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Evidence of the simultaneous replications of active viruses in specimens positive for multiple respiratory viruses

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ABSTRACT Genetic diagnostic assays for the detection of respiratory viruses sometimes show simultaneous multiple infections with low copy numbers. In such cases, the disease is considered caused by a single etiologic agent and others are nonspecific reactions and/or contaminations. Interferon-dependent interference is seen in dual infections of influenza and respiratory syncytial virus, which are the main causes of respiratory infections. Virus isolation is one of the answers in detecting other active viruses present in specimens, and the air–liquid interface culture of human bronchial/tracheal epithelial cells (HBTEC-ALI) is optimal for the isolation of respiratory viruses owing to its wide range of susceptibility. In this study, we successfully confirmed the replications of various viruses from specimens with low copy numbers and simultaneous passage of two to three viruses using HBTEC-ALI cultures, mainly including human bocavirus 1 and/or human rhinovirus.

IMPORTANCE Since the pandemic of coronavirus diseases 2019, the use of real-time PCR assay has become widespread among people who were not familiar with it in virus detection. As a result, whether a high real-time PCR value in one time test indicates virus transmissibly became a complicated social problem, regardless of the difference in assays and/or amplification conditions, the time and number of diagnostic test during the time course of infection. In addition, the multiple positives in the test of respiratory viruses further add to the confusion in the interpretation of the infection. To address this issue, we performed virus isolation using pediatric SARI (severe acute respiratory infections) specimens on air–liquid interface culture of human bronchial/tracheal epithelial cell culture. The result of this study can be a strong evidence that the specimens showing positivity for multiple agents in real-time PCR tests possibly contain infectious viruses.

KEYWORDS respiratory virus, co-cultivation, real-time RT-PCR, HBTEC-ALI culture

Acute respiratory infections (ARIs) are the leading cause of mortality in children worldwide (1), and numerous bacteria, viruses, and fungi are associated with disease development (2–4).

The development of multiple molecular assays, such as nucleic acid amplification tests, enables the simultaneous detection of these causative agents (5–8). The coronavirus disease pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which started in the end of 2019, accelerated the development of assays based on real-time polymerase chain reaction (PCR) techniques (9–11). Since then, its use has become widespread among people who were not familiar with real-time PCR in virus detection. As a result, whether a high Ct in one time test indicates the presence of infectious virus became a complicated social problem, regardless of the difference in

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assays and/or amplification conditions, the time and number of diagnostic test during the time course of infection (12–15).

Thus, the presence of active viruses in such multiple and/or low copy number infections should be assessed. However, in ARIs, especially in influenza (Flu) and respiratory syncytial viruses (RSV), viruses interfere with each other by inducing interferon-related genes; therefore, the virus that first caused an infection defeated the second virus (16–18). Therefore, the disease is considered caused by a single etiologic agent, and the effects of multiple infections on disease development are controversial (5, 8, 19).

Virus isolation is one of the methods of assessing the presence of active infectious agents in such suspicious situations. The air–liquid interface culture of human bronchial/tracheal epithelial cells (HBTEC-ALI) is an excellent tool for culturing various respiratory pathogens, particularly those that are hard to isolate, such as human bocavirus 1 (HBoV1) and human rubulavirus 4 [human parainfluenza virus (HPIV4)], due to the wide range of susceptibility for respiratory viruses (20, 21). In our laboratory, virus isolations using HBTEC-ALI cultures have been performed with clinical specimens obtained from pediatric in-patients with severe ARI (SARI) (22, 23).

In this study, we aimed to report that we successfully isolated various viruses from specimens with low copy number, and we detected different viruses simultaneously, mainly including HBoV1 and/or human rhinovirus (HRV).

MATERIALS AND METHODS

Clinical specimens

Clinical specimens were collected in acute phase from pediatric in-patients with SARIs in Fukushima, Japan, between 2018 and 2022 (22). Nasopharyngeal swabs were collected in the universal transport medium (Copan, Brescia, Italy) and stored at −80°C until use. In this study, any information of patient other than diseases criteria was not collected.

In this study, SARS-CoV-2-negative specimens were used to evaluate respiratory infections as common diseases enabling handling in biosafety level 2 (BSL-2) facilities.

