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Summary

Isolation of seasonal coronaviruses, which include human coronavirus (HCoV) OC43, HCoV-HKU1, and HCoV-NL63, from primary cultures is difficult because it requires experienced handling, an exception being HCoV-229E, which can be isolated using cell lines such as RD-18S and HeLa-ACE2-TMPRSS2. We aimed to isolate seasonal CoVs in Yamagata, Japan to obtain infective virions useful for further research and to accelerate fundamental studies on HCoVs and SARS-CoV-2. Using modified air-liquid interface (ALI) culture of the normal human airway epithelium from earlier studies, we isolated 29 HCoVs (80.6%: 16, 6, 6, and 1 isolates of HCoV-OC43, HCoV-HKU1, HCoV-NL63, and HCoV-229E, respectively) from 36 cryopreserved nasopharyngeal specimens. In ALI cultures of HCoV-OC43 and HCoV-NL63, the harvested medium contained more than $1 \times 10^4$ genome copies/µL at every tested time point during the more than 100 days of culture. Four isolates of HCoV-NL63 were further subcultured and successfully propagated in an LLC-MK2 cell line. Our results suggest that ALI culture is useful for isolating seasonal CoVs and sustainably obtaining HCoV-OC43 and HCoV-NL63 virions. Furthermore, the LLC-MK2 cell line in combination with ALI cultures can be used for the large-scale culturing of HCoV-NL63. Further investigations are necessary to develop methods for culturing difficult-to-culture seasonal CoVs in cell lines.
Introduction

The alphacoronaviruses human coronavirus (HCoV) 229E and HCoV-NL63 and the betacoronaviruses HCoV-OC43 and HCoV-HKU1, which we hereafter designate as seasonal CoVs, are known to be the causative agents of common cold that is mainly prevalent in winter (1–3). Earlier studies have indicated annual epidemics of the seasonal CoV infection (4–8). In contrast, fundamental studies related to the seasonal CoV isolates have not been performed sufficiently due to difficulties in virus isolation (9). To accelerate HCoV-related research, development of effective isolation methods for the seasonal CoVs is required.

Non-immortalized primary cells which are derived from the organs of animals or humans are suitable for the isolation of seasonal CoVs (10–13). Dijikman et al. reported that the air-liquid interface (ALI) culture of the primary human airway epithelium (HAE) can be used to isolate all seasonal CoVs (9). However, the ALI culture is cumbersome, time-consuming, and is not suitable for large-scale culturing. Therefore, the seasonal CoVs isolated by the ALI culture are rarely used in viral research (14–18).

To promote the study of coronaviruses, it is necessary to produce these viruses in large quantities using cell culture systems. However, cell culture systems capable of efficiently propagating coronaviruses are limited. Additionally, among the clinical
isolates, only HCoV-229E can be procured from several research institutes in Japan (19–21). Earlier studies have reported that HCoV-NL63 isolated using primary monkey kidney cells could also propagate in LLC-MK2, Vero, and CaCo2 cell lines (12, 22, 23). However, it is not clear if HCoV-NL63 isolated using ALI culture can propagate in cell lines. As for HCoV-OC43, a laboratory strain adapted to the HRT-18 cell line is commercially available; however, the genotype of this strain is different from that of the strains responsible for the current epidemic (24, 25). To the best of our knowledge, currently none of the available strains of HCoV-HKU1 can be cultured using cell lines.

The Yamagata Prefectural Institute of Public Health (YPIPH) in Yamagata City, Japan, has performed epidemiological studies on viral infectious diseases based on the virus strains isolated at the institute (26). To date, we have performed epidemiological studies on seasonal CoVs (5–7) and have tried to isolate seasonal CoVs (19, 20). Between April 2020 and September 2020, scientists from several institutes and commercial companies conducting research on SARS-CoV-2 asked us to share clinical isolates of seasonal CoVs. However, we could only provide clinical strains of HCoV-229E isolated using RD-18S or HeLa-ACE2-TMPRSS2 cells but no other seasonal CoVs (19, 20, 26).

