Research articles

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

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22

23 **Running title**

24 Viral co-cultivation in multiple infections

25

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

28

29 **Abstract**

30 Genetic diagnostic assays for the detection of respiratory viruses sometimes show

31 simultaneous multiple infections with low copy numbers. In such cases, the disease is

32 considered caused by a single etiologic agent and others are nonspecific reactions and/or

33 contaminations. Interferon-dependent interference is seen in dual infections of influenza

34 and respiratory syncytial virus, which are the main causes of respiratory infections.

35 Virus isolation is one of the solutions in detecting other active viruses present in

36 specimens, and the air–liquid interface culture of human bronchial/tracheal epithelial

37 cells (HBTEC-ALI) is optimal for the isolation of respiratory viruses owing to its wide
38 range of susceptibility. In this study, we successfully confirmed the replications of
39 various viruses from specimens with low copy numbers and passed 2–3 viruses
40 simultaneously using HBTEC-ALI cultures, mainly including human bocavirus 1 and/or
41 human rhinovirus.

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

45 The development of multiple molecular assays, such as nucleic acid amplification
46 tests, enable the simultaneous detection of these causative agents (5-8). The coronavirus
47 disease pandemic caused by severe acute respiratory syndrome coronavirus 2
48 (SARS-CoV-2), which occurred in the end of 2019, accelerated the development of
49 assays based on real-time polymerase chain reaction (PCR) techniques (9-11). Since
50 then, its use has become widespread among people who were not familiar with real-time
51 PCR in virus detection. As a result, whether a high Ct in one time test indicates virus
52 transmissibly became a complicated social problem, regardless of the difference in
53 assays and/or amplification conditions, the time and number of diagnostic test during
54 time course of infection (12-15).

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

60 agent, and the effects of multiple infections on disease development are controversial (5,
61 8, 19).

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

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

73

74 **Methods**

75 **Clinical specimens**

76 Clinical specimens were collected in acute phase from pediatric in-patients
77 with SARIs in Fukushima, Japan, between 2018 and 2022 (22). Nasopharyngeal swabs

78 were collected in the universal transport medium (Copan, Brescia, Italy) and stored at
79 −80°C until use.

80 The study protocol was approved by the Ethics Committees of the National
81 Institute of Infectious Diseases (Nos. 1001, 1087, and 1441) and Fukushima Medical
82 University (No. 29006). Informed consent was obtained verbally and was noted in the
83 medical records in each hospital. In this study, SARS-CoV-2-negative specimens were
84 used to evaluate respiratory infections as common diseases.

85

86 **Virus isolation**

87 Nucleic acids were extracted from specimens using QIAamp 96 Virus QIAcube
88 HT Kit (Qiagen, Hilden, Germany), QIAamp Viral RNA Mini Kit (Qiagen), or
89 NucleoSpin 96 Virus (Macherey-Nagel, Duren, Germany) following the manufacturer's
90 instruction, except that the elusion step was performed by centrifugation. Respiratory
91 viruses were detected from specimens by real-time PCR and reverse transcription
92 (RT)-PCR assays with LightCycler instruments (Roche, Basel, Switzerland) as
93 previously described (22, 24). The real-time PCR value was expressed as crossing point
94 (Cp). Primer/probe sequences are listed in Table S1. The following 17 viruses were
95 tested: human orthopneumovirus (RSV subgroups A and B); Flu A, B, and C; human