Virus isolation and real-time PCR

Nucleic acids were extracted from specimens using QIAamp 96 Virus QIAcube HT Kit (Qiagen, Hilden, Germany), QIAamp Viral RNA Mini Kit (Qiagen), or NucleoSpin 96 Virus (Macherey-Nagel, Duren, Germany) following the manufacturer's instruction, except that the elusion step was performed by centrifugation. Respiratory viruses were detected from specimens by real-time PCR and reverse transcription (RT)-PCR assays with LightCycler instruments (Roche, Basel, Switzerland) as previously described (22, 24). Briefly, cDNA was synthesized using SMART MMLV Reverse Transcriptase (Takara-Bio, Shiga, Japan) with Oligo(dT) primer (Qiagen) and primer random (Roche). The AgPath-ID One-Step RT-PCR reagents was used for one-step real-time RT-PCR and the LightCycler 480 Probes Master was used for two-step real-time PCR. Primer/probe sets with performance validated in previous studies were used, and the sequences are listed in Table S1 (6, 23–30). The following 17 viruses were tested: human orthopneumovirus (RSV subgroups A and B); Flu A, B, and C; human coronavirus (HCoV) (229E, OC43, NL63, and HKU1); human metapneumovirus (hMPV); HPIV (1, 2, 3, and 4); adenovirus (ADV) [2 (for A, C, D, and F) and 4 (B and E)]; HBoV1; and HRV (Table S1). The real-time PCR value was expressed as crossing point (Cp) and cut-off value was set following previous studies as needed (6, 23–30). Among the above specimens, 249 specimens were positive for respiratory viruses (single infection, n = 122; multiple infections, n = 127) and were subjected to virus isolation. The HBTEC-ALI was prepared as described previously (20, 21, 31). Briefly, HBTEC (FC-0035, LIFELINE Cell Technology, Frederick, MD, USA) were plated on 6.5 mm-diameter traswell (3470, Corning, One Riverfront Plaza, NY, USA). Upper medium was removed, and basal medium was replaced with differentiation medium

the next day, and human airway epithelium cultures were generated by culturing cells at an ALI for 4 weeks with every week medium change, resulting in well-differentiated, polarized cultures. Then, 20 µL of the specimen diluted with an equal volume of 1% fetal calf serum (FCS)-Dulbecco's modified Eagle's medium (DMEM) containing prescribed amount of antibiotics (penicillin-streptomycin, gentamicin, and fungizone) was inoculated onto the apical surface of HBTEC-ALI cultures. Cells were incubated at 34°C overnight and then washed with 1% FCS-DMEM four times. Because it took time to take effect, the fourth wash solution was kept as the starting control. The MC-210 (KAC Co Ltd., Hyogo, Japan), which was a quinolone antimicrobial for mycoplasmas, was added to the basolateral medium throughout the incubation periods to remove mycoplasmas and every buffer change a week. After 4, 7, 11, 18, and 25 days of incubation at 34°C (18 and 25 days were dependent on the condition of culture cells), cells were washed with 1% FCS-DMEM four times, and the cell-washes were stored at −80°C. Virus replications were confirmed by real-time RT-PCR assays as described above (22). During the time course and passage, when the specimen showed >3.3 Cp (≈1 $\log_{10}$) of increase relative to day 0 and/or 4, it was considered positive virus isolation. The copy number of each virus was determined using the calibration curve drawn by real-time RT-PCR assay with concentration-calculated synthesized control DNA or RNA containing the primer/probe targeted region. The copy numbers of the control DNA/RNA were calculated based on the molecular weight and absorbance at 260 nm of RNA that had been serially diluted (≥3 steps) and/or fluorescence using the Quantus Fluorometer (Promega, Madison, WI, USA) ($n = 3$).

Sequencing analysis

For next-generation sequencing, libraries were prepared using the NEBNext Ultra II RNA Library Prep Kit for Illumina [New England Biolabs (NEB), Ipswich, MA, USA] or the NEBNext Ultra II FS DNA Library Prep Kit for Illumina (NEB), following the manufacturer's instructions. The libraries for next generation sequencing (NGS) analysis were prepared using 4 to 11 days of virus depending on the replication kinetics and the amount of sample. Indexed libraries were analyzed for 2 × 150 cycles on a DNBSEQ-G400 sequencer at AZENTA/GENEWIZ (Chelmsford, MA, USA). Reads were trimmed and then *de novo* assembled and/or mapped to each reference sequence using the CLC Genomics Workbench (v21.0.4, Qiagen) with default settings.