In this study, we aimed to culture seasonal CoVs from cryopreserved clinical specimens using ALI culture and a LLC-MK2 cell line, with special focus on isolating
HCoV-OC43, HCoV-NL63, and HCoV-HKU1, which have never been previously isolated at YPIPH. This study was designed to isolate the clinical isolates of seasonal CoVs that would be useful for fundamental studies on HCoVs and SARS-CoV-2.

Materials and Methods

Specimens

Nasopharyngeal specimens positive for HCoV genomic RNA, which were collected during January to April of 2009, 2010–2019, and January to March of 2020, were used for this study (5–7).

Isolation of the seasonal CoVs using ALI culture

A modified method of ALI culture (9, 15) was used in this study. Seven steps and approximately 6 weeks were necessary to obtain the seasonal CoVs (Fig. 1).

1) Expansion of cells from frozen stock

Primary normal human bronchial epithelial (NHBE) cells (#CC-2540S, Lonza, Basel, Switzerland) were used as the HAE in this study. One cryovial of NHBE cells was seeded in a T-75 flask (#90076, TPP AG, Trasadingen, Switzerland) with expansion medium (preparation mentioned below). The cells were incubated at 37°C in a 5% CO₂ incubator, and the expansion medium was renewed every 2 days. The confluency of cells
was maintained at less than 80% to prevent loss of differentiation ability. The cells were detached using trypsin-EDTA (#07910, STEMCELL Technologies Inc., Vancouver, Canada) adjusted to 0.025% with Hanks balanced salt solution (HBSS) without Ca\(^{2+}\) and Mg\(^{2+}\) (#37250, STEMCELL Technologies) and then neutralized with Trypsin inhibitor (#T6522, Sigma-Aldrich, St. Louis, MO). Detached cells were passaged twice in a T-25 flask (#90026, TPP). The expanded cells were harvested for use in ALI culture. The rest of the cells were cryopreserved according to the instructions for the cryopreservation of Clonetics™ & Poietics™ primary cells (Lonza).

The expansion medium was prepared by mixing 500 mL of PneumaCult™-Ex (#05008, STEMCELL Technologies) with hydrocortisone (final concentration: 0.096 µg/mL, #07904, STEMCELL Technologies). Aliquots were refrigerated until use. For preparing the maintenance medium, two of the three components of PneumaCult™-ALI (#05001, STEMCELL Technologies), i.e., basal medium and 10× supplement, were mixed and refrigerated. At the time of use, hydrocortisone (final concentration: 0.48 µg/mL), heparin sodium (final concentration: 4 µg/mL, # 07980, STEMCELL Technologies), and the third PneumaCult™-ALI component (maintenance supplement) were added to the thawed aliquot to prepare the complete maintenance medium. Media were filtered using a 0.2 µm filter (#S-1302, Kurabo Industries Ltd., Osaka, Japan) before
(2) Seeding in inserts and renewing media

To establish the ALI culture, 40,000 cells in 100 μL expansion medium were seeded onto the apical chamber of a 6.5 mm Transwell-Clear polyester membrane insert containing 0.4 μm pores (#3740, Corning Incorporated, Corning, NY). The insert was placed in a well of 24-well tissue culture test plate (#92424, TPP) containing 550 μL expansion medium. Media of the apical and basal chambers were renewed three times per week until the cells became overly confluent.

(3) Airlifting and renewing basal medium

To initiate the ALI culture, media in the apical and basal chambers were removed and rinsed with Dulbecco’s phosphate buffered saline without Ca²⁺ and Mg²⁺ (#37350, STEMCELL Technologies). To expose the apical side of cells to air (airlifting), 550 μL of maintenance medium was added only to the basal chamber. The cells were incubated at 37°C in a 5% CO₂ incubator, and the medium was renewed three times (first week), then two times per week to allow the cells to differentiate into pseudostratified airway epithelial cells.

(4) Mucociliary differentiation

Approximately 2 weeks post-airlifting when movement of cilia became visible
microscopically, the apical surface of the cells was rinsed with HBSS to remove excess mucus. Media renewal and rinsing were repeated for 1 month post-airlifting.

(5) Inoculation of specimen

After centrifuging the specimen for 15 min at 400 \( \times \) g, 200 \( \mu \)L of the supernatant was inoculated onto the apical surface of ALI culture of the NHBE cells. After incubating for 2 h at 33°C in a 5% CO\(_2\) incubator, the inoculant was removed, and the apical surface was rinsed three times with 300 \( \mu \)L HBSS.

