1 **Molecular epidemiological analyses of *Cryptosporidium parvum* virus 1**
2 **(CSpV1), a symbiotic virus of *Cryptosporidium parvum*, in Japan**

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24 **Abstract**

25 We show that *Cryptosporidium parvum* virus 1 (CSpV1), a member of the
26 family *Partitiviridae*, genus *Cryspovirus* that can infect *Cryptosporidium parvum*,
27 is a new candidate for high-resolution tool for tracing *Cryptosporidium parvum*.
28 CSpV1 was detected in all *C. parvum*-positive samples tested. Phylogenetic
29 analysis of dsRNA1 sequence from CSpV1 can distinguish infected areas of *C.*
30 *parvum* on the national level. Sequences detected in samples from Iwate
31 prefecture and other islands (Tanegashima, and Okinawa) belonged to a single
32 clade. This system can differentiate the samples from Hokkaido and south part of
33 Japan as well as from other countries. Samples from Iwate, Tanegashima, and
34 Okinawa belonged to a single subclade respectively. Therefore, the CSpV1
35 dsRNA sequences reflect the regional distribution of their host and have potential
36 as a high-resolution tool to trace *C. parvum* IIaA15G2R1 subtype.

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38 Keywords : dsRNA viruses ; *Cryptosporidium parvum* ; *Cryptosporidium parvum*
39 virus 1; *Partitiviridae*; Epidemiology

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43 *Cryptosporidium parvum* belongs to the phylum Apicomplexa and has been
44 reported to be a zoonotic species that causes severe diarrhea (Sulaiman *et al.*,
45 2005) and no effective therapy has yet been established. It can cause waterborne
46 or foodborne outbreaks. Therefore, studies on the species/subspecies distribution
47 and transmission routes of *C. parvum* are important for public health and food
48 production. The 60-kDa glycoprotein (GP60) gene is the major subtyping gene of
49 *C. hominis* and *C. parvum* (Leave *et al.*, 2002; Glaberman *et al.*, 2002; Abe *et al.*,
50 2006) because the GP60 gene is the most polymorphic marker identified to date in
51 the *Cryptosporidium* genome. Moreover, GP60 is located on one of the dominant
52 targets for neutralizing antibody responses in humans (Cevallos *et al.*, 2000). On
53 the basis of this gene, the sequences of *C. parvum* and *C. hominis* have been
54 subtyped with following designations: Ia - Ig for *C. hominis*; and IIa - IIo for *C.*
55 *parvum* (Xiao, 2010; Valenzuela *et al.*, 2014; Wang *et al.*, 2014). The IIa subtype
56 is the most common subtype found in humans and cattle in many countries
57 (Santín *et al.*, 2008). GP60 based subtype can usually be further characterized
58 based on the number of trinucleotide repeats (TCA or TCG) coding for the amino
59 acid serine (Sulaiman *et al.*, 2005). In Japan, IIaA15G2R1 subtype is the only
60 subtype of *C. parvum* that has been detected to date (Wu *et al.*, 2003; Abe *et al.*,
61 2006; Murakoshi *et al.*, 2013, 2014; Ichikawa-Seki *et al.*, 2014; Aita *et al.*, 2015).
62 Therefore, there are no tools that can trace infection and contamination sources in
63 outbreaks.

64 Recently, double-stranded RNA (dsRNA) viruses, belonging to the family
65 *Partitiviridae*, have been identified in *Cryptosporidium* and classified as members
66 the genus *Cryspovirus* (Nibert *et al.*, 2009, 2014). These viruses contain two
67 unrelated, linear dsRNA segments of 1.7 kbp (dsRNA1) and 1.4 kbp (dsRNA2)