Statistical analysis

Statistical analysis was performed using SigmaPlot software (Ver. 14.5, Systat Software Inc., Palo Alto, CA, USA). The *z*-test and χ^2 test were used. The *t*-test or the Mann-Whitney test were also used depending on the normality test result. The *P*-value less than 0.05 was considered as significant.

RESULTS

Respiratory virus isolation using HBTEC-ALI cultures

The real-time (RT-)PCR techniques sometimes detect multiple positives for various targets in the tested specimen with suspicious respiratory infections. Virus isolation is the most suitable technique in identifying the "active" virus in specimens positive for multiple agents. Furthermore, HBTEC-ALI cultures are the best cells for respiratory virus isolation owing to the wide range of susceptibility. In this study, HBTEC-ALI cultures were used, and virus isolation was performed with clinical specimens obtained from pediatric in-patients with respiratory infections (22, 23). In HBTEC-ALI culture, cells generally did not show a cytopathic effect; therefore, the process of viral genomic replication was detected by real-time RT-PCR, and it was used as the indicator of successful virus isolation. The replication kinetics of virus should be monitored by repeated test during the time course of infection; in initial diagnosis, only one time test result can be available, generally. Therefore, the results of real-time (RT-)PCR in initial diagnosis were applied

following the experiment. The 249 specimens (122 single infection and 127 multiple infections) were subjected to virus isolation, and 131 specimens (52.6%) were positive for replication of some viruses. Moreover, 70 of 122 single-infection specimens were positive (57.3%) and 62 of 127 multiple-infection specimens were positive in at least one target (48.8%). No significant differences in virus isolation were found between single- and multiple-infection specimens by *z*-test ($P = 0.224$). For 17 respiratory viruses, 450 targets were tested, and 147 of these (32.7%) showed virus replication (Table 1). The rates of successful virus replication were low in RSV A ($P = 0.04$) and higher in Flu A ($P = 0.014$) and Flu B ($P = 0.002$) than average. Overall, successful virus replication was related to lower real-time RT-PCR value (25.7 vs 33.0, Cp value), i.e., high amounts of inoculated virus led to positive results (4.6 vs 2.5 $\log_{10}$ copies, $P < 0.001$) (Table 1). Yamada et al. reported that 87.5% of successful virus isolation was seen in less than 28.1 of Cp, which was corresponding to about log 3.5 copies determined by NIID-N2 set, in the isolation of SARS-CoV-2 using VeroE6/TMPRSS2 cells (32). The average (±confidence interval) copies of Cp = 28.1 in used real-time PCR assays is log 3.23 ± 0.36. Although the correlation between real-time PCR value and viral copy number is completely dependent on the performance of each primer/probe set (the same Cp value does not always show the same copy number in different primer/probe sets), data were sorted by the Cp value (≥28.1) to evaluate successful virus replication in low-copy specimens (Table 2). In total, among 296 targets, 54 showed virus replication (18.2%). The replication rates of Flu B, and HPIV1 were higher than average. In total, successful replications were also seen in specimens with lower Cp values (31.8 vs 34.9, $P < 0.001$) and the higher copy number (2.8 vs 1.9, $\log_{10}$ copies, $P < 0.008$). The performance of each real-time (RT-)PCR assay is different and same Cp value does not always show same copy number; therefore, the results have been converted to the copy number enable to compare altogether. In addition, the sensitivity of virus replication on HBTEC-ALI culture is different depending on the virus species. However, these results indicate the tendency that the higher the number of viral copies, the more successful is the virus replication. Although approximately several hundred of copies are required to lead to possible respiratory virus isolation in HBTEC-ALI cultures, intact virion (active virus) surely exists even in low copies specimens,