(6) Basal medium renewal

The inoculated culture was maintained at 33°C in a 5% CO\(_2\) incubator, and basal medium was renewed once a week.

(7) Harvesting

Apical harvesting was performed by adding 200 \( \mu \)L HBSS on the apical surface. After incubating for 10 min, HBSS was collected; 50 \( \mu \)L of the harvested HBSS was used to calculate viral yields. Rest of the HBSS was mixed with equal volumes of viral transport medium (minimum essential medium containing 0.5% gelatin, 100 units of penicillin, and 100 \( \mu \)g of streptomycin per mL) and stored at -80°C.

**Measurement of viral yields**

To confirm virus replication in the NHBE cells, we measured the number of viral
genome copies in harvested media as previously reported (6). Briefly, viral RNA was extracted and reverse transcribed into cDNA. Then, the cDNA was assayed by real-time PCR. We assumed that isolation was achieved when viral genome copies of a medium was higher than that of the inoculant.

**Subculturing HCoV-NL63 in LLC-MK2**

HCoV-NL63 isolated using the ALI culture was subcultured in the LLC-MK2-N cell line (26), which was seeded in a T-25 flask, and a confluent monolayer was maintained using 3 mL of minimum essential medium containing 5% fetal bovine serum. Medium was renewed 6-8 days post-infection. Infected cells were scraped and passaged to LLC-MK2-N in a 24-well tissue culture test plate 14-15 days after infection; passaging was performed weekly. Microscopic images of the cells were obtained using a microscope (ECLIPSE Ti-U; Nikon Corporation, Tokyo, Japan) and a software (NIS-Elements D version 4.20.00; Nikon). LLC-MK2-N cells were also passaged; these cells were grown on a round cover slip (#C015001, Matsunami glass industries limited, Osaka, Japan) in a 24-well tissue culture test plate for immunostaining.

**Immunofluorescence**

Immunofluorescence was performed as previously described to detect HCoV-NL63 in LLC-MK2-N (27). Cells were stained primarily with the anti-spike protein of
HCoV-NL63 polyclonal antibody, generated by immunizing rabbits with polypeptide, diluted 1:500 (SCRUM incorporated, Tokyo, Japan: courtesy of Dr. Shutoku Matsuyama), and secondarily with FITC-conjugated swine anti-rabbit IgG polyclonal antibody, diluted 1:40 (# F0205, Agilent Technologies inc., Santa Clara, CA).

**Statistical analysis**

To compare medians of viral yields of ALI culture between adjacent divisions of days’ post inoculation, Wilcoxon rank sum test adjusted with Holm’s method was applied to base 10 logarithm of the number of viral genome copies. We considered $P < 0.05$ as statistically significant. Statistical analysis was performed using R, version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria).

**Ethical considerations**

This study was approved by the ethics committees of the Yamagata University Faculty of Medicine (approval no. 2020-52) and the Yamagata Prefectural Institute of Public Health (approval no. YPIPHEC 20-08).

**Results**

**Isolation of the seasonal CoVs using ALI culture of NHBE cells**

Overall, seasonal CoVs were isolated from 29 (80.6%) out of 36 specimens using
ALI culture. Specifically, viral genomes were amplified in 16 of 20, 6 of 6, 6 of 9, and 1 of 1 cultures inoculated with HCoV-OC43, HCoV-HKU1, HCoV-NL63, and HCoV-229E positive specimens, respectively (Fig. 2). As for HCoV-229E, only one specimen was inoculated because we had previously established isolation methods using cell lines (19, 20). Among nasopharyngeal specimens with successful isolation, minimum genome copies/µL of medium were 3.1×10¹, 6.7×10¹, 6.9×10¹, and 3.5×10³ for HCoV-OC43, HCoV-HKU1, HCoV-NL63, and HCoV-229E, respectively. To confirm the production of infective virions using ALI culture of NHBE cells, we tested the harvested media obtained from 2018-0238, 2018-0029, 2020-0480, and 2018-0330 for representative specimens of HCoV-OC43, HCoV-HKU1, HCoV-NL63, and HCoV-229E, respectively. The harvested media were inoculated in another ALI culture of NHBE cells to verify the presence of virions, and the viral genomes were amplified (data not shown). Immunocytochemically, virus production was confirmed by immunofluorescent staining of HCoV-OC43 on the porous insert (data not shown).