68 that are encapsidated separately. dsRNA1 encodes the RNA-dependent RNA
69 polymerase (RdRp) and dsRNA2 encodes the capsid protein (CP). The molecular
70 mass of RdRp and CP are 62 kDa and 37 kDa, respectively (Khramtsov & Upton,
71 2000). *Cryspovirus* detected from *C. parvum* has been named *Cryptosporidium*
72 *parvum* virus 1 (CSpV1). This virus has also been detected from *C. felis*, *C.*
73 *hominis*, and *C. meleagridis* (Leoni *et al.*, 2003, 2006). Most species classified
74 with the family *Partitiviridae* infect fungi and plants, and are transmitted from
75 cell to cell during cell division. Therefore, CSpV1 is transmitted only by
76 intracellular routes, although its exact mode of transmission remains unclear.
77 There are a few studies of the host effects by CSpV1, and its virulence. Only a
78 correlation between CSpV1 dsRNA2 levels and parasite fecundity of *C. parvum*
79 has been reported (Jenkins *et al.*, 2008). RNA viruses show higher mutation
80 frequencies than their hosts because they lack proofreading enzymes (Holland *et*
81 *al.*, 1982). An attempt to subtype *C. parvum* using CSpV1 dsRNA sequences has
82 previously been reported by two research groups, suggesting that CSpV1
83 sequences may be useful for tracing *Cryptosporidium* infection sources because of
84 their sequence diversity (Xiao *et al.*, 2001; Leoni *et al.*, 2003). Consequently, we
85 were interested in whether CSpV1 dsRNA could serve as novel *C. parvum* trace
86 markers.

87 One hundred and seventeen calf fecal samples were collected from farms
88 located in Hokkaido (Kushiro and Ishikari) in 2014. *C. parvum* positive bovine
89 fecal samples from Ashoro (Hokkaido), Iwate, Okinawa, and Kagoshima
90 prefectures were kindly provided by numerous scientists (Ichikawa-Seki *et al.*,
91 2014; Aita *et al.*, 2015). The *C. parvum* HNJ-1 strain (kindly provided by Dr.
92 Yagita) was first isolated from an infected woman in Japan (Satoh *et al.*, 2005),

93 and passaged in our laboratory by using nude mice (BALB/c Slc-nu/nu, SLC
94 Japan). *C. parvum* was detected in the fecal samples using both sugar flotation
95 microscopic observation and a molecular procedure. For DNA extraction, 0.3–
96 0.4g of fecal specimen was used. *Cryptosporidium* spp. were detected and
97 subtyped by nested polymerase chain reaction (PCR) amplification targeting a
98 ~830 bp and a ~850 bp fragment of the small subunit (SSU) rRNA and 60-kDa
99 glycoprotein (GP60) genes, respectively, as described previously (Feng *et al.*,
100 2007).

101 Oocysts were purified from *C. parvum*-positive feces by using the sucrose
102 gradient method. To extract RNA easily, oocysts were excysted by modifying a
103 method previously described (Rochelle *et al.*, 2002). Total RNAs were extracted
104 from sporozoites with the TRIZOL reagent (Life Technologies) and total RNA
105 was used for cDNA synthesis with a SuperScript III First-Strand Synthesis
106 System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions
107 with the following primers: CPVL_ORF_R (5'-
108 TCCATAAATTTTGTGACTCCTG-3') and CPVS_ORF_R (5'-
109 ATGGGAGCGATCTGCGCTACAC-3'). The resulting cDNA was amplified
110 using KOD FX Neo (TOYOBO, Japan) with primers CPVL_ORF_F (5'-
111 AAGTTTGTCAATATCTATGAGATAC-3') and CPVL_ORF_R (5'-
112 TCCATAAATTTTGTGACTCCTG-3') for amplifying CspV1dsRNA1 ORF (-
113 1576bp), and CPVS_ORF_F (5'-ATTACAAGTTTTGAATCAATAGAG-3')
114 and CPVS_ORF_R (5'-ATGGGAGCGATCTGCGCTACAC-3') for
115 CspV1dsRNA2 ORF (-954bp). Then, all PCR products were cloned (Zero Blunt
116 TOPO PCR Cloning Kit, life technologies) and were sequenced.