Simultaneous virus replication in HBTEC-ALI cultures

In the virus isolation, 17 specimens showed simultaneous replications of two to three viruses in the first inoculation in the same HBTEC-ALI culture (Table S2). Eleven of them were in combination with HBoV1 or HRV, and four were in combination with ADV2. Combinations of envelope viruses were seen in two (HCoV229E/hMPV and HPIV1/HPIV4). Eleven of the co-replicated viruses could be simultaneously passed once (Tables 3 and 4). In the H739 specimen, ADV C, ADV B3, and HRV were replicated after 7 days in the first isolation (P0) despite a high Cp value in ADV C and HRV, and both ADV C and ADV B3 could be passed one time (P1). In the O898 specimen, ADV C and ADV B3 were replicated simultaneously in both P0 and P1 cultures. In the O876 specimen, HPIV1 and HPIV4 were replicated and passed in both P0 and P1 cultures despite high Cp values in the specimen. In the OR7 specimen, HCoVOC43, HRV, and HBoV1 were replicated in the P0 culture, and HCoVOC43 and HRV could be passed in the P1 culture. Simultaneous replications and passage of HBoV1 and HRV were most observed in specimens OR291, OR321, and OR331. The following four specimens could be simultaneously passed two times (Table 4). In the H257 specimen, HCoVNL63 and HBoV1 were replicated through the passage. In the H260 specimen, although HBoV1 was replicated in the P1 culture, HPIV3 and HRV were replicated during two passages. In the OR32 specimen, ADV C and HBoV1 were replicated and passed until the P2 culture. In the OR65 specimen, HBoV1 and HRV were co-replicated in all passages. In these passage-successful specimens, the nearly complete genomic sequencers of these replicated viruses could be decoded and were registered in the GenBank database (Table 5), proofing the presence of active viruses simultaneously

TABLE 1 Results of virus isolation from pediatric SARI specimen using HBTEC-ALI culture

	Target[a]	RSV A	RSV B	Flu A	Flu B	HCoV 229E	HCoV OC43	HCoV NL63	HCoV HKU1	hMPV	PIV1	PIV2	PIV3	PIV4	Flu C	ADV C	ADV B3	HBoV	HRV	Total
	Total test[b]	39	25	13	6	10	24	18	13	48	10	7	26	35	6	44	16	53	57	450
	Replicated	6	4	9	6	2	12	8	3	14	6	4	11	10	1	8	5	18	20	147
	Success rate (%)	15.4	16.0	69.2	100.0	20.0	50.0	44.4	23.1	29.2	60.0	57.1	42.3	28.6	16.7	18.2	31.3	34.0	35.1	32.7
	Replicated	22.4	20.7	24.2	29.5	24.2	23.7	26.0	27.6	26.9	31.2	25.6	27.2	31.1	21.1	24.7	23.0	22.9	31.2	25.7
Average Cp	Non-replicated	25.7	28.7	30.2	N/A[c]	36.4	32.1	30.6	35.6	31.2	29.4	37.1	36.7	35.3	31.3	35.5	37.1	34.8	33.8	33.0
	Replicated	5.6	6.2	4.3	3.2	5.4	4.3	4.5	3.6	5.2	2.2	4.6	5.6	2.8	6.1	4.6	5.6	5.2	4.2	4.6
	(Range)	3.6–6.8	5.6–6.5	2.0–6.1	2.5–3.6	4.3–6.5	2.4–6.8	2.9–6.0	1.3–6.5	4.3–6.5	0.9–3.6	2.7–6.0	4.3–7.3	0.8–4.5	N/A	1.1–6.7	1.5–7.8	2.3–7.7	2.4–6.6	
Average inoculated copies (log)	Non-replicated	4.6	4.0	2.5	N/A	1.4	1.7	3.2	1.1	3.9	2.7	1.2	2.8	1.7	3.1	1.2	1.6	1.5	3.5	2.5
	(Range)	1.3–6.9	1.8–7.3	2.0–3.7	N/A	0.6–2.9	0.4–5.0	1.2–5.5	0.2–2.6	1.4–6.7	1.0–4.7	0.8–1.8	1.8–5.3	0.4–4.3	1.5–5.3	−0.3–4.2	0.8–2.8	0.0–4.1	2.0–7.6	
	P-value[d]	0.33	<0.001	0.042	N/A	<0.151	<0.001	0.06	0.245	<0.001	0.595	0.018	<0.001	0.019	N/A	<0.001	0.015	<0.001	0.03	<0.001

[a]ADV2 set detects A, C, D, and F and ADV4 set detects B and E.
[b]Total test number was the total number of specimen shown positive for each target.
[c]N/A, not applicable.
[d]Statistical differences in copy number between replicated and non-replicated specimens were analyzed by *t*-test or Mann-Whitney test on SigmaPlot depending on the result of normalization test.

