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Title: Improved molecular diagnosis of COVID-19 by the novel, highly sensitive and specific COVID-19-RdRp/Hel real-time reverse transcription-polymerase chain reaction assay validated *in vitro* and with clinical specimens

Running Head: Development & evaluation of new COVID-19 RT-PCR assays

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ABSTRACT

On 31st December 2019, the World Health Organization was informed of a cluster of cases of pneumonia of unknown etiology in Wuhan, China. Subsequent investigations identified a novel coronavirus, now named as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), from the affected patients. Highly sensitive and specific laboratory diagnostics are important for controlling the rapidly evolving SARS-CoV-2-associated Coronavirus Disease 2019 (COVID-19) epidemic. In this study, we developed and compared the performance of three novel real-time RT-PCR assays targeting the RNA-dependent RNA polymerase (RdRp)/helicase (Hel), spike (S), and nucleocapsid (N) genes of SARS-CoV-2 with that of the reported RdRp-P2 assay which is used in >30 European laboratories. Among the three novel assays, the COVID-19-RdRp/Hel assay had the lowest limit of detection *in vitro* (1.8 $TCID_{50}$/ml with genomic RNA and 11.2 RNA copies/reaction with *in vitro* RNA transcripts). Among 273 specimens from 15 patients with laboratory-confirmed COVID-19 in Hong Kong, 77 (28.2%) were positive by both the COVID-19-RdRp/Hel and RdRp-P2 assays. The COVID-19-RdRp/Hel assay was positive for an additional 42 RdRd-P2-negative specimens [119/273 (43.6%) vs 77/273 (28.2%), P<0.001], including 29/120 (24.2%) respiratory tract specimens and 13/153 (8.5%) non-respiratory tract specimens. The mean viral load of these specimens was 3.21×10^4 RNA copies/ml (range, 2.21×10^2 to 4.71×10^5 RNA copies/ml). The COVID-19-RdRp/Hel assay did not cross-react with other human-pathogenic coronaviruses and respiratory pathogens in cell culture and clinical specimens, whereas the RdRp-P2 assay cross-reacted with SARS-CoV in cell culture. The highly sensitive and specific COVID-19-RdRp/Hel assay may help to improve the laboratory diagnosis of COVID-19.

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62 **KEYWORDS:** Coronavirus, COVID-19, diagnostic, emerging, PCR, SARS, Wuhan, virus.

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63 **INTRODUCTION**

64 On 31st December 2019, the World Health Organization was informed of a cluster of cases of
65 pneumonia of unknown etiology in Wuhan, Hubei Province, China
66 (https://www.who.int/westernpacific/emergencies/covid-19). Subsequent investigations
67 identified a novel coronavirus that was closely related to severe acute respiratory syndrome
68 coronavirus (SARS-CoV) from these patients (1-3). This new virus has been recently named as
69 severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by the Coronavirus Study
70 Group of the International Committee on Taxonomy of Viruses
71 (https://www.biorxiv.org/content/10.1101/2020.02.07.937862v1). Most patients with SARS-
72 CoV-2 infection, or Coronavirus Disease 2019 (COVID-19), present with acute onset of fever,
73 myalgia, cough, dyspnea, and radiological evidence of ground-glass lung opacities compatible
74 with atypical pneumonia (4-6). However, asymptomatic or mildly symptomatic cases have also
75 been reported (2, 7-9). Initial epidemiological investigations have indicated the Huanan seafood
76 wholesale market in Wuhan as a geographically linked source, but subsequent detailed
77 epidemiological assessment has revealed that up to 45% of the early cases with symptom onset
78 before 1st January 2020 were not linked to this market (4, 10). Person-to-person transmissions
79 among close family contacts and healthcare workers, including those without travel history to
80 Wuhan, have been reported (2, 6, 11, 12). Therefore, clinical features and epidemiological links
81 to Wuhan alone are not reliable for establishing the diagnosis of COVID-19.

82 As evidenced by previous epidemics caused by SARS-CoV and Middle East respiratory
83 syndrome coronavirus (MERS-CoV), highly sensitive and specific laboratory diagnostics for
84 COVID-19 are essential for case identification, contact tracing, animal source finding, and
85 rationalization of infection control measures (13-15). The use of viral culture for establishing

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86 acute diagnosis is not practical as it takes at least three days for SARS-CoV-2 to cause obvious
87 cytopathic effects in selected cell lines, such as VeroE6 cells (3). Moreover, isolation of the virus
88 requires biosafety level-3 facilities which are not available in most healthcare institutions. Serum
89 antibody and antigen detection tests have not yet been validated, and there may be cross-
90 reactivity with SARS-CoV which shares a high degree (~82%) of nucleotide identity with
91 SARS-CoV-2 (16). Because of these limitations, reverse transcription-polymerase chain reaction
92 (RT-PCR) remains the most useful laboratory diagnostic test for COVID-19 worldwide.

