Research articles

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

multiple respiratory viruses

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Running title

Viral co-cultivation in multiple infections

Keywords

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

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 solutions 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 passed 2–3 viruses

simultaneously using HBTEC-ALI cultures, mainly including human bocavirus 1 and/or

human rhinovirus.

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, enable 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 occurred 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 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

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 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 parainfluenzavirus (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).

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.

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. In this study, SARS-CoV-2-negative specimens were

used to evaluate respiratory infections as common diseases.

Virus isolation

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). The real-time PCR value was expressed as crossing point

(Cp). Primer/probe sequences are listed in Table S1. 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 (6, 23, 25-27). 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 air–liquid interface culture of human

bronchial/tracheal epithelial cells (HBTEC-ALI) was prepared as described previously

(20, 21, 28). Then, 20 μL of the specimen diluted with an equal volume of

1%FCS-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. The fourth wash solution was kept as the starting control. The MC-210

(KAC Co Ltd., Hyogo, Japan) 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 depended 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 ($\approx$ 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. 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 chi-square 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, genetic

replication detected by real-time RT-PCR was used as the indicator of successful virus

isolation. The replication kinetics of virus should be monitored by repeated test during

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 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 61 of 127 multiple-infection specimens were positive in at least

one target (48.0%). No significant differences in virus isolation were found between

single- and multiple-infection specimens by z-test (p = 0.177). 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 RSVA (p = 0.04) and

higher in FluA (p = 0.014) and FluB (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 (29). 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 ($\geq$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 FluB, 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 assays are 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 are 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, 16 specimens showed simultaneous replications of 2–3

viruses in the first inoculation in the same HBTEC-ALI culture (Table S2). Eleven of

them were in combination with HBoV1 or HRV, and three 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 (Table 3 and 4). In the H739 specimen, ADVC, ADVB3, and HRV were replicated

after 7 days in the first isolation (P0) despite a high Cp value in ADVC and HRV, and

both ADVC and ADVB3 could be passed one time (P1). In the O898 specimen, ADVC

and ADVB3 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 throw 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, ADVC and HBoV1 were replicated and passed till the P2

culture. In the OR65 specimen, HBoV1 and HRV were co-replicated in all passage. 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 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 2–3 viruses, mainly

including HBoV1 and/or human rhinovirus.

During the COVID-19 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

pandemic (30-32). 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 (33). 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, 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.

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 (34, 35). 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 report described persistent infection of HBoV1,

HRV, and ADV might cause high replication rate of these viruses (36-38). HBoV1 and

HRV cause severe respiratory tract infections with simultaneous infections caused by

other infectious agents (39-42). By contrast, some reports described that co-infections

with these viruses do not affect the disease severity (43, 44). 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. Furthermore, 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.

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Conflict of interest

The authors declare no conflict of interest.

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.

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Table 1. Results of virus isolation

	Target[a]	RSVA	RSVB	FluA	FluB	HCoV 229E	HCoV OC43	HCoV NL63	HCoV HKU1	hMPV	PIV1	PIV2	PIV3	PIV4	FluC	ADVC	ADVB3	HBoV	HRV	Total
	Total test	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
Average Cp	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
	Non-replicated	25.7	28.7	30.2	N.A[b]	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
Inoculated copies (log)	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	
	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[c]	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: N/A, not applicable

c: 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 (Cp ≥ 28.1)

	Target[a]	RSVA	RSVB	FluA	FluB	HCoV 229E	HCoV OC43	HCoV NL63	HCoV HKU1	hMPV	PIV1	PIV2	PIV3	PIV4	FluC	ADV2	ADV4	HBoV	Rhino	Total
	Total test	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
Average Cp	Replicated	N.A.[b]	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
	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
Inoculated copies (log)	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
	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: N/A, not applicable

Table 3. Simultaneous replication of multi-positive specimens.