TABLE 2 Results of virus isolation from pediatric SARI specimen using HBTEC-ALI culture sorted by Cp ≥28.1

	Target[a]	RSV A	RSV B	Flu A	Flu B	HCoV 229E	HCoV OC43	HCoV NL63	HCoV HKU1	hMPV	PIV1	PIV2	PIV3	PIV4	Flu C	ADV2	ADV4	HBoV	Rhino	Total
	Total test[b]	11	11	5	4	8	11	10	12	27	7	5	20	31	4	37	12	32	49	296
	Replicated	0	0	2	4	0	1	3	2	4	5	2	5	7	0	2	1	2	14	54
	Success rate (%)	0.0	0.0	40.0	100.0	0.0	9.1	30.0	16.7	14.8	71.4	40.0	25.0	22.6	0.0	5.4	8.3	6.3	28.6	18.2
	Replicated	N/A[c]	N/A	31.5	30.3	N/A	29.9	31.0	32.3	30.3	32.2	30.1	30.3	32.9	N/A	32.5	37.5	30.9	34.1	31.8
Average Cp	Non-replicated	33.9	34.2	31.5	N/A	36.4	33.8	33.5	35.6	33.8	33.9	37.1	36.7	35.6	33.1	35.8	37.1	36.1	34.6	34.9
	Replicated	N/A	N/A	2.1	3.0	N/A	2.4	3.1	2.2	4.2	1.9	3.3	4.7	2.3	N/A	2.1	1.5	2.7	3.5	2.8
Inoculated copies (log)	Non-replicated	2.1	2.6	2.1	N/A	1.4	1.2	2.4	1.1	3.2	1.4	1.2	2.8	1.6	2.6	1.1	1.6	1.1	3.3	1.9

[a]ADV2 set detects A, C, D, and F and ADV4 set detects B and E.
[b]Total test number was the total number of specimen shown positive for each target.
[c]N/A, not applicable.

TABLE 3 Kinetics of Cp value during serial passage of simultaneous replication of multi-positive specimens (P0 to P1)

Specimen number	Passage number	Target[a]	Inoculant	Cp value of inoculant	Days					
					0	4	7	11	18	25
		ADV2(C)		37.6	>40.0[b]	>40.0	>40.0	33.1	N.D.[c]	N.D.
		ADV4(B3)		21.6	26.4	>40.0	17.7[d]	18.1	N.D.	N.D.
	P0	HRV	Specimen	36.8	>40.0	>40.0	35.8	>40.0	N.D.	N.D.
		ADV2(C)		>40.0	>40.0	>40.0	30.0	22.6	N.D.	N.D.
		ADV4(B3)		17.7	>40.0	16.7	10.8	12.3	N.D.	N.D.
	P1-1	HRV	P0–7d	35.8	>40.0	>40.0	>40.0	>40.0	N.D.	N.D.
		ADV2(C)		>40.0	>40.0	>40.0	>40.0	>40.0	>40.0	>40.0
		ADV4(B3)		17.7	30.5	22.1	19.8	13.2	13.6	12.3
H739	P1-2	HRV	P0–7d	35.8	>40.0	>40.0	26.5	>40.0	39.1	36.0
		HPIV1		33.9	31.3	27.0	24.4	25.6	N.D.	N.D.
	P0	HPIV4	Specimen	36.5	33.5	30.3	25.8	27.1	N.D.	N.D.
		HPIV1		25.6	29.0	18.2	18.5	17.3	N.D.	N.D.
O876	P1	HPIV4	P0–11d	27.1	31.5	25.6	25.4	26.4	N.D.	N.D.
		ADV2(C)		32.9	25.9	34.8	18.1	18.1	N.D.	N.D.
	P0	ADV4(B3)	Specimen	29.1	>40.0	>40.0	27.7	27.2	N.D.	N.D.
		ADV2(C)		18.1	>40.0	>40.0	14.7	10.1	>40.0	N.D.
O898	P1	ADV4(B3)	P0–11d	27.2	>40.0	26.0	23.8	19.8	18.3	N.D.
		HCoVOC43		24.8	>40.0	21.6	23.4	N.D.	N.D.	N.D.
		HRV		31.7	29.7	19.7	21.1	N.D.	N.D.	N.D.
	P0	HBoV	Specimen	>40.0	>40.0	>40.0	29.6	N.D.	N.D.	N.D.
		HCoVOC43		21.6	25.2	>40.0	22.5	22.5	N.D.	N.D.
		HRV		19.7	23.6	18.6	21.9	19.7	N.D.	N.D.
OR7	P1	HBoV	P0–4d	>40.0	>40.0	>40.0	>40.0	>40.0	N.D.	N.D.
		HBoV		20.96	>40.0	22.2	25.2	23.6	20.4	15.6
	P0	HRV	Specimen	34.21	>40.0	12.4	15.0	17.5	18.7	20.4
		HBoV		23.6	>40.0	>40.0	>40.0	>40.0	>40.0	N.D.
	P1-1	HRV	P0–11d	17.47	30.1	19.7	15.7	18.9	18.1	N.D.
		HBoV		15.64	27.6	12.9	10.5	11.3	14.1	N.D.
OR291	P1-2	HRV	P0–25d	20.44	40.0	34.5	40.0	33.8	40.0	N.D.
		HBoV		14.6	>40.0	12.0	12.9	12.9	24.1	N.D.
		ADV2		37.3	>40.0	>40.0	>40.0	>40.0	>40.0	N.D.
	P0	HRV	Specimen	34.3	>40.0	18.3	22.9	28.3	24.2	N.D.
		HBoV		12.9	29.4	22.2	25.2	30.3	21.8	N.D.
		ADV2		>40.0	>40.0	>40.0	>40.0	>40.0	>40.0	N.D.
OR321	P1	HRV	P0–7d	22.9	25.0	15.8	21.9	22.5	22.2	N.D.
		HBoV		23.5	>40	20.9	18.7	14.5	11.3	12.2
	P0	HRV	Specimen	34.5	>40	23.0	19.0	18.1	21.8	26.4
		HBoV		14.5	28.0	14.8	15.8	12.5	10.9	11.41
OR331	P1	HRV	P0–11d	18.1	26.6	15.3	23.8	22.3	26.6	26.1