93 The availability of the complete genome of SARS-CoV-2 early in the epidemic facilitated
94 the development of specific primers and standardized laboratory protocols for COVID-19 (17,
95 18). The protocol of the first real-time RT-PCR assays targeting the RNA-dependent RNA
96 polymerase (RdRp), envelope (E), and nucleocapsid (N) genes of SARS-CoV-2 were published
97 on 23rd January 2020 (19). Among these assays, the RdRp assay had the highest analytical
98 sensitivity (3.8 RNA copies/reaction at 95% detection probability) (19). In this published RdRp
99 assay, probe 1 was a "pan Sarbeco-Probe" which would detect SARS-CoV-2, SARS-CoV, and
100 bat-SARS-related coronaviruses, whereas probe 2 (termed "RdRp-P2" assay in the present study)
101 was reported to be specific for SARS-CoV-2 and should not detect SARS-CoV (19). Notably,
102 these assays were designed and validated using synthetic nucleic acid technology and in the
103 absence of available SARS-CoV-2 isolates or original patient specimens (19). The reported
104 RdRp assays had been implemented in >30 laboratories in Europe (20). In this study, we
105 developed novel, highly sensitive and specific real-time RT-PCR assays for COVID-19 and
106 compared their performances with that of the established RdRp-P2 assay using both *in vitro* and
107 patient specimens. Clinical evaluation using different types of clinical specimens from patients

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108 with laboratory-confirmed COVID-19 showed that our novel assay targeting a different region of
109 the RdRp/Hel was significantly more sensitive and specific than the RdRp-P2 assay.
110

111 **MATERIALS AND METHODS**

112 ***Viruses and clinical specimens***

113 SARS-CoV-2 was isolated from a patient with laboratory-confirmed COVID-19 in Hong Kong
114 (21). The viral isolate was amplified by one additional passage in VeroE6 cells to make working
115 stocks of the virus (1.8×10^7 50% tissue culture infective doses [$TCID_{50}$]/ml). For *in vitro*
116 specificity evaluation, archived laboratory culture isolates (n=17) of other human-pathogenic
117 coronaviruses and respiratory viruses used were obtained from the Department of Microbiology,
118 The University of Hong Kong, as previously described (22). All experimental protocols
119 involving live SARS-CoV-2, SARS-CoV, and MERS-CoV followed the approved standard
120 operating procedures of the Biosafety Level 3 facility as previously described (23, 24). For the
121 clinical evaluation study, a total of 273 (120 respiratory tract and 153 non-respiratory tract)
122 clinical specimens were collected from 15 patients with laboratory-confirmed COVID-19 in
123 Hong Kong whose nasopharyngeal aspirate / swab, throat swab, and/or sputum specimens tested
124 positive for SARS-CoV-2 RNA by the RdRp2 assay (21). Additionally, the total nucleic acid
125 extracts of 22 archived (stored at -80°C until use) nasopharyngeal aspirates/swabs and throat
126 swabs collected from 22 adult patients who were managed at our hospitals in Hong Kong for
127 upper and/or lower respiratory tract symptoms that were tested positive for other respiratory
128 pathogens by FilmArray® RP (BioFire Diagnostics, Salt Lake City, UT, USA), were prepared
129 according to the manufacturer's instructions for assessing potential cross-reactivity of the assays

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130 with other respiratory pathogens in clinical specimens. The study was approved by Institutional
131 Review Board of The University of Hong Kong / Hospital Authority (UW 14-249).
132
133 ***Nucleic acid extraction***
134 Total nucleic acid (TNA) extraction of clinical specimens and laboratory cell culture of viral
135 isolates were performed using NucliSENS easyMAG extraction system (BioMerieux, Marcy-
136 l'Étoile, France) according to the manufacturer's instructions and as previously described (23).
137 The volume of the specimens used for extraction and the elution volume depended on the
138 specimen type and the available amount of the specimen. In general, 250µl of each respiratory
139 tract specimen, urine, rectal swab, and feces were subjected to extraction with an elution volume
140 of 55µl; and 100µl of each plasma specimen were subjected to extraction with an elution volume
141 of 25µl. The extracts were stored at -80°C until use. The same extracted product of each
142 specimen was used for all the RT-PCR reactions.
143
144 ***Primers and probes***
145 Primer and probe sets targeting different gene regions [RdRp/Helicase (Hel), Spike (S), and N]
146 of SARS-CoV-2 were designed and tested. The probes were predicted to specifically amplify
147 SARS-CoV-2 and had no homologies with human, other human-pathogenic coronaviruses or
148 microbial genes on BLASTn analysis that would potentially produce false-positive test results as
149 previously described (22). Primer and probe sets with the best amplification performance were
150 selected.
151
152 ***In vitro RNA transcripts for making positive controls and standards***

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153 Linearized pCR2.1-TOPO plasmid (Invitrogen, Carlsbad, CA, USA) with T7 promoter and a
154 cloned target region (RdRp/Hel, S, or N) of SARS-CoV-2 were used for *in vitro* RNA
155 transcription using MEGAscript T7 Transcription Kit (Ambion, Austin, TX, USA) for the
156 standards and limit of detection (LOD) as previously described (22, 25). Each linearized plasmid
157 template was mixed with 2µl each of ATP, GTP, CTP, and UTP, 10×reaction buffer, and enzyme
158 mix in a standard 20µl reaction mixture. The reaction mixture was incubated at 37°C for 16h,
159 followed by addition of 1µl of TURBO DNase, and was further incubated at 37°C for 15min.
160 The synthesized RNA was cleaned by RNeasy Mini Kit (Qiagen, Hilden, Germany) according to
161 the manufacturer's instructions. The concentration of purified RNA was quantified by BioDrop
162 µLITE (BioDrop, UK).
163
164 ***COVID-19 real-time RT-PCR assays***
165 Real-time RT-PCR assays for SARS-CoV-2 RNA detection were performed using QuantiNova
166 Probe RT-PCR Kit (Qiagen) in a LightCycler 480 Real-Time PCR System (Roche, Basel,
167 Switzerland) as previously described (25). Each 20µl reaction mixture contained 10µl of 2×
168 QuantiNova Probe RT-PCR Master Mix, 0.2µl of QN Probe RT-Mix, 1.6µl of each 10µM
169 forward and reverse primer, 0.4µl of 10µM probe, 1.2µl of RNase-free water and 5µl of TNA as
170 the template. The thermal cycling condition was 10min at 45°C for reverse transcription, 5min at
171 95°C for PCR initial activation, and 45 cycles of 5s at 95°C and 30s at 55°C. The RdRp-P2 assay
172 was performed as previously described (19).
173