96 coronavirus (HCoV) (229E, OC43, NL63, and HKU1); human metapneumovirus
97 (hMPV); HPIV (1, 2, 3, and 4); adenovirus (ADV) [2 (for A, C, D, and F) and 4 (B and
98 E)]; HBoV1; and HRV (6, 23, 25-27). Among the above specimens, 249 specimens
99 were positive for respiratory viruses (single infection, n = 122; multiple infections, n =
100 127) and were subjected to virus isolation. The air–liquid interface culture of human
101 bronchial/tracheal epithelial cells (HBTEC-ALI) was prepared as described previously
102 (20, 21, 28). Then, 20 μL of the specimen diluted with an equal volume of
103 1%FCS-DMEM containing prescribed amount of antibiotics (penicillin-streptomycin,
104 gentamicin, and fungizone) was inoculated onto the apical surface of HBTEC-ALI
105 cultures. Cells were incubated at 34°C overnight and then washed with 1%FCS-DMEM
106 four times. The fourth wash solution was kept as the starting control. The MC-210
107 (KAC Co Ltd., Hyogo, Japan) was added to the basolateral medium throughout the
108 incubation periods to remove mycoplasmas and every buffer change a week. After 4, 7,
109 11, 18, and 25 days of incubation at 34°C (18 and 25 days were depended on the
110 condition of culture cells), cells were washed with 1%FCS-DMEM four times, and the
111 cell-washes were stored at −80°C. Virus replications were confirmed by real-time
112 RT-PCR assays as described above (22). During the time course and passage, when the
113 specimen showed >3.3 Cp (≈ 1 $\log_{10}$) of increase relative to day 0 and/or 4, it was

114 considered positive virus isolation. The copy number of each virus was determined
115 using the calibration curve drawn by real-time RT-PCR assay with
116 concentration-calculated synthesized control DNA or RNA containing the primer/probe
117 targeted region. The copy numbers of the control DNA/RNA were calculated based on
118 the molecular weight and absorbance at 260 nm of RNA that had been serially diluted
119 (≥3 steps) and/or fluorescence using the Quantus Fluorometer (Promega, Madison, WI,
120 USA) (n = 3).
121
122 **Sequencing analysis**
123 For next-generation sequencing, libraries were prepared using the NEBNext
124 Ultra II RNA Library Prep Kit for Illumina [New England Biolabs (NEB), Ipswich,
125 MA, USA] or the NEBNext Ultra II FS DNA Library Prep Kit for Illumina (NEB),
126 following the manufacturer's instructions. Indexed libraries were analyzed for 2×150
127 cycles on a DNBSEQ-G400 sequencer at AZENTA/GENEWIZ (Chelmsford, MA,
128 USA). Reads were trimmed and then *de novo* assembled and/or mapped to each
129 reference sequence using the CLC Genomics Workbench (v21.0.4, Qiagen) with default
130 settings.
131

132 **Statistical analysis**

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

137

138 **Results**

139 **Respiratory virus isolation using HBTEC-ALI cultures**

140 The real-time (RT-)PCR techniques sometimes detect multiple positives for
141 various targets in the tested specimen with suspicious respiratory infections. Virus
142 isolation is the most suitable technique in identifying the "active" virus in specimens
143 positive for multiple agents. Furthermore, HBTEC-ALI cultures are the best cells for
144 respiratory virus isolation owing to the wide range of susceptibility. In this study,
145 HBTEC-ALI cultures were used, and virus isolation was performed with clinical
146 specimens obtained from pediatric in-patients with respiratory infections (22, 23). In
147 HBTEC-ALI culture, cells generally did not show a cytopathic effect; therefore, genetic
148 replication detected by real-time RT-PCR was used as the indicator of successful virus
149 isolation. The replication kinetics of virus should be monitored by repeated test during