[a]ADV2 set detects A, C, D, and F and ADV4 set detects B and E.
[b]>40, less than detection limit which means no virus replication.
[c]N.D., not done.
[d]The sample used for sequencing was underlined.

in the same cell cultures. These can be strong evidence that the specimens showing positivity for multiple agents in real-time PCR tests surely contain infectious viruses.

DISCUSSION

In this study, we show evidence on the presence of an active virus in specimens with low copies and/or positive for multiple respiratory infections. The results clearly show successful virus replication in HBTEC-ALI cultures inoculated with specimens with Cp >28.1 and simultaneous replications and serial passages of two to three viruses, mainly including HBoV1 and/or human rhinovirus.

TABLE 4 Kinetics of Cp value during serial passage of simultaneous replication of multi-positive specimens (P0 to P2)

Specimen number	Passage number	Target[a]	Inoculant	Cp value of inoculant	Days				
					0	4	7	11	18
		RSV		30.7	>40.0[b]	>40.0	>40.0	>40.0	N.D.[c]
		HCoVNL63		31.0	38.2	16.8[d]	17.7	15.6	N.D.
	P0	HBoV	Specimen	17.2	22.5	20.7	26.0	26.7	N.D.
		RSV		40.0	>40.0	>40.0	>40.0	>40.0	>40.0
		HCoVNL63		17.7	28.8	19.0	17.2	17.9	15.4
	P1	HBoV	P0–7d	26.0	39.5	36.7	31.4	38.9	>40.0
		RSV		40.0	>40.0	>40.0	>40.0	>40.0	>40.0
		HCoVNL63		17.2	40.0	19.6	17.8	23.0	22.4
H257	P2	HBoV	P1–7d	31.4	37.9	17.3	14.6	13.7	12.2
		HPIV3		29.9	35.3	37.5	19.0	19.2	N.D.
		HBoV		33.0	37.9	>40.0	>40.0	>40.0	N.D.
	P0	HRV	Specimen	38.1	38.9	27.9	21.7	24.0	N.D.
		HPIV3		19.0	29.1	22.3	22.1	23.3	N.D.
		HBoV		>40.0	>40.0	>40.0	>40.0	29.6	N.D.
	P1	HRV	P0–4d	21.7	27.6	24.7	24.1	23.6	N.D.
		HPIV3		23.3	31.6	25.5	28.4	N.D.	N.D.
		HBoV		29.6	31.8	>40.0	>40.0	N.D.	N.D.
H260	P2	HRV	P1–11d	23.6	38.5	33.1	>40.0	N.D.	N.D.
		ADV2 (C)		21.8	28.9	17.4	24.3	N.D.	N.D.
		HBoV		27.7	>40.0	37.8	>40.0	N.D.	N.D.
	P0	HRV	Specimen	36.1	>40.0	>40.0	>40.0	N.D.	N.D.
		ADV2 (C)		17.4	27.8	38.3	21.6	19.1	N.D.
		HBoV		37.8	>40.0	23.2	>40.0	>40.0	N.D.
	P1	HRV	P0–4d	>40.0	>40.0	38.5	>40.0	>40.0	N.D.
		ADV2 (C)		38.3	>40.0	>40.0	14.0	11.0	>40.0
		HBoV		23.2	>40.0	>40.0	>40.0	25.3	16.2
OR32	P2	HRV	P1–4d	38.5	>40.0	>40.0	>40.0	38.1	>40.0
		HBoV		23.4	30.0	17.7	12.7	N.D.	N.D.
	P0	HRV	Specimen	29.6	28.5	20.6	21.5	N.D.	N.D.
		HBoV		12.7	22.1	>40.0	14.7	16.4	N.D.
	P1	HRV	P0–7d	21.5	30.6	>40.0	28.8	29.3	N.D.
		HBoV		10.1	29.1	17.1	10.1	10.8	N.D.
OR65	P2	HRV	P1–7d	24.2	33.1	23.2	24.2	30.6	N.D.