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174 ***Confirmation of discrepant results in different COVID-19 real-time RT-PCR assays by the***
175 ***LightMix® Modular SARS and Wuhan CoV E-gene kit with LightCycler Multiplex RNA Virus***
176 ***Master***

177 Discrepant results were confirmed by additional testing with the LightMix® Modular SARS and
178 Wuhan CoV E-gene kit (TIB Molbiol, Berlin, Germany) with LightCycler Multiplex RNA Virus
179 Master (Roche) which could detect SARS-CoV-2, SARS-CoV, and bat SARS-like coronaviruses
180 (*Sarbecovirus*) (LOD = 10 genome equivalent copies or less per reaction) without cross-
181 reactivity with other human-pathogenic coronaviruses according to the manufacturer's
182 instructions. Briefly, each 20µl reaction mixture contained 4µl of Roche Master, 0.1µl of RT
183 Enzyme, 0.5µl of reagent mix, 10.4µl of water and 5µl of TNA as the template. The thermal
184 cycling condition was 5min at 55°C for reverse transcription, 5min at 95°C for denaturation, and
185 45 cycles of 5s at 95°C, 15s at 60°C and 15s at 72°C.

186

187 ***Statistical analysis***

188 The Fisher's exact test was used to compare the performance of the assays. $P<0.05$ was
189 considered statistically significant. Computation was performed using Predictive Analytics
190 Software (v18.0).

191

192 **RESULTS**

193 ***Design of novel COVID-19 real-time RT-PCR assays targeting different gene regions of the***
194 ***SARS-CoV-2 genome***

195 Three novel real-time COVID-19 RT-PCR assays targeting the RdRp/Hel, S, and N genes of
196 SARS-CoV-2 were developed (Supplementary Table 1). To avoid cross-reactivity with human

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197 SARS-CoV, we purposely designed the probes of our assays to contain 7 to 9 nucleotide
198 differences with those of human SARS-CoV (strains HKU-39849 and GZ50) (Supplementary
199 Figure 1). In comparison, the probe of the RdRp-P2 assay contained only 3 nucleotide
200 differences with those of human SARS-CoV (strains Frankfurt-1, HKU-39849, and GZ50) (19)
201 (Supplementary Figure 1).
202
203 ***Analytical sensitivity of the novel COVID-19 real-time RT-PCR assays***
204 To determine the analytical sensitivity of the COVID-19 assays, we first evaluated their LODs
205 using viral genomic RNA extracted from culture lysate and clinical specimen. Serial 10-fold
206 dilutions of SARS-CoV-2 RNA extracted from culture lysate were prepared and tested in
207 triplicate with each corresponding assay in two independent runs. The LOD of COVID-19-
208 RdRp/Hel, COVID-19-S, and COVID-19-N was 1.8×10^{0} $TCID_{50}$/ml, while the LOD of RdRp-
209 P2 was 1-log higher (1.8×10^{1} $TCID_{50}$/ml) (Table 1). Serial 10-fold dilutions of SARS-CoV-2
210 RNA extracted from a laboratory-confirmed patient's nasopharyngeal aspirate were also prepared
211 and tested in triplicate with each corresponding assay in two independent runs. The LOD of
212 COVID-19-RdRp/Hel and COVID-19-N (10^{-5} fold dilution) was 1-log lower than that of
213 COVID-19-S and RdRp-P2 (10^{-4} fold dilution) (Table 1). Based on these results, we then
214 selected the COVID-19-RdRp/Hel and COVID-19-N assays for further evaluation and
215 determined their LODs using *in vitro* viral RNA transcripts (Table 2). The LODs of the COVID-
216 19-RdRp/Hel and COVID-19-N assays using serial dilutions of *in vitro* viral RNA transcripts as
217 calculated with probit analysis were 11.2 RNA copies/reaction (95% confidence interval = 7.2-
218 52.6 RNA copies/reaction) and 21.3 RNA copies/reaction (95% confidence interval = 11.6-177.0
219 copies/reaction), respectively.

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221 ***Comparative performance of the COVID-19-RdRp/Hel and RdRp-P2 for the detection of***
222 ***SARS-CoV-2 RNA in different types of clinical specimens***

223 Based on the lower LOD of the COVID-19-RdRp/Hel assay than the COVID-19-N assay, we
224 then evaluated the performance of COVID-19-RdRp/Hel assay in the detection of SARS-CoV-2
225 RNA in clinical specimens and compared it with that of the RdRp-P2 assay. A total of 120
226 respiratory tract (nasopharyngeal aspirates/swabs, throat swabs, saliva, and sputum) and 153
227 non-respiratory tract (plasma, urine, and feces / rectal swabs) specimens were collected from 15
228 patients with laboratory-confirmed COVID-19 in Hong Kong (positive nasopharyngeal aspirate /
229 swab, throat swab, or sputum by the RdRp-P2 assay). The median number of specimens
230 collected per patient was 13. There were a total of 8 males and 7 females. Their median age was
231 63 years (range: 37 to 75 years). All of them had clinical features compatible with acute
232 community-acquired atypical pneumonia and radiological evidence of ground-glass lung
233 opacities. At the time of writing, 11 patients were in stable condition, 3 were in critical condition,
234 and 1 patient had succumbed.

