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Isolation of Saffold Virus Type 2 from Children with Acute Respiratory Infections by Using the RD-18S-Niigata Cell Line

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In 2007, Saffold virus (SAFV) was recovered from the fecal specimens obtained from an 8-month-old girl with fever of unknown origin and was classified as a member of the genus Cardiovirus, family Picornaviridae (1–3). Since then, there have been a number of reports of new SAFV genotypes. Naeem et al. recently characterized 11 genotypes, including 3 new genotypes, based on analysis of the complete virus protein (VP) 1 region (4–7). Among these 11 genotypes, SAFV types 2 and 3 (SAFV-2 and SAFV-3) are particularly common, causing widespread human viral infections in early childhood (2,3,8–10). SAFVs have been detected in children presenting with respiratory tract infections, gastroenteritis, neurological diseases, and non-polio acute flaccid paralysis (2–6,11–14). However, the epidemiological characteristics, distribution, prevalence, diversity, disease spectrum, and pathogenic role of SAFVs are still not fully understood; indeed, the description of SAFVs only after the addition of monoclonal anti-cytokine antibodies (20). Although they analyzed viral tropism in vitro by using the HTCV-UC6 strain and 11 cell lines, they observed that viral replication was quite limited (20). Hertzler et al. reported the successful adaptation of SAFV-2 to a limited number of cell lines, such as HeLa and U118MG, after repeated passaging in 14 human, non-human, and rodent cell lines (19). Galama et al. and Blomqvist et al. reported that the FIN2008 (or FIN08-13B) strain, which was the first SAFV-2 strain to grow well in green monkey kidney and HeLa cell lines, was needed to carry out seroepidemiological studies because most SAFV-2 isolates from their and other laboratories grew poorly in cell cultures, thus hampering the detection of neutralization (8,18).

In Niigata in 2013, we inoculated 539 clinical specimens onto 6 cell lines: MDCK, Caco-2, LLC-MK2, Vero9013, RD-18S-Niigata (RD-18S-N), and A549. We succeeded in isolating 5 strains of SAFV by using the RD-18S-N cell line alone (21). The culture conditions for the RD-18S-N cell line are described in Table 1. Subsequently, we confirmed the isolates as SAFV-2 by reverse transcription PCR (RT-PCR) and sequencing (21).

We next used the RD-18S-N cell line to try to isolate SAFV-2 from the RT-PCR-positive specimens analyzed in Yamagata in 2009. Prior to this attempt, we had cultured the RD-18S-Y and RD-18S-N cell lines independently in Yamagata and Niigata.

In Yamagata, we prepared a 96-well microplate (Greiner Bio-One, Frickenhausen, Germany) of the RD-18S-N cell line. The RD-18S-N cell line was cultured using the growth medium for the RD-18S-Y cell line (Table 1). When the cell monolayer was ready for specimen inoculation, we discarded the growth medium, washed the monolayer with phosphate-buffered saline without calcium or magnesium (−PBS), and added the maintenance medium, which was identical to the growth medium, except that the final concentration of fetal bovine serum was 2%. We then inoculated the cells with the frozen-stocked nasopharyngeal specimens found to be SAFV-2-positive by RT-PCR in our previous study (13). We observed the specimen-inoculated cells 2–3 times per week. When we found picornavirus-like CPEs, we moved on to the next RT-PCR step for sequence analysis. When no CPE was observed, we transferred the inoculated culture fluid onto a freshly prepared plate until the 4th passage.

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were incubated at 37°C for 60 min at 37°C with a multiplicity of infection (MOI) of 0.001 and incubated were infected with the representative SAFV-2 isolate at a multiplicity of infection (MOI) of 0.001 and incubated were infected with the representative SAFV-2 isolate (1801-Yamagata-2009). In 2009, demonstrating that it is difficult to distinguish between the RD-18S-N and RD-18S-Y cell lines by using a phylogenetic tree that was constructed using the neighbor-joining method as reported previously (13,22). For sequence analysis, we used not only primers 315F, 738R, 316F, and 621R, which were used in the previous study (13,22) but also CardioVP1-1F and CardioVP1-4R (16) to obtain the whole VP1 sequences. Sequence data were added to GenBank under accession numbers LC026114–LC026131. When the sequence could not be amplified using the above-mentioned primers for SAFVs, we tried to amplify it with primers 9565-reverse, 9895-forward, AN88, AN89, 011, 101, 012, and 040 for rhinovirus and enterovirus (23–25).

We thereby inoculated a total of 49 specimens into the RD-18S-N cells. Using these cells, we found picornavirus-like CPEs and finally succeeded in isolating SAFV-2 from 18 of the 33 specimens (54.5%) found to be positive by the 1st PCR in the previous study (13), whereas we failed to isolate any SAFV-2 strain from the 16 specimens found to be positive by nested PCR in the previous study (13).