Specimen number	Passage number	Target[a]	Inoculant	Cp value of inoculant	0	4	7	11	18	25
							Days			
H739	P0	ADV2(C)	Specimen	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	18.1	N.D.	N.D.
		HRV		36.8	> 40.0	> 40.0	35.8	> 40.0	N.D.	N.D.
	P1	ADV2(C)	P0-7d	> 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.
		HRV		35.8	> 40.0	> 40.0	> 40.0	> 40.0	N.D.	N.D.
O876	P0	HPIV1	Specimen	33.9	31.3	27.0	24.4	25.6	N.D.	N.D.
		HPIV4		36.5	33.5	30.3	25.8	27.1	N.D.	N.D.
	P1	HPIV1	P0-11d	25.6	29.0	18.2	18.5	17.3	N.D.	N.D.
		HPIV4		27.1	31.5	25.6	25.4	26.4	N.D.	N.D.
O898	P0	ADV2(C)	Specimen	32.9	25.9	34.8	18.1	18.1	N.D.	N.D.
		ADV4(B3)		29.1	> 40.0	> 40.0	27.7	27.2	N.D.	N.D.
	P1	ADV2(C)	P0-11d	18.1	> 40.0	> 40.0	14.7	10.1	> 40.0	N.D.
		ADV4(B3)		27.2	> 40.0	26.0	23.8	19.8	18.3	N.D.
OR7	P0	HCoVOC43	Specimen	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.
		HBoV		> 40.0	> 40.0	> 40.0	29.6	N.D.	N.D.	N.D.
	P1	HCoVOC43	P0-4d	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.
		HBoV		> 40.0	> 40.0	> 40.0	> 40.0	> 40.0	N.D.	N.D.
OR291	P0	HBoV	Specimen	20.96	> 40.0	22.2	25.2	23.6	20.4	15.6
		HRV		34.21	> 40.0	12.4	15.0	17.5	18.7	20.4
	P1-1	HBoV	P0-11d	23.6	> 40.0	> 40.0	> 40.0	> 40.0	> 40.0	N.D.
		HRV		17.47	30.1	19.7	15.7	18.9	18.1	N.D.
	P1-2	HBoV	P0-25d	15.64	27.6	12.9	10.5	11.3	14.1	N.D.
		HRV		20.44	40.0	34.5	40.0	33.8	40.0	N.D.
OR321.	P0	HBoV	Specimen	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.
		HRV		34.3	> 40.0	18.3	22.9	28.3	24.2	N.D.
	P1	HBoV	P0-7d	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.
		HRV		22.9	25.0	15.8	21.9	22.5	22.2	N.D.
OR331	P0	HBoV	Specimen	23.5	> 40	20.9	18.7	14.5	11.3	12.2
		HRV		34.5	> 40	23.0	19.0	18.1	21.8	26.4
	P1	HBoV	P0-11d	14.5	28.0	14.8	15.8	12.5	10.9	11.41
		HRV		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.

Table 4. Serial passage of multi-positive specimens.

Specimen number	Passage number	Target[a]	Inoculant	Cp value of inoculant	Days				
					0	4	7	11	18
H257	P0	RSV	Specimen	30.7	> 40.0[b]	> 40.0	> 40.0	> 40.0	N.D.[c]
		HCoVNL63		31.0	38.2	16.8	17.7	15.6	N.D.
		HBoV		17.2	22.5	20.7	26.0	26.7	N.D.
	P1	RSV	P0-7d	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
		HBoV		26.0	39.5	36.7	31.4	38.9	> 40.0
	P2	RSV	P1-7d	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
		HBoV		31.4	37.9	17.3	14.6	13.7	12.2
H260	P0	HPIV3	Specimen	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.
		HRV		38.1	38.9	27.9	21.7	24.0	N.D.
	P1	HPIV3	P0-4d	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.
		HRV		21.7	27.6	24.7	24.1	23.6	N.D.
	P2	HPIV3	P1-11d	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.
		HRV		23.6	38.5	33.1	> 40.0	N.D.	N.D.
OR32	P0	ADV2 (C)	Specimen	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.
		HRV		36.1	> 40.0	> 40.0	> 40.0	N.D.	N.D.
	P1	ADV2 (C)	P0-4d	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.
		HRV		> 40.0	> 40.0	38.5	> 40.0	> 40.0	N.D.
	P2	ADV2 (C)	P1-4d	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
		HRV		38.5	> 40.0	> 40.0	> 40.0	38.1	> 40.0
OR65	P0	HBoV	Specimen	23.4	30.0	17.7	12.7	N.D.	N.D.
		HRV		29.6	28.5	20.6	21.5	N.D.	N.D.
	P1	HBoV	P0-7d	12.7	22.1	> 40.0	14.7	16.4	N.D.
		HRV		21.5	30.6	> 40.0	28.8	29.3	N.D.
	P2	HBoV	P1-7d	10.1	29.1	17.1	10.1	10.8	N.D.
		HRV		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.

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