235 Among the 273 specimens collected from these 15 patients, 77 (28.2%) were positive by
236 the RdRp-P2 assay (Table 3). The novel COVID-2019-RdRp/Hel assay was positive for all of
237 these 77 specimens. Additionally, the COVID-2019-RdRp/Hel assay was positive for another 42
238 [total positive specimens = 119/273 (43.6%) by COVID-2019-RdRp/Hel vs 77/273 (28.2%) by
239 RdRp-P2, P<0.001] specimens, including 29/120 (24.2%) respiratory tract specimens and 13/153
240 (8.5%) non-respiratory tract specimens that were negative by the RdRp-P2 assay. All of these
241 42/273 (15.4%) additional positive specimens were confirmed to be positive by the LightMix®
242 Modular SARS and Wuhan CoV E-gene kit with the LightCycler Multiplex RNA Virus Master.

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243 The mean viral load of these specimens was 3.21×10^4 RNA copies/ml (range, 2.21×10^2 to
244 4.71×10^5 RNA copies/ml) and was about 6 folds higher in the respiratory tract specimens
245 (4.33×10^4 RNA copies/ml) than the non-respiratory tract specimens (7.06×10^3 RNA copies/ml).
246 The COVID-19-RdRp/Hel assay was significantly more sensitive than the RdRp-P2
247 assay for the detection of SARS-CoV-2 RNA in nasopharyngeal aspirates/swabs or throat swabs
248 (P=0.043), saliva (P<0.001), and plasma (P=0.001) specimens. As shown in Figure 1, the
249 COVID-19-RdRp/Hel assay consistently detected SARS-CoV-2 RNA in these samples than the
250 RdRp-2 assay throughout the patients' course of illness up to day 12 (nasopharyngeal
251 aspirates/swabs and/or throat swabs) to day 18 (saliva). The sensitivity of the two assays did not
252 differ significantly for sputum and feces / rectal swabs.
253
254 ***Cross-reactivity of the novel COVID-19-RdRp/Hel and COVID-19-N assays with other***
255 ***human-pathogenic coronaviruses and respiratory viruses***
256 The SARS-CoV-2 genome is highly similar to that of human SARS-CoV, with an overall ~82%
257 nucleotide identity (16). RT-PCR assays that target gene fragments that are homologous in both
258 viruses may therefore be non-specific. To investigate whether the novel COVID-19-RdRp/Hel
259 and COVID-19-N assays cross-react with SARS-CoV, other human-pathogenic coronaviruses,
260 and respiratory viruses, we used the assays to test 17 culture isolates of coronaviruses (SARS-
261 CoV, MERS-CoV, HCoV-OC43, HCoV-229E, and HCoV-NL63), adenovirus, human
262 metapneumovirus, influenza A (H1N1 and H3N2) viruses, influenza B virus, influenza C virus,
263 parainfluenza viruses types 1 to 4, rhinovirus, and respiratory syncytial virus. As shown in Table
264 4, no cross-reactivity with these viruses was found in either assay. Unlike what was previously
265 reported, we found that the RdRp-P2 assay cross-reacted with SARS-CoV culture lysate (19).

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266 This cross-reactivity was consistently observed in two independent runs conducted on different
267 days with each run having three technical replicates of each biological replicate (two biological
268 replicates: SARS-CoV strains HKU-39849 and GZ50) and stringent compliance with the
269 published protocol.

270 To investigate whether the COVID-19-RdRp/Hel assay was specific for SARS-CoV-2 in
271 clinical specimens, we used the assay to test 22 archived nasopharyngeal aspirates/swabs and
272 throat swabs that were positive for other respiratory pathogens by FilmArray RP from 22 patients
273 with upper and/or lower respiratory tract symptoms. As shown in Table 5, none of these
274 specimens was positive by the COVID-19-RdRp/Hel assay, suggesting that the assay was
275 specific for the detection of SARS-CoV-2 RNA in nasopharyngeal aspirates/swabs and throat
276 swabs containing DNA/RNA of other human-pathogenic coronaviruses and respiratory
277 pathogens.

278

279 **DISCUSSION**

280 The positive-sense, single-stranded RNA genome of SARS-CoV-2 is ~30 kilobases in size and
281 encodes ~9860 amino acids (2, 16, 17, 26). Like other betacoronaviruses, the SARS-CoV-2
282 genome is arranged in the order of 5'-replicase (ORF1a/b)-S-E-Membrane-N-poly(A)-3' (16).
283 Traditionally, the preferred targets of coronavirus RT-PCR assays included the conserved and/or
284 abundantly expressed genes such as the structural S and N genes, and the non-structural RdRp
285 and replicase open reading frame (ORF) 1a/b genes (15, 27). For COVID-19, the protocols of a
286 number of RT-PCR assays used by different institutes have recently been made available online
287 (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-
288 guidance/laboratory-guidance). These assays target the ORF1a/b, ORF1b-nsp14, RdRp, S, E, or

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289 N genes of SARS-CoV-2 and some are non-specific assays that would detect SARS-CoV-2 and
290 other related betacoronaviruses such as SARS-CoV (19, 28). Importantly, the in-use evaluation
291 data of these assays using a large number of clinical specimens from patients with confirmed
292 COVID-19 are lacking. In this study, we developed and evaluated three novel real-time RT-PCR
293 assays that target different gene regions of the SARS-CoV-2 genome. We showed that the novel
294 COVID-19-RdRp/Hel assay was highly sensitive and specific for the detection of SARS-CoV-2
295 RNA *in vitro* and in COVID-19 patient specimens.