150 time course of infection, in initial diagnosis, only one time test result can be available,
151 generally. Therefore, the results of real-time (RT-)PCR in initial diagnosis were applied
152 following experiment. The 249 specimens (122 single infection and 127 multiple
153 infections) were subjected to virus isolation, and 131 specimens (52.6%) were positive
154 for replication of some viruses. Moreover, 70 of 122 single-infection specimens were
155 positive (57.3%) and 61 of 127 multiple-infection specimens were positive in at least
156 one target (48.0%). No significant differences in virus isolation were found between
157 single- and multiple-infection specimens by *z*-test (p = 0.177). For 17 respiratory
158 viruses, 450 targets were tested, and 147 of these (32.7%) showed virus replication
159 (Table 1). The rates of successful virus replication were low in RSVA (p = 0.04) and
160 higher in FluA (p = 0.014) and FluB (p = 0.002) than average. Overall, successful virus
161 replication was related to lower real-time RT-PCR value (25.7 vs 33.0, Cp value), i.e.,
162 high amounts of inoculated virus led to positive results (4.6 vs 2.5 $\log_{10}$ copies, $p <$
163 0.001) (Table 1). Yamada et al., reported that 87.5 % of successful virus isolation was
164 seen in less than 28.1 of Cp, which was corresponding to about log 3.5 copies
165 determined by NIID-N2 set, in the isolation of SARS-CoV-2 using VeroE6/TMPRSS2
166 cells (29). The average (±confidence interval) copies of Cp = 28.1 in used real-time
167 PCR assays is log 3.23±0.36. Although the correlation between real-time PCR value and

168 viral copy number is completely dependent on the performance of each primer/probe set
169 (the same Cp value does not always show the same copy number in different
170 primer/probe sets), data were sorted by the Cp value (≥28.1) to evaluate successful virus
171 replication in low-copy specimens (Table 2). In total, among 296 targets, 54 showed
172 virus replication (18.2%). The replication rates of FluB, and HPIV1 were higher than
173 average. In total, successful replications were also seen in specimens with lower Cp
174 values (31.8 vs 34.9, $p < 0.001$) and the higher copy number (2.8 vs 1.9, $\log_{10}$ copies, p
175 < 0.008). The performance of each real-time (RT-)PCR assays are different and same Cp
176 value does not always show same copy number; therefore, the results have been
177 converted to the copy number enable to compare altogether. In addition, the sensitivity
178 of virus replication on HBTEC-ALI culture are different depending on the virus species.
179 However, these results indicate the tendency that the higher the number of viral copies,
180 the more successful is the virus replication. Although approximately several hundred of
181 copies are required to lead to possible respiratory virus isolation in HBTEC-ALI
182 cultures, intact virion (active virus) surely exists even in low copies specimens,
183

184 **Simultaneous virus replication in HBTEC-ALI cultures**

185 In the virus isolation, 16 specimens showed simultaneous replications of 2–3

186 viruses in the first inoculation in the same HBTEC-ALI culture (Table S2). Eleven of
187 them were in combination with HBoV1 or HRV, and three were in combination with
188 ADV2. Combinations of envelope viruses were seen in two (HCoV229E/HMPV and
189 HPIV1/HPIV4). Eleven of the co-replicated viruses could be simultaneously passed
190 once (Table 3 and 4). In the H739 specimen, ADVC, ADVB3, and HRV were replicated
191 after 7 days in the first isolation (P0) despite a high Cp value in ADVC and HRV, and
192 both ADVC and ADVB3 could be passed one time (P1). In the O898 specimen, ADVC
193 and ADVB3 were replicated simultaneously in both P0 and P1 cultures. In the O876
194 specimen, HPIV1 and HPIV4 were replicated and passed in both P0 and P1 cultures
195 despite high Cp values in the specimen. In the OR7 specimen, HCoVOC43, HRV, and
196 HBoV1 were replicated in the P0 culture, and HCoVOC43 and HRV could be passed in
197 the P1 culture. Simultaneous replications and passage of HBoV1 and HRV were most
198 observed in specimens OR291, OR321, and OR331. The following four specimens
199 could be simultaneously passed two times (Table 4). In the H257 specimen, HCoVNL63
200 and HBoV1 were replicated throw the passage. In the H260 specimen, although HBoV1
201 was replicated in the P1 culture, HPIV3 and HRV were replicated during two passages.
202 In the OR32 specimen, ADVC and HBoV1 were replicated and passed till the P2
203 culture. In the OR65 specimen, HBoV1 and HRV were co-replicated in all passage. In

204 these passage-successful specimens, the nearly complete genomic sequencers of these
205 replicated viruses could be decoded and were registered in the GenBank database (Table
206 5), proofing the presence of active viruses simultaneously in the same cell cultures.
207 These can be strong evidence that the specimens showing positivity for multiple agents
208 in real-time PCR tests surely contain infectious viruses.
209

210 **Discussion**

211 In this study, we show evidence on the presence of an active virus in specimens
212 with low copies and/or positive for multiple respiratory infections. The results clearly
213 show successful virus replication in HBTEC-ALI cultures inoculated with specimens
214 with Cp >28.1 and simultaneous replications and serial passages of 2–3 viruses, mainly
215 including HBoV1 and/or human rhinovirus.