[a]ADV2 set detects A, C, D, and F and ADV4 set detects B and E.
[b]>40, less than detection limit which means no virus replication.
[c]N.D., not done.
[d]The sample used for sequencing was underlined.

During the coronavirus disease 2019 pandemic, real-time RT-PCR-based diagnostic assays have been used to detect SARS-CoV-2; however, the correlation between positive signals in real-time RT-PCR and the possibility of virus spread from patients became a problem because PCR-negative results had been required for discharge in the early phase of the pandemic (33–35). For SARS-CoV-2, the assays that targeted structural proteins such as nucleocapsid gene were well-used (9, 11); however, these assays also detect subgenomic mRNAs of coronaviruses, causing confusion on the presence of intact virions. In a previous report, the relation between real-time RT-PCR and successful virus isolation was assessed using the ORF1a assay, which could detect mainly genomic RNA, and several dozens of copies of genomic RNA were required for possible virus isolation (36). In the present study, various real-time RT-PCR assays were used, and comparing their results using the value of real-time PCR (such as Ct and Cp) was difficult. However, it enables conversion of the results to copy numbers, and the presence of approximately several hundred copies of viruses possibly leads to replications in HBTEC-ALI cultures for all respiratory viruses tested in this study. In suspicious specimens, the copy number,

TABLE 5 GenBank accession numbers for nearly complete genomic sequences of simultaneous passaged viruses

Specimen number	Isolate name	GenBank accession number
H739	ADV2_Fukushima_H739_2019	LC756665
	ADVB3_Fukushima_H739_2019	LC703523
	HRV_A15_Fukushima_H739_2019	LC756672
O876	PIV1_Fukushima_O876_2019	LC720863
	PIV4b_Fukushima_O876_2019	LC720882
O898	ADV2_Fukushima_O898_2019	LC756666
	ADVB3_Fukushima_O898_2019	LC757030
OR7	HCoVOC43_Fukushima_OR7_2020	LC720427
	HRV_A40_Fukushima_OR7_2020	LC720413
	HBoV_Fukushima_OR7_2020	LC720423
OR291	HBoV_Fukushima_OR291_2021	LC720417
	HRV_A80_Fukushima_OR291_2021	LC720411
OR321	HBoV_Fukushima_OR321_2021	LC720419
	HRV_C6_Fukushima_C6_2021	LC720414
OR331	HBoV_Fukushima_OR331_2021	LC720422
	HRV_A80_Fushima_OR331_2021	LC720412
H257	HCoVNL63_Fukushima_H257_2018	LC687394
	HBoV_Fukushima_H257_2018	LC720416
H260	PIV3_Fukushima_H260_2018	LC720871
	HRV_A78_Fukushima_H260_2018	LC699414
OR32	HAdVC_Fukushima_OR32_2020	LC720426
	HBoV_Fukushima_OR32_2020	LC720421
OR65	HBoV_Fukushima_OR65_2020	LC651178
	HRV_A80_Fukushima_OR65_2020	LC699423

not the RT-PCR value, should be used as a guide for possible infectivity. Note that the frozen specimens were used in virus isolation in this study due to the convenience of transportation, and it might affect the result of virus replication like RSV.