296 Among the three assays developed in this study, the COVID-19-RdRp/Hel assay has the
297 lowest LOD with *in vitro* viral RNA transcripts (11.2 RNA copies/reaction, 95% confidence
298 interval = 7.2-52.6 RNA copies/reaction). The LOD with genomic RNA was also very low (1.80
299 $TCID_{50}$/ml). Importantly, the COVID-19-RdRp/Hel assay was significantly more sensitive
300 ($P \leq 0.001$) than the established RdRp-P2 assay for the detection of SARS-CoV-2 RNA in both
301 respiratory tract and non-respiratory tract clinical specimens. The COVID-19-RdRp/Hel assay
302 detected SARS-CoV-2 RNA in 42/273 (15.4%) additional specimens that were tested negative
303 by the RdRp-P2 assay. These findings are clinically and epidemiologically relevant because
304 asymptomatic and mildly symptomatic cases of COVID-19 have been increasingly recognized
305 and these patients with cryptic pneumonia may serve as a potential source for propagating the
306 epidemic (2, 7). Given the large number of patients (>60,000 cases in China at the time of
307 writing) involved in this expanding epidemic, the additional positivity specimens detected by the
308 COVID-19-RdRp/Hel assay might translate into thousands of specimens that would otherwise be
309 considered as SARS-CoV-2-negative by the less sensitive RdRp-P2 assay.

310 Regarding the different types of clinical specimens, the COVID-19-RdRp/Hel assay was
311 significantly more sensitive than the RdRp-P2 assay for the detection of SARS-CoV-2 RNA in

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312 nasopharyngeal aspirate/swab or throat swab, saliva, and plasma specimens. False-negative
313 results might arise from testing nasopharyngeal aspirate/swabs or throat swabs with low viral
314 loads in COVID-19, SARS, and MERS patients (2, 29-32). RT-PCR assays with higher
315 sensitivity, such as the COVID-19-RdRp/Hel assay, might help to reduce the false-negative rate
316 among these specimens which are frequently the only specimens available for establishing the
317 diagnosis of COVID-19. We have previously shown that saliva has a high concordance rate with
318 nasopharyngeal aspirates for the detection of influenza viral RNA and might also be a suitable
319 specimen for diagnosing COVID-19 (21, 33). The use of the highly sensitive COVID-19-
320 RdRp/Hel assay to test saliva from suspected cases of COVID-19 might be a simple and rapid
321 way to avoid the need of aerosol-generating procedures during collection of nasopharyngeal
322 aspirates and suction of sputum, especially in regions most heavily affected by the ongoing
323 COVID-19 outbreak where full personal protective equipment are insufficient (12). SARS-CoV-
324 2 RNAemia has been reported in a small proportion of COVID-19 patients (2, 4). However, as
325 shown in our clinical evaluation in which the RdRp-P2 assay was negative for all the 10 plasma
326 specimens that were tested positive by the COVID-19-RdRp/Hel assay, the genuine incidence of
327 SARS-CoV-2 RNAemia might be underestimated by less sensitive RT-PCR assays. We have
328 previously shown that high serum viral loads in SARS patients were associated with more severe
329 disease as evidenced by higher incidence of oxygen desaturation, need for mechanical
330 ventilation, hepatic dysfunction, and death (34). Thus, serial monitoring of the plasma viral load
331 in COVID-19 patients with the highly sensitive COVID-19-RdRp/Hel assay should be
332 considered to provide prognostic insights and facilitate treatment decisions.

333 The COVID-19-RdRp/Hel assay was highly specific and exhibited no cross-reactivity
334 with other common respiratory pathogens *in vitro* and in nasopharyngeal aspirates. Interestingly,

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335 our evaluation showed that the RdRp-P2 assay cross-reacted with SARS-CoV *in vitro*, which is
336 different from what was previously reported (19). We postulated that this might be due to the
337 small number (n=3) of nucleotide differences between the probe used in the RdRp-P2 assay with
338 at least 3 strains of SARS-CoV (19). This cross-reactivity would be especially important for
339 laboratories in areas where SARS-CoV might re-emerge and co-circulate with SARS-CoV-2, as
340 the clinical progressions of SARS and COVID-19 remain incompletely understood at this stage.

341 The main limitation of this study was that the COVID-19-RdRp/Hel and RdRp-P2 assays
342 were performed using different commercially available reagents, primer/probe concentrations,
343 and cycling conditions, which made it challenging to determine the root of the difference in
344 sensitivity. Nevertheless, our data showed that the newly established COVID-19-RdRp/Hel assay
345 was highly sensitive and specific for the detection of SARS-CoV-2 RNA *in vitro* and in
346 respiratory and non-respiratory tract clinical specimens. The use of this novel RT-PCR assay
347 might be especially useful for detecting COVID-19 cases with low viral loads and when testing
348 upper respiratory tract, saliva, and plasma specimens of patients. Development of COVID-19-
349 RdRp/Hel into a multiplex assay which can simultaneously detect other human-pathogenic
350 coronaviruses and respiratory pathogens may further increase its clinical utility in the future.

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TRANSPARENCY DECLARATION

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517 **FIGURE LEGENDS**

518

519 **FIG 1** The number of clinical specimens that were positive for SARS-CoV-2 RNA by the
520 COVID-19-RdRp/Hel (red circles) assay or RdRp-P2 (blue triangles) assay on different days
521 after symptom onset: (A) nasopharyngeal aspirates/swabs and/or throat swabs, (B) saliva
522 specimens, (C) sputum specimens, (D) plasma specimens, and (E) feces or rectal swabs.