216 During the COVID-19 pandemic, real-time RT-PCR-based diagnostic assays have
217 been used to detect SARS-CoV-2; however, the correlation between positive signals in
218 real-time RT-PCR and the possibility of virus spread from patients became a problem
219 because PCR-negative results had been required for discharge in the early phase of
220 pandemic (30-32). For SARS-CoV-2, the assays that targeted structural proteins such as
221 nucleocapsid gene were well-used (9, 11); however, these assays also detect

222 subgenomic mRNAs of coronaviruses, causing confusion on the presence of intact
223 virions. In a previous report, the relation between real-time RT-PCR and successful
224 virus isolation was assessed using the ORF1a assay, which could detect mainly genomic
225 RNA, and several dozens of copies of genomic RNA were required for possible virus
226 isolation (33). In the present study, various real-time RT-PCR assays were used, and
227 comparing their results using the value of real-time PCR (such as Ct and Cp) was
228 difficult. However, it enables conversion of the results to copy numbers, and the
229 presence of approximately several hundred copies of viruses possibly leads to
230 replications in HBTEC-ALI cultures for all respiratory viruses tested in this study. In
231 suspicious specimens, the copy number, not the RT-PCR value, should be used as a
232 guide for possible infectivity. Note that the frozen specimens were used in virus
233 isolation in this study due to the convenience of transportation, and it might affect the
234 result of virus replication like RSV.

235 Multiple respiratory viruses infect respiratory tissues one after another and induce
236 reciprocal interference in one another, i.e., Flu, RSV, HMPV, and HRV interfere with
237 other viruses by the signaling of intracellular RNA sensors or inducing
238 interferon-related genes (16, 17). This is also a major theory in hepatitis, i.e., hepatitis C
239 virus infection inhibits the infections of other hepatitis viruses (34, 35). Therefore, we

240 tended to think that the first virus suppresses the second virus, and the infectious
241 diseases were mainly caused by the first virus. Certainly, only one virus could be
242 replicated in most specimens with multiple infections (73.8%, 45 of 61, Table S2);
243 however, simultaneous virus replications were observed, although mainly with HBoV1,
244 HRV, and/or ADV infections. Several report described persistent infection of HBoV1,
245 HRV, and ADV might cause high replication rate of these viruses (36-38). HBoV1 and
246 HRV cause severe respiratory tract infections with simultaneous infections caused by
247 other infectious agents (39-42). By contrast, some reports described that co-infections
248 with these viruses do not affect the disease severity (43, 44). Thus, the relationship
249 between the synergistic replication of infected dual viruses and disease severity must be
250 investigated. In addition, the mechanism of acceptable dual virus replication is unclear.
251 Although many reports describe one virus infection disturbs other virus infections
252 (16-18), there is no report for viral symbiotic. Therefore, it is necessary to elucidate the
253 acceptable combination of respiratory virus species for dual virus replication and the
254 mechanisms. Furthermore, in this study, although the co-cultivative cases are minority,
255 it is due to the limit of the sensitivity of virus isolation system. Improvement of
256 HBTEC-ALI culturing system might increase the number of dual replication cases.
257

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265

266 **Conflict of interest**

267 The authors declare no conflict of interest.

268

269 **Data availability**

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

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References

1. Williams BG, Gouws E, Boschi-Pinto C, Bryce J, Dye C. Estimates of world-wide distribution of child deaths from acute respiratory infections. Lancet Infect Dis. 2002 Jan;2(1):25-32.