To investigate long-term virus production, we observed the course of ALI culture for the representative specimens HCoV-OC43, HCoV-HKU1, and HCoV-NL63 (Fig. 3). We maintained one generation of HAE and harvested from it repeatedly. The results showed that HCoV-OC43 and HCoV-NL63 viral yields of more than 1×10⁴ genome
copies/µL of medium were maintained for more than 100 days. In contrast, HCoV-HKU1 showed decreasing viral yield after the 12th day post inoculation.

**Subculturing of HCoV-NL63 isolated by ALI culture using cell lines**

Media of HCoV-NL63 isolates obtained from specimens 2012-1966, 2016-0854, 2018-0597, 2018-0501, 2019-0554, and 2020-0480 by ALI culture was subcultured in LLC-MK2-N. The genome of the virions from the harvested media of the LLC-MK2-N cells was amplified after the first passage, except from the cultures of the specimens 2018-0501 and 2019-0554 (data not shown). In the second passage, viral yield of each strain was between 5.9×10⁵ and 1.8×10⁸ genome copies/µL of medium on the 6th day. The number of passages after which syncytium formation was observed first was different for each specimen. After the third passage of 2018-0597, syncytium formation appeared on the 4th day post passage and spread over the entire culture with progress in time (Fig. 4A). Immunofluorescent staining revealed that some LLC-MK2-N cells were stained positive with anti-HCoV-NL63 antibodies on the 5th day post-infection (Fig. 4B).

**Isolation and propagation of seasonal CoVs in YPIPH**

Table 1 shows the summary of the isolation and propagation of seasonal CoVs in YPIPH as of August 2020. We have isolated four seasonal CoVs using the ALI culture and further cultured HCoV-NL63 using the LLC-MK2-N cell line in this study. HCoV-
229E isolated using the ALI culture was also subcultured in the HeLa-ACE2-TMPRSS2 cell line and viral genome was amplified (data not shown). Additionally, we have already established the isolation of HCoV-229E using RD-18S and HeLa-ACE2-TMPRSS2 cell lines in earlier studies (19, 20). As of September 2020, we have not found a cell line that can be used to isolate HCoV-OC43, HCoV-HKU1, or HCoV-NL63 from clinical specimens. We also have not found a cell line that can propagate HCoV-OC43 or HCoV-HKU1 that was isolated using ALI culture.

**Discussion**

From this study, we demonstrated that ALI culture is a remarkable method to isolate seasonal CoVs from clinical specimens. ALI cultures that were inoculated with a HCoV-OC43- or HCoV-NL63-positive specimen continued to produce virus for more than 100 days. Moreover, HCoV-NL63 isolated using ALI culture could propagate in LLC-MK2-N cell lines. Our results showed that the combination of ALI culture and subculturing using cell lines might be applicable for culturing difficult-to-culture HCoVs. These results could accelerate research on HCoVs and SARS-CoV-2 (e.g., developing highly specific rapid antigen detection tests for SARS-CoV-2 and studying cross-immunity between seasonal CoVs and SARS-CoV-2), which is currently the need of the hour.
The ALI culture of NHBE cells was useful for isolating seasonal CoVs from clinical specimens. In this study, NHBE cells, which were differentiated into pseudostratified airway epithelium, could multiply the seasonal CoVs with a high success rate, and viral genome copies of HCoV-OC43 and HCoV-NL63 were significantly amplified in cultures showing the presence of these viruses in the first week (Fig. 2). Furthermore, our passaging and immunocytochemical approaches suggested the replication of these infective virions. Generally, virus isolation aids in developing diagnostic methods for detecting viral diseases, antiviral drugs, vaccines, etc. (26). Although preparation of differentiated NHBE cells needs experience, delicate handling, and long periods, optimized ALI culture of NHBE cells will be a candidate for performing further HCoV research using infective virions. Our method, which uses commercially available NHBE cells and media, might be practical for laboratories in which obtaining resected lung tissues to prepare HAE is difficult. Moreover, our detailed protocols, which were designed for the materials used in this study, might help researchers reproduce the isolation of HCoVs.