117 The sequences were aligned using Clustal X2 (Larkin *et al.* 2007) and the

118 computed sequences were edited by hand with BioEdit 7.0.5.3 (Hall, 1999). All
119 gaps were eliminated and dsRNA1 sequences were used for the phylogenetic
120 analysis. A maximum likelihood (ML) analysis was performed using MEGA 6.0.6
121 (Tamura *et al.*, 2011). The used substitution model and optional parameter sets
122 were evaluated by MEGA 6.0.6, and the most suitable sets were selected
123 according to the Akaike information criterion (AIC). To calculate the bootstrap
124 values, 500 ML trees were constructed using the same datasets. To investigate
125 whether CSpV1 dsRNA1 sequence can trace sampling region, in Fig. 1, we
126 constructed phylogenetic tree which substitution model and optional parameters
127 used is T92 (Tavaré, 1986) + Gamma distribution (five categories) option. CSpV1
128 sequences from America and Australia were retrieved from the NCBI database,
129 and Japanese CSpV1 sequences were obtained in this study. Most of CSpV1
130 reference sequences registered with NCBI were partial. Because of the short
131 reference sequences, Fig. 1 has a small amount of information (low-resolution).
132 To acquire more detailed information, we reconstructed a phylogenetic tree with a
133 focus on Japanese samples using longer sequences (1446 bp) with the T92
134 substitution model incorporating the invariable site and Gamma distribution (five
135 categories) options (Fig. 2). We remove LC014998 sequence because we cannot
136 read full length of its ORF.

137 Of the 117 fecal samples collected from Ishikari and Kushiro (Hokkaido), 4
138 samples (3.4%) were positive for *Cryptosporidium*. The parasites in all of the
139 PCR-positive specimens were identified as *C. parvum* and all of the GP60
140 nucleotide sequences were identical to each other and had similarity with *C.*
141 *parvum* subtype IIaA15G2R1. Ashoro (Hokkaido) (7 samples), Iwate (5 samples),
142 Okinawa (5 samples) prefectures and Tanegashima Island (2 samples) samples

were previously reported and also subtyped *C. parvum* IlaA15G2R1 (Ichikawa-Seki *et al.*, 2014; Aita *et al.*, 2015). In total, all 24 *C. parvum*-positive specimens were CSpV1 dsRNA-positive by RT-PCR. *Partitivirus* sequences were found integrated into host chromosomes and some of them were transcribed (Liu *et al.*, 2010; Chiba *et al.*, 2011). Therefore, we did PCR using *C. parvum* genomic DNA as a template with CSpV1-specific primers. As a result, no DNA was amplified. We also performed Local BLAST search using whole genome of *Cryptosporidium parvum*, however, CSpV1 sequence was not detected. Therefore, CSpV1 seems not to be integrated in the host genome. This extremely high prevalence is consistent with a previous report (Khrantsov *et al.*, 2000). Together, these findings suggest that CSpV1 is available as a trace marker of *C. parvum*. CSpV1 dsRNA1 and dsRNA2 ORF sequences (1576 bp and 954 bp) acquired in this study were deposited in the GenBank database under the following accession numbers: LC014992–LC015015 and LC085158–LC085179. The dsRNA1 sequence identities between the HNJ-1 strain (LC015008) and a Chinese strain (EU183403) and between the HNJ-1 strain and other Japanese strains were 95.5% and 96.6 – 99.3% respectively. There were many nucleotide substitutions in the dsRNA1 sequences, however most of these substitutions were synonymous, preserving the amino acid sequence. Fifteen amino acid substitutions were detected when the partial dsRNA1 genes were compared with the *C. parvum* KSU strain (AAC47805) and HNJ-1 strain, whereas ~8 amino acid substitutions were detected when the partial dsRNA1 genes were compared to HNJ-1 strain and other Japanese samples. The dsRNA2 sequence identities between the HNJ-1 strain (LC085170) and a Chinese strain (EU183404) and

167 between the HNJ-1 strain and other Japanese strains were 97.7% and 98.4 –
168 99.4% respectively.