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1 **TABLE 1** Test results used for the calculation of limits of detection of the COVID-19 real-time RT-PCR assays with genomic RNA
2 for SARS-CoV-2 in culture lysate and clinical specimen
3

COVID-19-RdRp/Hel

Culture lysate							Clinical specimen						
Virus quantity ($TCID_{50}$/ml)	*Cp* (Intra-run)			*Cp* (Inter-run)			RNA extract (fold dilution)	*Cp* (Intra-run)			*Cp* (Inter-run)		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3		Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
1.8×10^{1}	34.03	33.64	33.63	33.89	33.67	33.80	10^{-4}	34.86	34.97	34.79	35.34	35.20	34.89
1.8×10^{0}	36.90	36.43	36.41	36.94	36.61	37.25	10^{-5}	37.74	38.05	39.45	37.95	37.96	37.83
1.8×10^{-1}	40.00	40.00	40.00	38.52	40.00	-	10^{-6}	-	40.00	-	40.00	38.55	-
1.8×10^{-2}	-	-	-	-	-	-	10^{-7}	-	-	-	-	-	-

4

COVID-19-S

Culture lysate							Clinical specimen						
Virus quantity ($TCID_{50}$/ml)	*Cp* (Intra-run)			*Cp* (Inter-run)			RNA extract (fold dilution)	*Cp* (Intra-run)			*Cp* (Inter-run)		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3		Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
1.8×10^{1}	34.88	34.96	35.08	36.32	35.94	35.64	10^{-4}	37.15	37.46	36.86	37.38	37.59	37.32
1.8×10^{0}	36.79	36.99	37.60	38.33	39.25	38.71	10^{-5}	-	40.00	-	-	40.00	40.00
1.8×10^{-1}	40.00	40.00	40.00	40.00	-	-	10^{-6}	-	40.00	-	-	-	-
1.8×10^{-2}	-	-	-	-	-	-	10^{-7}	-	-	-	-	-	-

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COVID-19-N

Culture lysate							Clinical specimen						
Virus quantity ($TCID_{50}$/ml)	*Cp* (Intra-run)			*Cp* (Inter-run)			RNA extract (fold dilution)	*Cp* (Intra-run)			*Cp* (Inter-run)		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3		Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
1.8×10^{1}	31.88	31.73	31.67	32.72	32.61	32.85	10^{-4}	35.64	35.01	35.10	35.52	35.38	35.62
1.8×10^{0}	34.14	34.26	34.57	35.69	35.86	35.86	10^{-5}	39.16	40.00	39.09	40.00	38.12	37.12
1.8×10^{-1}	38.32	37.29	36.9	40.00	38.42	-	10^{-6}	-	-	40.00	-	-	-
1.8×10^{-2}	-	-	-	-	-	-	10^{-7}	-	-	-	-	-	-

RdRp-P2

Culture lysate							Clinical specimen						
Virus quantity ($TCID_{50}$/ml)	*Cp* (Intra-run)			*Cp* (Inter-run)			RNA extract (fold dilution)	*Cp* (Intra-run)			*Cp* (Inter-run)		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3		Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
1.8×10^{1}	33.46	33.74	33.49	33.53	33.45	33.46	10^{-4}	33.63	33.31	33.65	33.68	33.34	33.62
1.8×10^{0}	34.05	34.64	34.12	33.78	33.83	-	10^{-5}	34.15	34.00	33.95	-	-	34.01
1.8×10^{-1}	-	-	-	-	-	-	10^{-6}	-	-	-	-	-	-
1.8×10^{-2}	-	-	-	-	-	-	10^{-7}	-	-	-	-	-	-

Abbreviations: +, positive; -, negative; *Cp*, cycle number at detection threshold.

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8 **TABLE 2** Test results used for the calculation of limits of detection of COVID-19 real-time RT-
9 PCR assays with *in vitro* RNA transcripts for SARS-CoV-2

10

	No. of positive tests / no. of replicates (%)	
Predicted no. of RNA copies/reaction	**COVID-19-RdRp/Hel**	**COVID-19-N**
40	8/8 (100.0)	8/8 (100.0)
20	8/8 (100.0)	7/8 (87.5)
10	8/8 (100)	7/8 (87.5)
5	3/8 (37.5)	5/8 (62.5)
2.5	2/8 (25.0)	2/8 (25.0)
0	0/8 (0.0)	0/8 (0.0)

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12 **TABLE 3** Comparison between the COVID-19-RdRp/Hel and RdRp-P2 real-time RT-PCR assays for the detection of SARS-CoV-2
13 RNA in different types of clinical specimens from 15 patients with laboratory-confirmed COVID-19
14

Specimen type	COVID-19-RdRp/Hel	RdRp-P2	P value	Mean (range) viral load in RdRp-P2-negative but COVID-19-RdRp/Hel-positive specimens, RNA copies/ml
Respiratory tract:	102/120 (85.0%)	73/120 (60.8%)	<0.001	4.33×10^4 (2.85×10^3 to 4.71×10^5)
NPA/NPS/TS	30/34 (88.2%)	22/34 (64.7%)	0.043	1.74×10^4 (2.85×10^3 to 8.40×10^4)
Saliva	59/72 (81.9%)	38/72 (52.8%)	<0.001	5.32×10^4 (1.74×10^3 to 4.71×10^5)
Sputum	13/14 (92.9%)	13/14 (92.9%)	NS	NA
Non-respiratory tract:	17/153 (11.1%)	4/153 (2.6%)	0.005	7.06×10^3 (2.21×10^2 to 1.67×10^4)
Plasma	10/87 (11.5%)	0/87 (0.0%)	0.001	7.86×10^3 (2.21×10^2 to 1.67×10^4)
Urine	0/33 (0.0%)	0/33 (0.0%)	NS	NA
Feces / rectal swabs	7/33 (21.2%)	4/33 (12.1%)	NS	4.38×10^3 (1.54×10^3 to 6.69×10^3)
Total	119/273 (43.6%)	77/273 (28.2%)	<0.001	3.21×10^4 (2.21×10^2 to 4.71×10^5)

