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Sequencing and Phylogenetic Analyses of Saffold Cardiovirus (SAFV) Genotype 3 Isolates from Children with Upper Respiratory Infection in Gunma, Japan

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Saffold cardiovirus (SAFV) is a newly discovered virus that belongs to family **Picornaviridae** and genus **Cardiovirus** (1). Recent studies suggest that SAFV is relatively common in children with various diseases, with PCR-positive rates varying from 0.5–12% (2–6). For example, SAFV has been detected in stool samples from children with respiratory tract infections, gastroenteritis, and non-polio acute flaccid paralysis cases (2–6). Recently, we showed that SAFV genotype 2 was associated with typical exudative tonsillitis in Japanese children (7). However, as this virus is also detected in asymptomatic humans, the pathogenicity of SAFV is not yet clearly understood (5). In addition, Zoll et al. suggested that different SAFV strains may also exhibit different in vitro tropisms (6), thus suggesting that it may be relatively difficult to isolate SAFV using culture methods, which may explain why few studies have been conducted to date on its isolates (6). Herein we report the isolation of two strains of SAFV genotype 3 from children with acute respiratory infections, and the results of sequence and phylogenetic analyses (VP1 coding region) of these strains.

We analyzed nasopharyngeal samples from two male patients, aged 5 and 6 years, who presented with fever (>38°C), canker sores, and upper respiratory inflammation. A pediatrician had diagnosed upper respiratory infection (URI) with sinusitis, pharyngitis, and laryngitis (8). The samples were obtained by the local health authority of Gunma Prefecture in 2008 for the surveillance of viral diseases in Japan. Informed consent was obtained from the parents of both subjects for donation of the nasopharyngeal samples used in this analysis.

The throat swab samples were centrifuged at 3,000 × g at 4°C for 30 min and the supernatants used for virus isolation. Cell lines (HEp-2, Vero, MDCK, A549, and RD18s cells) were inoculated and subjected to three freeze/thaw cycles.

The observation of enteroirus-like cytopathic effects (CPEs), such as collapsed balloons and small fragmented cells floating in the medium inoculated with HEp-2 cells (Fig. 1), led us to initially suspect another virus such as an enteroirus. However, we failed to amplify the VP1 and VP4 coding region of the hypothetical enteroirus in culture supernatants by RT-PCR with broad detectable primers (9,10). Next, we carried out a
Fig. 1. Cytopathic effects of Saffold cardiovirus (SAFV) type 3 in HEp-2 cells. (A) No infection. (B) SAFV type 3 infected-HEp–2 cells. Images were taken following three passages after specimen (throat swab) inoculation.

Fig. 2. A phylogenetic tree of SAFV type 2 based on the VP1 coding region (327 nt). The phylogenetic tree was constructed using the VP1 coding region. Numbers in parentheses are the GenBank accession numbers.

comprehensive sequencing (using the RDV method) for viral RNA determination, as described previously (11). The partial viral nucleotide sequences obtained upon analysis of the sequence data with BLAST (11) suggested SAFV. Finally, we analyzed the VP1 coding region of SAFV, as described previously (7). Briefly, viral RNA was extracted from 140 μL of the supernatants using a QIAamp Viral RNA Mini Kit (Qiagen, Germantown, Md., USA) and the RT-PCR procedure performed according to the manufacturer’s instructions (One-step RT PCR kit; Qiagen). The primers for RTPCR (for amplification of the VP1 coding region) were as follows: 5’-HAA RCA RGR YTG GAR YTT YNT NAT GTT-3’ (primer 315F) and 5’-DGG BCK DGG RCA RWA VAC YCT CAT-3’ (primer 738R) (7). The following protocol was used: incubation for 30 min at 50°C, 15 min at 95°C, followed by 40 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, ending with elongation for an additional 5 min at 72°C. The DNA fragments were purified using a QIAquick PCR Purification kit (Qiagen) and the nucleotide sequence determined using an automated DNA sequencer (7). Amplificons were sequenced and aligned (327 bp), then analyzed phylogenetically using the CLUSTAL W program on the website of DNA Data Bank of Japan (DDBJ; http://hypermag.nig.ac.jp/homology/clustalw-e.shtml) and TreeExplorer (version 2.12) (http://evolgen.biometro-u.ac.jp/TE/). Evolutionary distances were estimated using Kimura’s two-parameter method and the phylogenetic trees were constructed using the neighbor-joining (N-J) method (12). The reliability of the tree was estimated using 1,000 bootstrap replications.

Phylogenetic analysis based on the VP1 coding region revealed two strain clusters in the SAFV genotype 3 (Fig. 2), with an interstrain nucleotide identity of 100%. The high diversity of SAFV is highlighted by the identification of at least eight VP1 coding regions (5). No pathogen other than SAFV was isolated using the cell-culture method.

Although a wide range of viruses is known to cause respiratory infections, the etiology of 20–30% of these currently remains unidentified (13). This may be due to the lack of sensitivity of some of the detection methods used, and it has also been suggested that unrecognized infectious agents, including other viruses, have yet to be discovered (14). Viral diagnosis is mostly based on clinical symptoms; thus, it is important to collect appropriate clinical samples early in the infection process, and the type of sample collected is somewhat dependent on the clinical symptoms. In this study, we succeeded in isolating SAFV from the throat swabs of two URI patients, thus we suppose that the fever and URI were associated with SAFV. Using the tissue culture method, we isolated 67 viruses from 124 samples taken during 2008 in Gunma Prefecture for the surveillance of viral diseases. Although 39 strains of influenza virus were isolated, only two of these were SAFV. This could suggest that SAFV is seldom the cause of URI, although much larger numbers of strains will be required to address its epidemiology more comprehensively.

In conclusion, SAFV might be a causative agent of acute respiratory infection. A previous report has suggested that SAFVs are ubiquitous human viruses with a global prevalence, therefore SAFV might be the cause in samples with enterovirus-like CPEs (6). The growth characteristics of our strains are similar to those seen
previously in other slow-growing SAFVs (5), and the HEp-2 cell line has been shown to be an effective cell line for further research into SAFV, including clarification of its epidemiology and etiology, although blind passages may be necessary to isolate the virus (15). A more detailed analysis, including serological surveys of SAFV, is needed to determine its exact role in human disease.

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Conflict of interest None to declare.

REFERENCES