical to each other and belonged to group (iii). Similarly, nucleotide sequences of CSpV1 for *C. ryanae* showed complete identity and clustered within group (iv) (**Fig. 4A, 4B**). However, the GP60-based sequence network revealed identical sequences with only one C nucleotide replaced with A at base pair positions 395. Taken together, these findings indicate that the partial dsRNA2 sequence that we targeted in this study could serve as an important tracking marker for *C. parvum*.

Discussion

In the present study, we demonstrated that a PCR method targeting a partial sequence of CSpV1 dsRNA2 can be used for virus detection from *Cryptosporidium* oocysts. Previous studies have shown that a PCR-based CSpV1 detection method has advantages for sensitive detection of *Cryptosporidium*, for example, detection of fewer than 5 oocysts have been reported[19]. However, these studies used various detection conditions, including various PCR protocols, different target regions of the viral RNA, and different primer pairs. Accordingly, the relationship between the efficiency of virus detection and the detection method has remained unclear. In the present study, we found that PCR amplification efficiency is specific for the target region of the viral RNA; whereas the first half of dsRNA2 is difficult to detect, the second half of dsRNA2 is effectively amplified by PCR. In addition, the 3-prime end of the coding sequence can lead to non-specific amplification, possibly because the first half of dsRNA2 contains a highly AT-rich region. In the present study, we clearly demonstrated that the second half of dsRNA2, which lacks the 3-prime end of the coding sequence, is effectively amplified by using our PCR method.

It has been reported that 100 μg of total RNA can be extracted from 10^9 *Cryptosporidium* oocysts[28]. Given that one *Cryptosporidium* oocyst contains 0.1 pg of total RNA, CSpV1 RNA may be present as less than 0.1 pg in one oocyst. Our study showed that 0.01 pg of RNA extracted from a *Cryptosporidium* oocyst is enough for CSpV1 detection, suggesting that our method has the potential to detect a single oocyst. Moreover, it has been reported that a short-conserved region within dsRNA2 is shared among all known virus sequences that have been identified in different *Cryptosporidium* species[18]. Importantly, amplicons produced by our method include this conserved region, which suggests that our method may be useful for tracking sources of *Cryptosporidium* outbreaks.

Most previous CSpV1 detection studies used RNA extracted not from faeces but from oocysts[17, 20]. However, it is difficult to purify *Cryptosporidium* oocysts from the faeces of hosts with a low oocyst load[3]. In addition, degradation of oocysts due to excystation or storage can result in a significant decrease in the efficiency of purification of *Cryptosporidium* oocysts[11]. In fact, the PCR sensitivity with clinical faecal samples stored for 5 weeks was found to be less than 20%[11]. Of note, our method successfully detected CSpV1 from clinical samples that had been stored for more than a year and subjected to frequent freezing-thawing cycles (at least one) before RNA extraction. Thus, we have established a highly sensitive method for CSpV1 detection from clinical samples without the need for purification of *Cryptosporidium* oocysts.

A previous study found that 50 out of 62 samples contained *Cryptosporidium* oocysts based on a *Cryptosporidium* 18S rRNA detection method (personal communication). In contrast, using our detection method, we demonstrated that 59 of those 62 samples are, in fact, CSpV1-positive. Importantly, these CSpV1-positive samples included the 50 *Cryptosporidium* 18S rRNA-positive samples, thereby demonstrating that our PCR method targeting the partial CSpV1 dsRNA2 can be used as an alternative *Cryptosporidium* detection method. Of interest, our current study identified 9 of the *Cryptosporidium* 18S rRNA-negative samples as CSpV1-positive, suggesting that CSpV1 may be a more sensitive marker of *Cryptosporidium* than the 18S rRNA gene of the *Cryptosporidium* genome. Some studies have reported that CSpV1-based methods show greater sensitivity in detecting *Cryptosporidium* in clinical samples than do techniques that use oocyst or sporozoite antigens; however, these studies involved serologic techniques or colloidal gold-based immunochromatographic tests[15, 16, 31]. In contrast, here we demonstrated that a PCR-based method targeting the partial CSpV1 dsRNA2 can detect for *Cryptosporidium* in clinical samples with high sensitivity. *Cryptosporidium* has been

isolated all over the world including rural areas and developing countries[6, 10, 23].Improving PCR-based CSpV1 detection methods for use with clinical samples that have been preserved is important, because such methods are widely applicable for tracking of *Cryptosporidium* infections. Thus, our PCR-based method targeting the partial CSpV1 dsRNA2 could contribute to various characterization studies of *Cryptosporidium* without temporal and spatial constraints.

Although 29 *Cryptosporidium* species have been recognized to date[4], CSpV1 has been reported in only four species: *C. parvum, C. felis, C. hominis, and C. meleagridis*[17, 18]. In the present study, we found CSpV1 in *C. bovis* and *C. ryanae* for the first time. The lack of information about CSpV1 in *C. bovis* and *C. ryanae* may be due to the pathology of these *Cryptosporidium* species; infection with these *Cryptosporidium* species is usually without clinical symptoms or diarrhoea[22]. Therefore, it may be more difficult to detect *C. bovis* and *C. ryanae* from clinical samples compared with *C. parvum*. In addition, most studies have examined clinical samples collected from young stock because of the pathology of *C. parvum*. In contrast, *C. bovis* and *C. ryanae* are found mainly in older calves and stock aged 3–11 months[27], and the clinical samples used in this study came from animals across a wide age range. Whatever the case, our data show that this method can be used to detect the presence of CSpV1 in *C. bovis* and *C. ryanae*. Clearly, this first CSpV1 detection spurs interest in the *Cryptosporidium*-virus interaction, in particular, the host factors that determine or modulate CSpV1 infection. Further studies regarding the identification of animal factors and host interactions in relation to CSpV1 infection are warranted.

Our current study showed that there is no correlation between the presence of CSpV1 and the breed of calf, suggesting that *Cryptosporidium* is widespread regardless of host factors such as breed. Most subtyping studies have focused on the *C. parvum* GP60 gene[21, 26]; therefore, additional high-resolution tools for tracking different *Cryptosporidium* species and subtypes may be required. Here, sequence analysis of 25 samples using the partial dsRNA2 sequence rather than the GP60 gene sequence revealed greater genetic diversity among the virus sequences from several isolates even though the partial dsRNA2 sequence is shorter than the GP60 gene sequence. In addition, CSpV1 seems to be non-species specific based on the similarity observed among the CSpV1 sequences from samples infected with *C. bovis, C. ryanae,* or *C. parvum*. Taken together, our data show that this partial dsRNA2 sequence could be used as an alternative marker for *Cryptosporidium* detection and identification.

In summary, we have shown that a partial dsRNA2 sequence from CSpV1 is a useful PCR target for the detection of *Cryptosporidium* and CSpV1 from clinical samples without the need for purification of *Cryptosporidium* oocysts. This method will be of value in *Cryptosporidium* and CSpV1 detection and identification.

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