15 Abbreviations: NA, not applicable; NPA, nasopharyngeal aspirate; NPS, nasopharyngeal swab; NS, not significant; TS, throat swab.
16

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17 **TABLE 4** Cross-reactivity between the COVID-19 real-time RT-PCR assays and other
18 respiratory viruses in cell culture
19

Virus	Viral titer ($TCID_{50}$/ml)[a]	COVID-19-RdRp/Hel	COVID-19-N	RdRp-P2
SARS-CoV	1.0×10^3	-	-	+
MERS-CoV	5.6×10^3	-	-	-
HCoV-OC43	3.2×10^3	-	-	-
HCoV-229E	5.0×10^2	-	-	-
HCoV-NL63	3.2×10^1	-	-	-
Adenovirus	1.0×10^2	-	-	-
hMPV	3.2×10^2	-	-	-
IAV (H1N1)	4.2×10^3	-	-	-
IAV (H3N2)	5.6×10^3	-	-	-
IBV	3.2×10^3	-	-	-
ICV	5.6×10^2	-	-	-
PIV1	1.0×10^2	-	-	-
PIV2	1.0×10^3	-	-	-
PIV3	1.0×10^3	-	-	-
PIV4	1.0×10^3	-	-	-
Rhinovirus	7.9×10^3	-	-	-
RSV	1.0×10^3	-	-	-

20 [a]The same viral titers were used for all the assays.
21 Abbreviations: +, positive; -, negative; HCoV, human coronavirus; hMPV, human
22 metapneumovirus; IAV, influenza A virus; IBV, influenza B virus; ICV, influenza C virus;
23 MERS-CoV, Middle East respiratory syndrome coronavirus; PIV, parainfluenza virus; RSV,
24 respiratory syncytial virus; SARS-CoV, severe acute respiratory syndrome coronavirus; $TCID_{50}$,
25 50% tissue culture infective doses.

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26 **TABLE 5** Lack of cross-reactivity between the COVID-19-RdRp/Hel assay and other respiratory
27 pathogens in clinical specimens[a]
28

FilmArray RP2 result	No. COVID-19-RdRp/Hel-positive specimens / No. of total specimens
HCoV-OC43	0/2
HCoV-HKU1	0/1
HCoV-229E	0/1
Adenovirus	0/3
IAV	0/7
PIV	0/3
Rhinovirus/EV	0/4
Mycoplasma pneumoniae	0/1
Total	0/22

29 [a]These included nasopharyngeal aspirates, nasopharyngeal swabs, and throat swabs tested by
30 FilmArray RP2.
31 Abbreviations: EV, enterovirus; HCoV, human coronavirus; IAV, influenza A virus; PIV,
32 parainfluenza virus.

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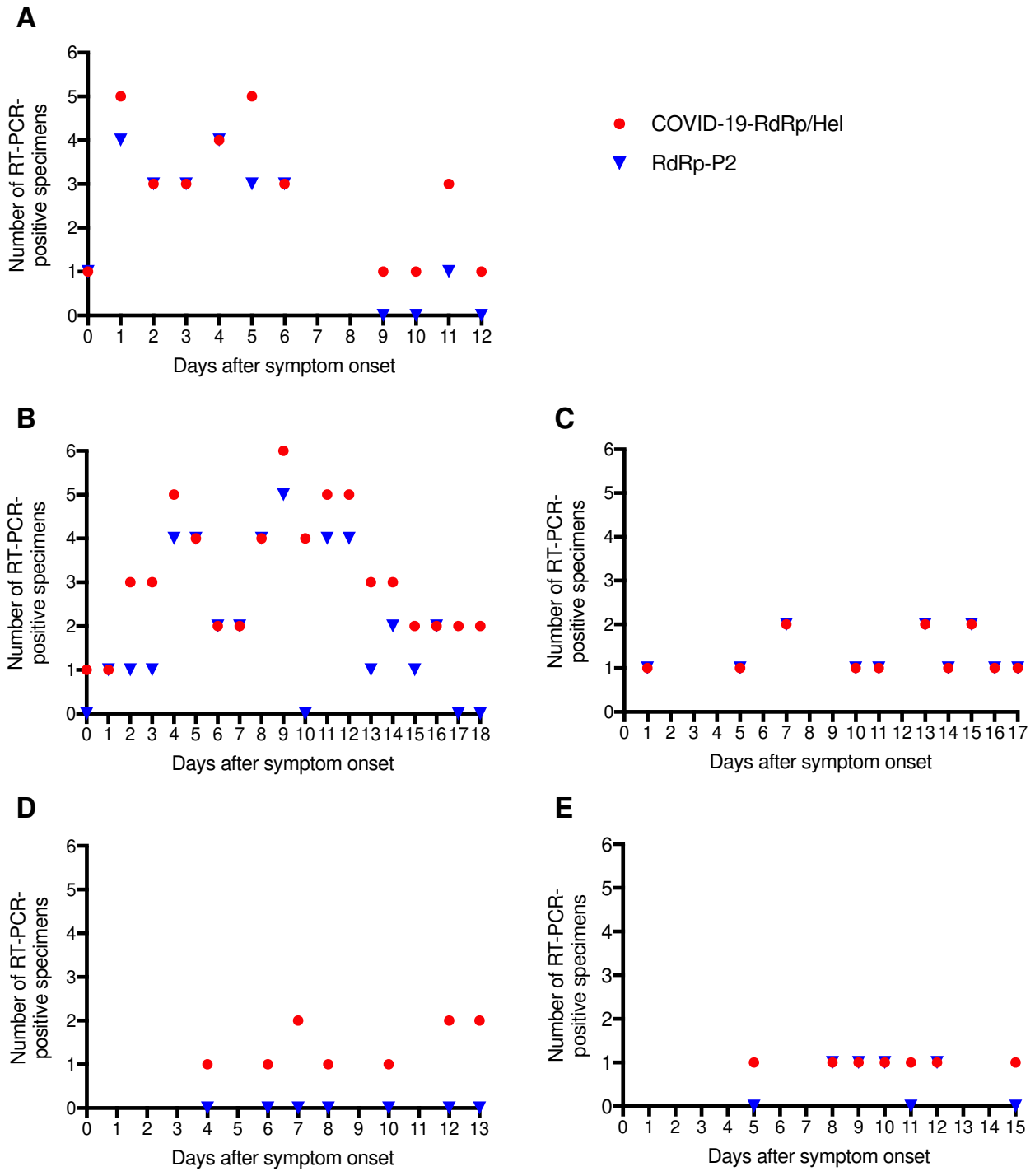