ALI culture of NHBE cells might supply HCoV-OC43 and HCoV-NL63 clinical isolates over 100 days (Fig. 3). Given that the seasonal CoVs are not likely to kill NHBE cells (9), NHBE cells may survive for a long period. An earlier study suggested that
HCoV-OC43 infection did not affect the integrity of mature HAE, which was constructed using nasal epithelium, because HCoV-OC43 induces the production of cytokines weaker than the cytokine production induced by other respiratory viruses tested (28). In contrast, viral yields of ALI culture with a HCoV-HKU1 positive specimen decreased within a short period (Fig. 3). Further investigation is needed to elucidate the different methods of culturing seasonal CoVs.

LLC-MK2-N cell line in combination with NHBE cells can be utilized for large-scale culturing of HCoV-NL63. Given that HCoV-NL63 isolation using ALI culture had some advantages (e.g., increase in the number of infective virions, changes in viral properties) over propagation using cell lines, isolation using primary cells might be necessary for the large-scale culturing of HCoV-NL63. HCoV-NL63-induced syncytium formation in LLC-MK2-N cells (a read-out of viral amplification), can lead to easy culturing of HCoV-NL63 (Fig. 4). Hence, we can culture the alphacoronaviruses, HCoV-229E and HCoV-NL63 on a large-scale using cell lines (Table 1). However, we cannot culture the betacoronaviruses HCoV-OC43 and HCoV-HKU1 using cell lines, in contrast to another betacoronavirus, SARS-CoV-2, which can be isolated using cell lines (29). Cryopreserved HCoV-OC43 and HCoV-HKU1 isolated using ALI culture are expected to be useful for further experiments to explore permissive cell lines for propagation.
Hereafter, HCT-8, Huh-7, Calu-3, and other kidney-originated cell lines could be used as target cells for culturing HCoV-OC43 and HCoV-HKU1 (24, 27, 30, 31).

This study has three limitations. First, genomic mutations in the seasonal CoVs during ALI culture was not checked. Comparison of viral properties between different time points was also not done. Whole genome sequencing is expected to elucidate the course of viral genome mutation, especially for the long-term observation of HCoV-OC43 and HCoV-NL63 isolates in ALI culture (Fig. 3). Second, we did not evaluate infectious titers of specimens and harvested media. To evaluate the efficiency of isolation by ALI culture more precisely, infectious titer of viral yields should be unified. Finally, the sampling days for the ALI cultures varied due to difficulties in obtaining and maintaining optimized ALI cultures (Fig. 2). Accordingly, we measured the viral yields of the seasonal CoVs by dividing days post inoculation into sets of every 3 days.

In conclusion, the ALI culture of NHBE cells was useful for isolating four seasonal CoVs, i.e., HCoV-OC43, HCoV-HKU1, HCoV-NL63, and HCoV-229E from nasopharyngeal specimens and might be also used to produce HCoV-OC43 and HCoV-NL63 over 100 days. Moreover, the LLC-MK2-N cell line in combination with ALI cultures can be utilized for the large-scale culturing of HCoV-NL63. Further experiments aimed at isolating (and propagating) seasonal CoVs from clinical specimens must be
performed to accelerate fundamental infective virion-based studies on HCoVs.

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**Conflict of Interest**

None to declare.
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Figure legends

**Fig. 1** Schematic representation of air-liquid interface (ALI) culture procedure of normal human bronchial epithelial (NHBE) cells. Porous insert with 0.4 µm pores in the membrane is the key component of ALI culture. Approximately 4–6 weeks are necessary for the optimization of NHBE cell growth, and inoculation and harvesting of human coronaviruses OC43, HKU1, NL63, and 229E.

**Fig. 2** Time course of viral yields of air-liquid interface (ALI) culture of normal human bronchial epithelial cell inoculated with human coronavirus (HCoV) positive nasopharyngeal specimens: A; HCoV-OC43 (n = 16), B; HCoV-HKU1 (n = 6), C; HCoV-NL63 (n = 6), D; HCoV-229E (n = 1). *P < 0.05.

