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NOTE

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Discovery of fur seal feces-associated circular DNA virus in swine feces in Japan

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ABSTRACT. Fur seal feces-associated circular ssDNA virus (FSfaCV) was discovered in a pig for the first time in Japan using a next-generation sequencer with duplex-specific nuclease. Full genome of the virus showed approximately 92% similarity to FSfaCVs from New Zealand fur seals. Furthermore, we investigated the prevalence of the ssDNA virus in 85 piglets in Japan, and 65 piglets were positive (76%) for the virus.

KEY WORDS: duplex-specific nuclease, fur seal feces-associated circular ssDNA virus, next-generation sequencer, pig, ssDNA

Recently, novel viruses have been discovered using high-throughput sequencing. Many types of samples are used to detect novel viral genomes, such as whole blood, tissue, feces and cell culture supernatant. It is difficult to obtain viral genomic information from such samples using next-generation sequencing without the appropriate pre-treatment of samples, because the abundant host genome prevents the detection of small amounts of viral genome. Particularly, single-stranded (ss) DNA viral genomes are difficult to detect using next-generation sequencing, because a step of adaptor-ligation to double-stranded DNA ends is required. Duplex-specific nuclease (DSN) is an enzyme extracted from the pancreas of Red King crab (Kamchatka crab) that cleaves the double-stranded (ds) structure of DNA and RNA, but does not cleave single-stranded (ss) DNA, ssRNA and dsRNA [13]. This enzyme can degrade the dsDNA viral genome after DNA extraction, enriching the ssDNA viral genome in the samples. After enrichment of the ssDNA viral genome, phi 29 DNA polymerase, which can conduct template-independent amplification, is useful for amplifying ssDNA viral genomes as dsDNA products. In this study, we used DSN and phi 29 DNA polymerase to enrich ssDNA viral genomes and discovered ssDNA viruses in swine fecal samples. Furthermore, we investigated the prevalence of the ssDNA virus in pigs in Japan.

The swine fecal samples were collected from nine farms in Japan from January to February 2014. We reported discovery of novel porcine rotavirus, astrovirus and posavirus from the same samples [5, 7, 12]. DNA samples were extracted from the supernatants of 10% fecal suspension in sterile phosphate buffer saline using a High Pure Viral Nucleic Acid Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. Eight DNA samples were pooled, and the total amount of DNA was 500 *n*g. In order to digest dsDNA, 1 μl of DSN solution (Evrogen JSC, Moscow, Russia) per each sample was used, and the reaction mixture was incubated for 10 min at 35°C. After DSN treatment, DNA was purified using the Monofas DNA purification Kit I (GL Sciences, Tokyo, Japan) and was eluted with 10 μl of nuclease-free water. The DNA was amplified using the GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Little Chalfont, U.K.) according to the manufacturer's protocol. After purification, a Nextera XT DNA Sample Prep Kit (Illumina, San Diego, CA, U.S.A.) was used for library construction for next-generation sequencing according to the manufacturer's protocol. The constructed DNA library was sequenced in MiSeq bench-top sequencer (Illumina) using the MiSeq Reagent Kit v2 (50 cycles). The reads obtained by deep sequencing were *de novo* assembled using the CLC Genomics Workbench 6.5.1 (CLC, CLC bio, Aarhus, Denmark) with a word size of 50 and threshold for a minimum contig length of 200 bp. Homology searching of contigs was conducted using the BLASTn program on the NCBI website.

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Table 1. As a result of BLASTn of contigs. It shows the highest homology to each contig (Cut off E-value10^{-5})

Contig No.	Length	Description	Accession No.	E-value
Contig 1002	361	Fur seal faeces associated circular DNA virus isolate as50, complete genome	KF246569	1.10E-147
Contig 287	352	Fur seal faeces associated circular DNA virus isolate as50, complete genome	KF246569	4.00E-145
Contig 913	251	Fur seal faeces associated circular DNA virus isolate as50, complete genome	KF246569	1.70E-114
Contig 314	322	Porcine stool-associated circular virus 2 isolate TP3, complete genome	KJ577818	1.70E-112
Contig 262	361	Porcine stool-associated circular virus 3 isolate L2T, complete genome	KC545230	1.75E-63
Contig 249	1,185	Porcine stool-associated circular virus 2 isolate TP3, complete genome	KJ577818	3.09E-60
Contig 691	269	Fur seal faeces associated circular DNA virus isolate as50, complete genome	KF246569	8.37E-33
Contig 415	837	Odonata-associated circular virus-17 isolate OdasCV-17-US-1619LM1-12, complete genome	KM598400	5.12E-24
Contig 98	663	Po-Circo-like virus 21, complete genome	JF713716	1.95E-22
Contig 1	254	Fur seal faeces associated circular DNA virus isolate as50, complete genome	KF246569	7.85E-21
Contig 80	889	Dromedary stool-associated circular ssDNA virus isolate DcSCV_c1566, complete genome	KM573776	8.36E-13
Contig 280	556	Fur seal faeces associated circular DNA virus isolate as50, complete genome	KF246569	9.10E-13
Contig 79	884	Dromedary stool-associated circular ssDNA virus isolate DcSCV_c1566, complete genome	KM573776	4.33E-10
Contig 269	355	Fur seal faeces associated circular DNA virus isolate as50, complete genome	KF246569	1.64E-09