2. Bhuyan GS, Hossain MA, Sarker SK, Rahat A, Islam MT, Haque TN, et al. Bacterial and viral pathogen spectra of acute respiratory infections in under-5 children in hospital settings in Dhaka city. PloS one. 2017;12(3):e0174488.

3. Aman AT, Wibawa T, Kosasih H, Asdie RH, Safitri I, Intansari US, et al. Etiologies of severe acute respiratory infection (SARI) and misdiagnosis of influenza in Indonesia, 2013-2016. Influenza Other Respir Viruses. 2021 Jan;15(1):34-44.

4. Li J, Song CL, Wang T, Ye YL, Du JR, Li SH, et al. Etiological and epidemiological characteristics of severe acute respiratory infection caused by multiple viruses and Mycoplasma pneumoniae in adult patients in Jinshan, Shanghai: A pilot hospital-based surveillance study. PloS one. 2021;16(3):e0248750.

5. Stefanska I, Romanowska M, Donevski S, Gawryluk D, Brydak LB. Co-infections with

301 influenza and other respiratory viruses. Adv Exp Med Biol. 2013;756:291-301.
302 6. Kaida A, Kubo H, Takakura K, Sekiguchi J, Yamamoto SP, Kohdera U, et al.
303 Associations between co-detected respiratory viruses in children with acute respiratory
304 infections. Japanese journal of infectious diseases. 2014;67(6):469-75.
305 7. Yoshida LM, Suzuki M, Nguyen HA, Le MN, Dinh Vu T, Yoshino H, et al. Respiratory
306 syncytial virus: co-infection and paediatric lower respiratory tract infections. The
307 European respiratory journal. 2013 Aug;42(2):461-9.
308 8. Kouni S, Karakitsos P, Chranioti A, Theodoridou M, Chrousos G, Michos A.
309 Evaluation of viral co-infections in hospitalized and non-hospitalized children with
310 respiratory infections using microarrays. Clin Microbiol Infect. 2013 Aug;19(8):772-7.
311 9. CDC. Research Use Only 2019-Novel Coronavirus (2019-nCoV) Real-time RT-PCR
312 Primers and Probes. 2020 June 6, 2020 [cited 2022 21 Sep]; Available from:
313 https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html
314 10. Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DK, et al. Detection
315 of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 2020 Jan;25(3).
316 11. Shirato K, Nao N, Katano H, Takayama I, Saito S, Kato F, et al. Development of
317 Genetic Diagnostic Methods for Detection for Novel Coronavirus 2019(nCoV-2019) in Japan.
318 Jpn J Infect Dis. 2020 Jul 22;73(4):304-7.
319 12. Borger P, Malhorta BR, Yeadon M, Craig C, McKernan K, Steger K, et al. External
320 peer review of the RTPCR test to detect SARS-CoV-2 reveals 10 major scientific flaws at
321 the molecular and methodological level: consequences for false positive results. Review
322 report Corman-Drosten et al Eurosurveillance 2020 2020 Nov. 27. 2020 [cited 2021 OCT.
323 27]; Available from: https://cormandrostenreview.com/report/
324 13. Platten M, Hoffmann D, Grosser R, Wisplinghoff F, Wisplinghoff H, Wiesmuller G,
325 et al. SARS-CoV-2, CT-Values, and Infectivity-Conclusions to Be Drawn from Side
326 Observations. Viruses. 2021 Jul 27;13(8).
327 14. Al Bayat S, Mundodan J, Hasnain S, Sallam M, Khogali H, Ali D, et al. Can the
328 cycle threshold (Ct) value of RT-PCR test for SARS CoV2 predict infectivity among close
329 contacts? J Infect Public Health. 2021 Sep;14(9):1201-5.
330 15. Singanayagam A, Patel M, Charlett A, Lopez Bernal J, Saliba V, Ellis J, et al.
331 Duration of infectiousness and correlation with RT-PCR cycle threshold values in cases
332 of COVID-19, England, January to May 2020. Euro Surveill. 2020 Aug;25(32).
333 16. Essaidi-Laziosi M, Geiser J, Huang S, Constant S, Kaiser L, Tapparel C.
334 Interferon-Dependent and Respiratory Virus-Specific Interference in Dual Infections of
335 Airway Epithelia. Sci Rep. 2020 Jun 24;10(1):10246.
336 17. Piret J, Boivin G. Viral Interference between Respiratory Viruses. Emerg Infect