169 Fig. 1 shows the phylogenetic relationships based on partial dsRNA1
170 sequences (113 bp). The Japanese samples formed a single Japanese clade. These
171 results suggest that in *C. parvum* IIAA15G2R1 subtype, CSpV1 dsRNA1 sequence
172 can distinguish sampling areas on the national level. By contrast, based on the
173 dsRNA2 sequence, there was no regional distribution of each clade because of
174 their few polymorphism. To acquire more detailed information, we constructed a
175 phylogenetic tree based on the CSpV1 dsRNA1 using longer sequences (1446 bp).
176 The CSpV1 dsRNA1 sequences detected from Iwate, Tanegashima (Kagoshima)
177 and Okinawa prefectures formed a single clade (Honshu and other islands clade),
178 and samples from Iwate, Tanegashima (Kagoshima), and Okinawa prefectures
179 formed a single subclade respectively (Fig. 2). The monophyly among these
180 branches was supported moderately (50%–100%). Sequence from the HNJ-1
181 strain (LC015008) was more closely related to those of samples from Hokkaido
182 compared with samples from Iwate, Okinawa, and Tanegashima (Kagoshima)
183 prefectures (Fig. 2). LC015000 and LC015006 sampled from Hokkaido were
184 classified in Honshu and other islands clade, but branches were not well
185 supported. Therefore, it cannot be discussed at present. Hokkaido is north part of
186 Japan and this area thrives with agriculture, livestock and dairy industries.
187 Therefore, farmers export many calves very often to other regions and prefectures.
188 The same situation happens in importing calves. We predict this is the main cause
189 that CSpV1 detected from Hokkaido have sequence variation. The fact that
190 LC015012 samples collected from Tanegashima Island were classified into the
191 Hokkaido clade with branches well supported by the bootstrap value. Interestingly

192 the farmers in Tanegashima Island imported calves from Hokkaido, raising the
193 possibility that CSpV1-infected *C. parvum* are transferred from Hokkaido to
194 Tanegashima Island depending on the movement of the host.

195 As a result, the CSpV1 dsRNA1 sequences reflected geographic origin.
196 Therefore, CSpV1 dsRNAs appear to have potential as a high-resolution tool for
197 tracing *C. parvum* IIaA15G2R1 subtype in Japan, possibly also other subtypes in
198 the world.

199

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210

211 **Figure legends**

212 **Fig. 1. Phylogenetic tree based on partial sequences of the *Cryspovirus***
213 **dsRNA1 sequence from around the world.**

214 Phylogenetic tree based on partial sequences of the *Cryspovirus* dsRNA1
215 sequence constructed by using the maximum likelihood method using 130
216 nucleotides without gaps. Substitution model and optional parameters = T92+Γ.
217 Only bootstrap values > 50% from 500 pseudo-replicates are shown.

218

219 **Fig. 2. Geographic origins of the calf fecal samples and phylogenetic tree**
220 **based on partial sequences of the *Cryspovirus* dsRNA1 sequence from Japan.**

221 Geographic origins of the calf fecal samples infected with *C. parvum*
222 used in this study. We compared all of the CSpV1 sequences detected from the
223 northernmost prefecture (Hokkaido), Okinawa, Iwate prefectures, and
224 Tanegashima island in Japan. Phylogenetic tree based on partial sequences of the
225 *Cryspovirus* dsRNA1 sequence constructed by using the maximum likelihood
226 method using 1446 nucleotides without gaps. Substitution model and optional
227 parameters = T92+Γ+I. 500 bootstrap replications were used to estimate the
228 percentage of trees.

229

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Figure 1

Figure 2