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Short communication

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

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ABSTRACT

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

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

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

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

One hundred and seventeen calf fecal samples were collected from farms located in Hokkaido (Kushiro and Ishikari) in 2014. *C. parvum* positive bovine fecal samples from Ashoro (Hokkaido), Iwate, Okinawa, and Tanegashima (Kagoshima) prefectures were kindly provided by numerous scientists (Ichikawa-Seki et al., 2014; Aita et al., 2015). The *C. parvum* HNJ-1 strain (kindly provided by Dr. Yagita) was first isolated from an infected woman in Japan (Satoh et al., 2005), and passaged in our laboratory by using nude mice (BALB/c Slc-nu/nu, SLC Japan). *C. parvum* was detected in the fecal samples using both sugar flotation microscopic observation and a molecular procedure. 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 a ~850 bp fragment of the small subunit (SSU) rRNA and 60-kDa glycoprotein (GP60) genes, respectively, as described previously (Feng et al., 2007).

Oocysts were purified from *C. parvum*-positive feces by using the sucrose gradient method. To extract RNA easily, oocysts were excysted by modifying a method previously described (Rochelle et al., 2002). Total RNAs were extracted from sporozoites with the TRIZOL reagent (Life Technologies) and total RNA was used for cDNA synthesis with a SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions with the following primers: CPVL_ORF_R (5′-TCCATAAATTTTGTGACTCCTG-3′) and CPVS_ORF_R (5′-ATGGGAGCGATCTGCGCTACAC-3′). The resulting cDNA was amplified using KOD FX Neo (TOYOBO, Japan) with primers CPVL_ORF_F (5′-AAGTTTGTCAATATCTATGAGATAC-3′) and CPVL_ORF_R (5′-TCCATAAATTTTGTGACTCCTG-3′) for amplifying CSpV1 dsRNA1 ORF (−1576 bp), and CPVS_ORF_F (5′-ATTACAAGTTTTGAATCAATAGAG-3′) and CPVS_ORF_R (5′-ATGGGAGCGATCTGCGCTACAC-3′) for CSpV1 dsRNA2 ORF (−954 bp). Then, all PCR products were cloned (Zero Blunt TOPO PCR Cloning Kit, life technologies) and were sequenced.

The sequences were aligned using Clustal X2 (Larkin et al., 2007) and the computed sequences were edited by hand with BioEdit 7.0.5.3 (Hall, 1999). All gaps were eliminated and dsRNA1 sequences were used for the phylogenetic analysis. A maximum likelihood (ML) analysis was performed using MEGA 6.0.6 (Tamura et al., 2011). The used substitution model and optional parameter sets were evaluated by MEGA 6.0.6, and the most suitable sets were selected according to the Akaike information criterion (AIC). To calculate the bootstrap values, 500 ML trees were constructed using the same datasets. To investigate whether CSpV1 dsRNA1 sequence

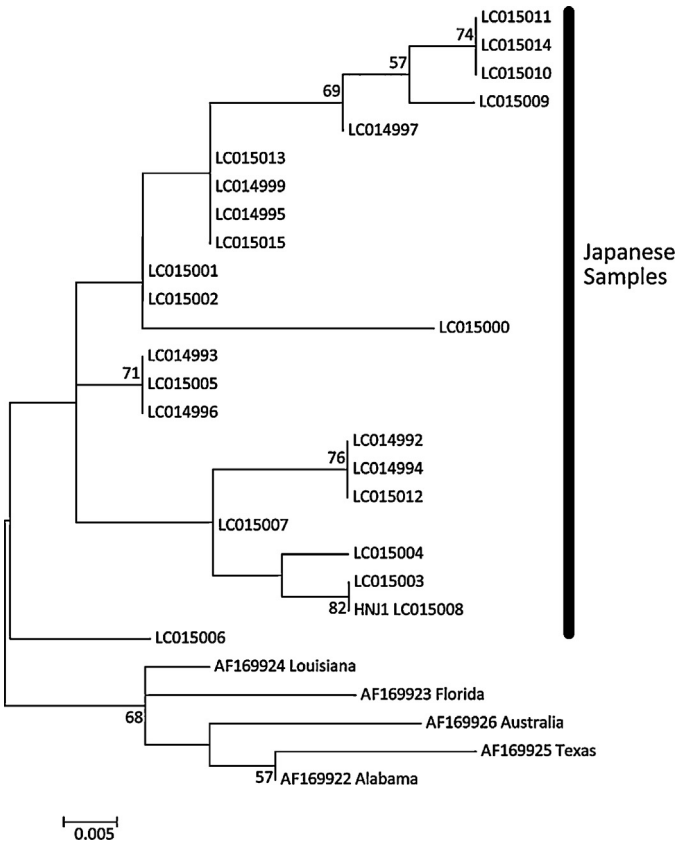

Fig. 1. Phylogenetic tree based on partial sequences of the CSpV1 dsRNA1 sequence from around the world.
Phylogenetic tree based on partial sequences of the CSpV1 dsRNA1 sequence constructed by using the maximum likelihood method using 130 nucleotides without gaps. Substitution model and optional parameters = T92 + Γ. Only bootstrap values >50% from 500 pseudo-replicates are shown.

can trace sampling region, in Fig. 1, we constructed phylogenetic tree which substitution model and optional parameters used is T92 (Tavaré, 1986) + Gamma distribution (five categories) option. CSpV1 sequences from America and Australia were retrieved from the NCBI database, and Japanese CSpV1 sequences were obtained in this study. Most of CSpV1 reference sequences registered with NCBI were partial. Because of the short reference sequences, Fig. 1 has a small amount of information (low-resolution). To acquire more detailed information, we reconstructed a phylogenetic tree with a focus on Japanese samples using longer sequences (1446 bp) with the T92 substitution model incorporating the invariable site and Gamma distribution (five categories) options (Fig. 2). We remove LC014998 sequence because we cannot read full length of its ORF.

Of the 117 fecal samples collected from Ishikari and Kushiro (Hokkaido), 4 samples (3.4%) were positive for *Cryptosporidium*. The parasites in all of the PCR-positive specimens were identified as *C. parvum* and all of the GP60 nucleotide sequences were identical to each other and had similarity with *C. parvum* subtype IIaA15G2R1. Ashoro (Hokkaido) (7 samples), Iwate (5 samples), Okinawa (5 samples) prefectures and Tanegashima Island (2 samples) samples were previously reported and also subtyped *C. parvum* IIaA15G2R1 (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 *C. parvum*, however, CSpV1 sequence