**Fig. 3** Long-term observation of viral yields of air-liquid interface culture of normal human bronchial epithelial cells inoculated with human coronaviruses’ OC43, HKU1, and NL63 positive nasopharyngeal specimens.

**Fig. 4** Cytopathic effect observed in LLC-MK2-N caused by human coronavirus (HCoV) NL63. A, Syncytium formation observed in LLC-MK2-N cells infected with HCoV-NL63. Live cells were observed consecutively for 4 days. B, Immunostaining of LLC-MK2-N infected with HCoV-NL63. Cells of second passage culture were fixed at 5 days post-infection. Cells were primarily stained with 4’,6-diamidino-2-phenylindole to
visualize nuclei (blue). Cells were stained with primary antibodies targeted to spike proteins of HCoV-NL63 and with FITC-conjugated secondary antibodies to detect HCoV-NL63+ cells (green). *Differential interference contrast. Bar indicates 100 µm.
Table 1: Summary of isolation of human coronaviruses at the Yamagata Prefectural Institute of Public Health

| Checked categories                                      | Results                                      |
|---------------------------------------------------------|----------------------------------------------|
|                                                         | Human coronaviruses                          |
|                                                         | OC43          | HKU1         | NL63          | 229E          |
| Isolation by ALI\(^1\) culture                        | Achieved     | Achieved     | Achieved     | Achieved     |
| Subculturing in cell line after isolation using ALI culture | Achieved     | Achieved     | (LLC-MK2-N\(^2\)) | (HeLa-ACE2-TMPRSS2) |
| Isolation in cell line                                 | N\(^3\)       |              |              | Achieved\(^3\) |

\(^1\) ALI: Air liquid interface.  
\(^2\) LLC-MK2-N: LLC-MK2 cells transfected with N from SARS-CoV-2.  
\(^3\) RD-18S, HeLa-ACE2-TMPRSS2: RD-18S cells transfected with ACE2 and TMPRSS2 from SARS-CoV-2.
| Year-1 | Year-2 | Year-3   | Year-4   | Year-5   | Year-6   |
|--------|--------|----------|----------|----------|----------|
| 2009-  | 2015-  |          |          |          |          |
| 0206   | 0151   |          |          |          |          |
| 2010-  | 2016-  |          |          |          |          |
| 0241   | 0980   |          |          |          |          |
| 2010-  | 2016-  |          |          |          |          |
| 2388   | 0999   | 2015-2302| 2012-1966|          |          |
| Abbreviated names | 2010-  | 2017-  | 2017-2621| 2016-0854|          |
| of strains isolated by | 2594   | 0193   | 2018-0029| 2018-0501|          |
| ALI culture in this study | 2012-  | 2017-  | 2018-0036| 2018-0597|
|          | 2416   | 0410   | 2019-1029| 2019-0554|          |
|          | 2013-  | 2018-  | 2020-0258| 2020-0480|
|          | 0300   | 0238   |          |          |          |
|          | 2014-  | 2018-  |          |          |          |
|          | 0375   | 0347   |          |          |          |
|          | 2014-  | 2020-  |          |          |          |
|          | 0378   | 0316   |          |          |          |
1) Air-liquid interface

2) An LLC-MK2 cell line derived from Niigata Prefectural Institute of Public Health and Environmental Sciences

3) Matoba Y et al. (19, 20)

4) The official names of representative HCoV-OC43, HCoV-HKU1, HCoV-NL63, and HCoV-229E strains are OC43/Yamagata.JPN/2009-0206, HKU1/Yamagata.JPN/2015-2302, NL63/Yamagata.JPN/2012-1966, and 229E/Yamagata.JPN/2018-0330, respectively.

5) Isolates obtained by ALI culture were propagated in the LLC-MK2-N cell line.
1. Expansion of cells from frozen stock

2. Seeding in inserts and renewing media

3. Air-lifting and renewing basal medium

4. Mucociliary differentiation

5. Inoculation

6. Renewing basal medium

7. Harvesting

Fig. 1
Fig. 3

Number of viral genome copies per μL of medium

Days post inoculation

OC43
HKU1
NL63

10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Fig. 4

A

Day 3 Day 4 Day 5 Day 6

B

Nuclei Anti-HCoV-NL63 DIC* Merge

HCoV-NL63

Virus mock