We failed to amplify viral genomes using primers for SAFVs in 4 specimens that showed a picornavirus-like CPE. However, we succeeded in amplifying the viral genomes by using primers for rhinovirus and enterovirus, and we finally identified these viruses as rhinovirus B (1 case), coxsackievirus A3 (1 case), and coxsackievirus A4 (2 cases). Among these 4 viruses, the rhinovirus B, the coxsackievirus A3, and 1 coxsackievirus A4 had already been isolated and identified using the RD-18S-Y cell line (Fig. 2D), whereas no picornavirus-like CPEs were observed in the RD-18S-Y cell line (Fig. 1). Our findings suggest that the RD-18S-N cell line has higher sensitivity to SAFV-2 than does the RD-18S-Y cell line, although the reason for this is unknown. The failure to isolate virus from clinical specimens using the RD-18S-Y cell line in 2009 (13) may have been due to low titers in the specimens and the low sensitivity of the RD-18S-Y cell line.

Himeda et al. (27) reported that the susceptibility of HeLa cells to SAFV-3 was variable under different culture conditions because of differences in receptor expression. We have cultured the RD-18S-N and RD-18S-Y cell lines under different conditions, with different antibiotics (Table 1), independently in Yamagata and Niigata for a long time. Therefore, as reported previously (27), it is possible that there are differences in receptor expression between the RD-18S cell lines, which in turn may alter the sensitivity to SAFV-2.

Here, we report the successful isolation of SAFV-2 strains that have been generally difficult to grow using tissue culture methods and which we failed to isolate in our previous trial in 2009. We strongly agree that the availability of SAFV isolates that can be propagated to high titers is crucial to future epidemiological studies (18), as we ourselves have experienced in another epidemiological study related to human parechovirus type 3 infections (28). Indeed, the isolation of SAFV-2 enabled analysis of the whole VP1 region, whereas we observed cells under a light microscope, and supernatants were collected at 24, 48, 72, 96, 120, 144, and 168 h post-inoculation (p. i.) and kept at −80°C until they were used for infectivity assays. Infectivity assays were performed using the end-point dilution method with the RD-18S-N cell line. Briefly, 50 μl aliquots of 10-fold serial dilutions of each supernatant were inoculated onto the monolayer in a 96-well plate, and cells were incubated and observed for the presence of a CPE. We used 4 wells per dilution, and the 50% tissue culture infectious dose (TCID50) of each supernatant was calculated by the Kärber method (26).

As shown in Fig. 1, virus titers in the RD-18S-N cell line increased more rapidly than those in the RD-18S-Y cell line. The yields of SAFV-2 in the RD-18S-N cell line were more than 100-fold higher than those in the RD-18S-Y cell line at 120 h p.i. We observed clear CPEs in the RD-18S-N cell line (Fig. 2A and 2B), whereas no picornavirus-like CPEs were observed in the RD-18S-Y cell line (Fig. 2D); however, SAFV-2 grew less efficiently in the RD-18S-Y cell line (Fig. 1). Our findings suggest that the RD-18S-N cell line has higher sensitivity to SAFV-2 than does the RD-18S-Y cell line, although the reason for this is unknown. The failure to isolate virus from clinical specimens using the RD-18S-Y cell line in 2009 (13) may have been due to low titers in the specimens and the low sensitivity of the RD-18S-Y cell line.

For sequence analysis, viral RNA was extracted from 200 μl of culture fluid by using a High Pure Viral RNA Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. RT and subsequent amplification of the VP1 region of SAFV-2 were performed as previously described (13,22). Amplicons were sequenced and the genotype of each amplicon was determined from a phylogenetic tree that was constructed using the neighbor-joining method as reported previously (13,22). For sequence analysis, we used not only primers 315F, 738R, 316F, and 621R, which were used in the previous study (13,22) but also CardioVP1-1F and CardioVP1-4R (16) to obtain the whole VP1 sequences. Sequence data were added to GenBank under accession numbers LC026114–LC026131. When the sequence could not be amplified using the above-mentioned primers for SAFVs, we tried to amplify it with primers 9565-reverse, 9895-forward, AN88, AN89, 011, 012, and 040 for rhinovirus and enterovirus (23–25).
Fig. 1. Growth of SAFV-2 on RD-18S-N and RD-18S-Y cell lines. RD-18S-N (solid line) and RD-18S-Y (broken line) cell lines were infected with the SAFV-2 isolate (1801-Yamagata-2009) at an MOI of 0.001 and incubated at 37°C for the indicated periods. The virus yield in the supernatants was titrated on RD-18S-N cell line.

Fig. 2. The cytopathic effect (CPE) in the RD-18S-N cell line (A, B) and a non-infected control (C). After inoculation with the SAFV-2 isolate (1801-Yamagata-2009) at an MOI of 0.001, the CPE progressed and picornavirus-like changes, such as rounding formation, cell destruction, and detachment, were observed (A: 72 h, B: 168 h post-inoculation). Image of control cells without inoculation at 168 h (C). In contrast, no CPE was observed in the RD-18S-Y cell line at 168 h post-inoculation, even after inoculation with the same amount of viral fluid (D). Magnification ×100.

analyzing clinical specimens directly (13,22).

Now that we can isolate SAFV-2 using the RD-18S-N cell line, we can continue to analyze the longitudinal epidemiology of SAFV-2 based on virus isolation, plan seroepidemiological studies using the SAFV-2 isolates as a challenge virus antigen, and attempt to isolate other SAFV serotypes.

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Conflict of interest None to declare.

REFERENCES