In this study, nearly complete sequences of simultaneous replicated viruses could be decoded, and it proofed the presence of active viruses simultaneously in the same cell cultures. However, although it was the limitation of this study, viral sequences in specimen were not determined because direct sequencing of specimen had an ethical problem from the viewpoint of personal information of patient and viral sequencing protocol using PCR amplicons was not established for all respiratory viruses. Certainly, the HBTEC-ALI culture is useful for virus isolation for respiratory viruses due to the wide range of susceptibility, but there was possibility that isolation and passage on HBTEC-ALI culture might induce adaptation and artificial mutation in genome. In order to show the availability of HBTEC-ALI culture for the propagation of clinical isolates of respiratory viruses maintaining original characteristics, genetic stability of isolates on HBTEC-ALI culture must be evaluated.

Multiple respiratory viruses infect respiratory tissues one after another and induce reciprocal interference in one another, i.e., Flu, RSV, hMPV, and HRV interfere with other viruses by the signaling of intracellular RNA sensors or inducing interferon-related genes (16, 17). This is also a major theory in hepatitis, i.e., hepatitis C virus infection inhibits the infections of other hepatitis viruses (37, 38). Therefore, we tended to think that the first virus suppresses the second virus, and the infectious diseases were mainly caused by the first virus. Certainly, only one virus could be replicated in most specimens with multiple infections (73.8%, 45 of 61, Table S2); however, simultaneous virus replications were observed, although mainly with HBoV1, HRV, and/or ADV infections. Several reports described persistent infection of HBoV1, HRV, and ADV might cause high replication rate of these viruses (39–41). HBoV1 and HRV cause severe respiratory tract infections with simultaneous infections caused by other infectious agents (42–45). By contrast,

some reports described that co-infections with these viruses do not affect the disease severity (46, 47). This study was also limited that SARS-CoV-2 positive specimens were excluded from the study for handling in BSL-2 facilities. However, co-infection with other respiratory pathogens was reported and, especially, the increased severity of co-infection with influenza virus is focused on SARS-CoV-2 infection (48). Thus, the relationship between the synergistic replication of infected dual viruses and disease severity must be investigated. In addition, the mechanism of acceptable dual virus replication is unclear. Although many reports describe one virus infection disturbs other virus infections (16–18), there is no report for viral symbiotic. Therefore, it is necessary to elucidate the acceptable combination of respiratory virus species for dual virus replication and the mechanisms including host cell factors using clinical isolates of viruses and HBTEC-ALI culturing system. In this study, although the co-cultivative cases are minority, it is due to the limit of the sensitivity of virus isolation system. Improvement of HBTEC-ALI culturing system might increase the number of dual replication cases. Nonetheless, the wide range of virus susceptibility of HBTE-ALI culture is useful for possible virus isolation from specimens, even from health children with no symptom.

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AUTHOR CONTRIBUTIONS

Miyuki Kawase, Data curation, Investigation, Methodology, Resources | Reiko Suwa, Investigation, Resources | Satoko Sugimoto, Investigation, Methodology, Resources, Software | Masatoshi Kakizaki, Investigation, Methodology, Resources, Software | Yohei

Kume, Resources | Mina Chishiki, Resources | Takashi Ono, Resources | Hisao Okabe, Resources | Sakurako Norito, Resources | Makoto Ujike, Data curation, Validation, Visualization, Writing – review and editing | Mitsuaki Hosoya, Resources | Koichi Hashimoto, Resources, Supervision, Writing – review and editing | Kazuya Shirato, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing

DATA AVAILABILITY

The nearly complete genome sequences of isolates have been deposited in GenBank under the following accession numbers (Table 5): LC756665, LC703523, LC756672, LC720863, LC720882, LC756666, LC757030, LC720427, LC720413, LC720423, LC720417, LC720411, LC720419, LC720414, LC720422, LC720412, LC687394, LC720416, LC720871, LC699414, LC720426, LC720421, and LC699423.

ETHICS APPROVAL

The study protocol was approved by the Ethics Committees of the National Institute of Infectious Diseases (nos. 1001, 1087, and 1441) and Fukushima Medical University (no. 29006). Informed consent was obtained verbally and was noted in the medical records in each hospital.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Table S1 (Spectrum01920-23-S0001.docx). Primer/probe list.
Table S2 (Spectrum01920-23-S0002.docx). Virus detection and isolation pattern in specimen with multiple infections.

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