Dis. 2022 Feb;28(2):273-81.
18. Wu A, Mihaylova VT, Landry ML, Foxman EF. Interference between rhinovirus and influenza A virus: a clinical data analysis and experimental infection study. Lancet Microbe. 2020 Oct;1(6):e254-e62.
19. Asner SA, Science ME, Tran D, Smieja M, Merglen A, Mertz D. Clinical disease severity of respiratory viral co-infection versus single viral infection: a systematic review and meta-analysis. PloS one. 2014;9(6):e99392.
20. Kakizaki M, Kume Y, Suwa R, Kawase M, Ono T, Chishiki M, et al. Thirteen Nearly Complete Genome Sequences of Human Bocavirus 1 Isolated from Pediatric Inpatients in Fukushima, Japan. Microbiol Resour Announc. 2022 Jan 20;11(1):e0102721.
21. Sugimoto S, Kume Y, Suwa R, Kawase M, Kakizaki M, Egawa K, et al. Ten Nearly Complete Genome Sequences of Human Orthorubulavirus 4 Isolated from Pediatric Inpatients in Fukushima, Japan. Microbiol Resour Announc. 2022 Jun 9:e0041122.
22. Kume Y, Hashimoto K, Chishiki M, Norito S, Suwa R, Ono T, et al. Changes in virus detection in hospitalized children before and after the severe acute respiratory syndrome coronavirus 2 pandemic. Influenza Other Respir Viruses. 2022 Apr 29.
23. Kume Y, Hashimoto K, Shirato K, Norito S, Suwa R, Chishiki M, et al. Epidemiological and clinical characteristics of infections with seasonal human coronavirus and respiratory syncytial virus in hospitalized children immediately before the coronavirus disease 2019 pandemic. J Infect Chemother. 2022 Jul;28(7):859-65.
24. Suwa R, Kume Y, Kawase M, Chishiki M, Ono T, Norito S, et al. Practical Validation of United States Centers for Disease Control and Prevention Assays for the Detection of Human Respiratory Syncytial Virus in Pediatric Inpatients in Japan. Pathogens. 2022;11(7).
25. Wang L, Piedra PA, Avadhanula V, Durigon EL, Machablishvili A, Lopez MR, et al. Duplex real-time RT-PCR assay for detection and subgroup-specific identification of human respiratory syncytial virus. J Virol Methods. 2019 Sep;271:113676.
26. WHO. WHO information for the molecular detection of influenza viruses. 2021 [cited 2022 14 July]; Available from: https://cdn.who.int/media/docs/default-source/influenza/molecular-detention-of-influenza-viruses/protocols_influenza_virus_detection_feb_2021.pdf?sfvrsn=df7d268a_5
27. Dare RK, Fry AM, Chittaganpitch M, Sawanpanyalert P, Olsen SJ, Erdman DD. Human coronavirus infections in rural Thailand: a comprehensive study using real-time reverse-transcription polymerase chain reaction assays. J Infect Dis. 2007 Nov 1;196(9):1321-8.
28. Shirato K, Kawase M, Matsuyama S. Wild-type human coronaviruses prefer cell-surface TMPRSS2 to endosomal cathepsins for cell entry. Virology. 2018 Apr;517:9-15.