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1 **SUPPLEMENTARY TABLE 1** Primer and probe sequences of the novel COVID-19 real-time RT-PCR assays in this study

Assay	Gene target	Genome location[a]	Primer/probe	Sequence (5' to 3')
COVID-19-RdRp/Hel	RdRp/Helicase	16220-16239	Forward	CGCATACAGTCTTRCAGGCT
		16330-16353	Reverse	GTGTGATGTTGAWATGACATGGTC
		16276-16303	Probe	FAM-TTAAGATGTGGTGCTTGCATACGTAGAC-lABkFQ
COVID-19-S	Spike	22712-22741	Forward	CCTACTAAATTAAATGATCTCTGCTTTACT
		22849-22869	Reverse	CAAGCTATAACGCAGCCTGTA
		22792-22813	Probe	HEX-CGCTCCAGGGCAAACTGGAAAG-lABkFQ
COVID-19-N	Nucleocapsid	29210-29227	Forward	GCGTTCTTCGGAATGTCG
		29284-29306	Reverse	TTGGATCTTTGTCATCCAATTTG
		29257-29278	Probe	FAM-AACGTGGTTGACCTACACAGST-lABkFQ

2 [a]The genome sequence of SARS-CoV-2 (strain HKU-SZ-005b 2020) was used as reference (GenBank accession no. MN975262).
3 Abbreviations: RdRp, RNA-dependent RNA polymerase.

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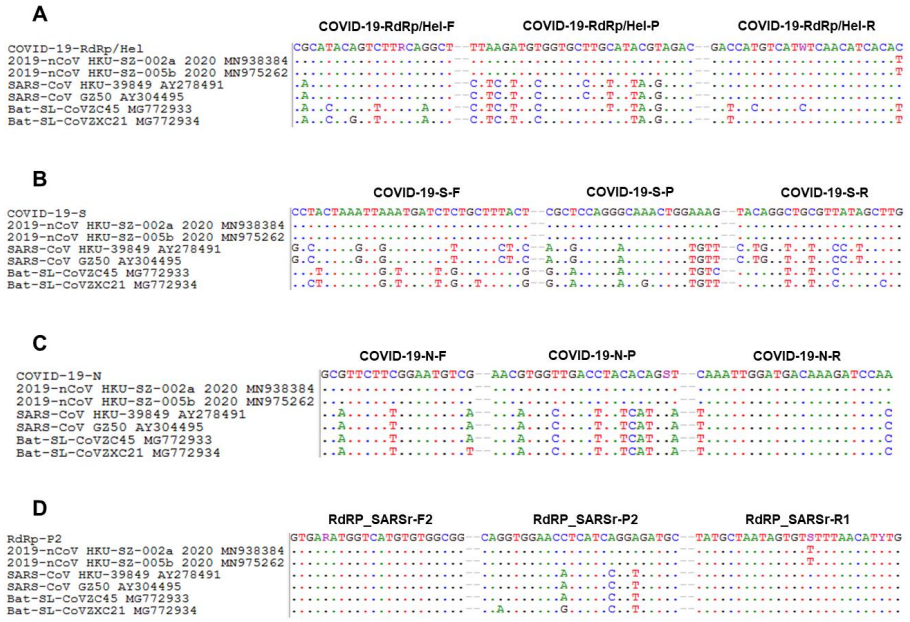
SUPPLEMENTARY FIGURE



SUPPLEMENTARY FIGURE 1

Partial alignment of the oligonucleotide regions of SARS-CoV-2 (2019-nCoV HKU-SZ-002a 2020, GenBank accession no. MN938384; and 2019-nCoV HKU-SZ-005b 2020, GenBank accession no. MN975262), human SARS-CoV (SARS-CoV HKU-39849, GenBank accession no. AY278491; and SARS-CoV GZ50, GenBank accession no. AY304495), and bat SARS-like coronaviruses (Bat-SL-CoVZC45, GenBank accession no. MG772933; and Bat-SL-CoVZXC21, GenBank accession no. MG772934) predicted to bind with the novel (A) COVID-19-RdRp/Hel, (B) COVID-19-S, (C) COVID-19-N, and the published (D) RdRp-P2 real-time RT-PCR assays. The sequences of COVID-19-RdRp/Hel-R, COVID-19-S-R, COVID-19-N-R, and RdRP-SARSr-R1 represent the reverse complements of the reverse primers shown in Supplementary Table 1.