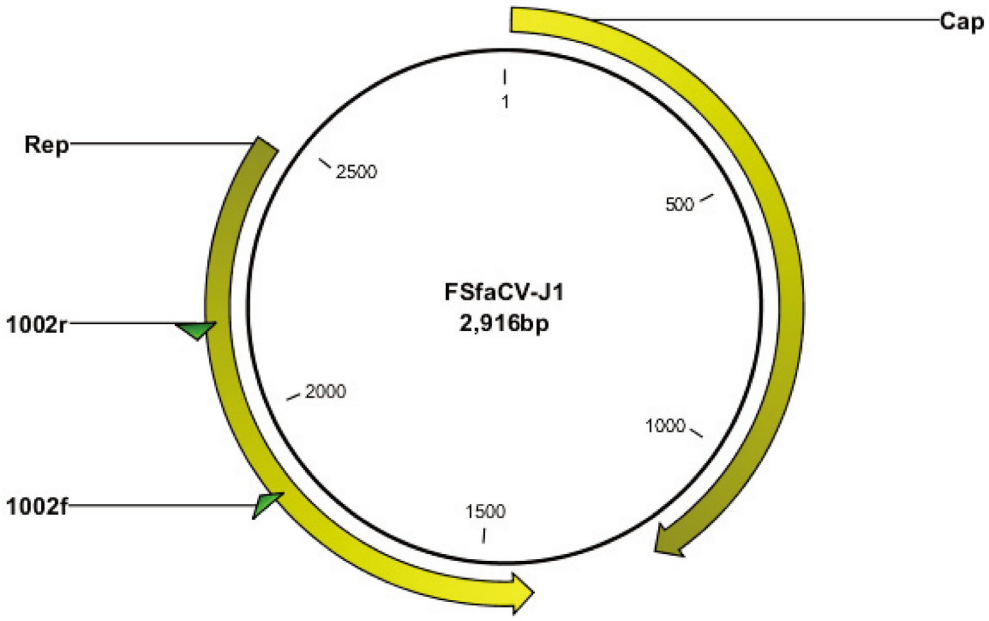

Fig. 1. Genome of FSfaCV-J1. Two open reading frames are indicated by yellow arrows. Two primers designed from the NGS contigs are indicated green arrows. To determine the whole genome sequence of the ssDNA virus, five primer sets were used for overlapping PCR (data not shown). The nucleotide sequences of 1002f and 1002r primers for screening were shown in the text.

In this study, 1,767,619 single reads and 1,113 contigs were obtained. Fourteen contigs were homologous to ssDNA virus with a cut off E-value of 10^{-5} (Table1). The contig with the lowest E-value showed homology to fur seal feces-associated circular ssDNA virus (FSfaCV) (Accession No. KF246569) as shown in Table 1 (the "description" representing the most highly homologous virus for each contig). Overlapping PCR was performed using newly designed primers based on the nucleic acid information obtained from next-generation sequencing (data not shown). The full genome of ssDNA virus showed two open reading frames, which encode replication associated protein and capsid protein (Fig. 1). The sequence was approximately 92% similar to that of FSfaCVs. This result indicates that a virus isolated from the fecal matter of New Zealand fur seals was discovered in a pig for the first time in Japan. This virus was named fur seal feces-associated circular DNA virus JPN1 (FSfaCV-J1), and its nucleotide sequence was deposited in GenBank (Accession No. LC133373).

The prevalence of FSfaCV-J1 in Japanese pigs was investigated by conventional PCR as shown in Fig. 1 (primers: 1002f 5′-ctgtatccgctcgccttgaa-3′ and 1002r 5′-cggagaatttaaagtcattgtcaac-3′). DNA samples, which were extracted from the feces of the 85 unweaned piglets used above, were evaluated for the presence of FSfaCV-J1 by PCR. Sixty-five samples were deemed positive (76%), and all obtained bands confirmed the nucleic acid sequences. There were no differences in the prevalence rate and nucleic acid sequences among the farms (data not shown).

Circovirus is ssDNA virus belonging to the Family *Circoviridae* and has been isolated from various mammals, birds, reptiles, fish and environments [1, 6, 8, 11, 14, 15]. Among porcine circovirus, studies have shown that the porcine circovirus-2 (PCV-2) causes postweaning multisystemic wasting syndrome in weaning pigs [3, 4, 9]. However, there are few reports of other porcine circovirus, because they are thought to have no pathogenicity. In this study, we showed the FSfaCV-J1 infects pigs in Japan. Horizontal infection via the feces is known to be a major transmission route of porcine circovirus 2 [10], and the possibility of the vertical contagion was also supported experimentally [2, 10]. Vertical transmission may be more likely to occur, as FSfaCV-J1 was detected in a pig of day age 0 in this study.

In this study, we discovered fur seal feces-associated circular DNA virus in swine feces. The detection of this virus is reported for the first time in Japan.

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