29. Yamada S, Fukushi S, Kinoshita H, Ohnishi M, Suzuki T, Fujimoto T, et al. Assessment of SARS-CoV-2 infectivity of upper respiratory specimens from COVID-19 patients by virus isolation using VeroE6/TMPRSS2 cells. BMJ Open Respir Res. 2021 Feb;8(1):e000830.
30. Suri T, Mittal S, Tiwari P, Mohan A, Hadda V, Madan K, et al. COVID-19 Real-Time RT-PCR: Does Positivity on Follow-up RT-PCR Always Imply Infectivity? Am J Respir Crit Care Med. 2020 Jul 1;202(1):147.
31. Kim MC, Cui C, Shin KR, Bae JY, Kweon OJ, Lee MK, et al. Duration of Culturable SARS-CoV-2 in Hospitalized Patients with Covid-19. N Engl J Med. 2021 Feb 18;384(7):671-3.
32. Manzulli V, Scioscia G, Giganti G, Capobianchi MR, Lacedonia D, Pace L, et al. Real Time PCR and Culture-Based Virus Isolation Test in Clinically Recovered Patients: Is the Subject Still Infectious for SARS-CoV2? J Clin Med. 2021 Jan 15;10(2).
33. Shirato K, Kakizaki M, Tomita Y, Kawase M, Takeda M. Detection of the ORF1 Gene Is an Indicator of the Possible Isolation of Severe Acute Respiratory Syndrome Coronavirus 2. Pathogens. 2022 Feb 27;11(3).
34. Zhang K, Lai X, Song J, He L, Wang L, Ou G, et al. A novel cell culture model reveals the viral interference during hepatitis B and C virus coinfection. Antiviral Res. 2021 May;189:105061.
35. Burkard T, Proske N, Resner K, Collignon L, Knegendorf L, Friesland M, et al. Viral Interference of Hepatitis C and E Virus Replication in Novel Experimental Co-Infection Systems. Cells. 2022 Mar 8;11(6).
36. Radke JR, Cook JL. Human adenovirus infections: update and consideration of mechanisms of viral persistence. Curr Opin Infect Dis. 2018 Jun;31(3):251-6.
37. Xu M, Perdomo MF, Mattola S, Pyoria L, Toppinen M, Qiu J, et al. Persistence of Human Bocavirus 1 in Tonsillar Germinal Centers and Antibody-Dependent Enhancement of Infection. mBio. 2021 Feb 2;12(1).
38. Esneau C, Duff AC, Bartlett NW. Understanding Rhinovirus Circulation and Impact on Illness. Viruses. 2022 Jan 13;14(1).
39. Ji K, Sun J, Yan Y, Han L, Guo J, Ma A, et al. Epidemiologic and clinical characteristics of human bocavirus infection in infants and young children suffering with community acquired pneumonia in Ningxia, China. Virol J. 2021 Oct 29;18(1):212.
40. Sun H, Sun J, Ji W, Hao C, Yan Y, Chen Z, et al. Impact of RSV Coinfection on Human Bocavirus in Children with Acute Respiratory Infections. J Trop Pediatr. 2019 Aug 1;65(4):342-51.
41. Costa LF, Queiroz DA, Lopes da Silveira H, Bernardino Neto M, de Paula NT, Oliveira TF, et al. Human rhinovirus and disease severity in children. Pediatrics. 2014 Feb;133(2):e312-21.

409 42. Goikhman Y, Drori Y, Friedman N, Sherbany H, Keller N, Mendelson E, et al.
410 Adenovirus load correlates with respiratory disease severity among hospitalized pediatric
411 patients. Int J Infect Dis. 2020 Aug;97:145-50.
412 43. Calvo C, Garcia-Garcia ML, Pozo F, Paula G, Molinero M, Calderon A, et al.
413 Respiratory Syncytial Virus Coinfections With Rhinovirus and Human Bocavirus in
414 Hospitalized Children. Medicine (Baltimore). 2015 Oct;94(42):e1788.
415 44. Morikawa S, Kohdera U, Hosaka T, Ishii K, Akagawa S, Hiroi S, et al. Seasonal
416 variations of respiratory viruses and etiology of human rhinovirus infection in children.
417 J Clin Virol. 2015 Dec;73:14-9.
418

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	Days 0	4	7	11	18	25
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