

1 **Title: COVID-19 re-infection by a phylogenetically distinct SARS-coronavirus-2 strain**
2 **confirmed by whole genome sequencing**

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38 **ABSTRACT**

39 **Background**

40 Waning immunity occurs in patients who have recovered from COVID-19. However, it
41 remains unclear whether true re-infection occurs.

42 **Methods**

43 Whole genome sequencing was performed directly on respiratory specimens collected
44 during two episodes of COVID-19 in a patient. Comparative genome analysis was conducted to
45 differentiate re-infection from persistent viral shedding. Laboratory results, including RT-PCR
46 Ct values and serum SARS-CoV-2 IgG, were analyzed.

47 **Results**

48 The second episode of asymptomatic infection occurred 142 days after the first
49 symptomatic episode in an apparently immunocompetent patient. During the second episode,
50 there was evidence of acute infection including elevated C-reactive protein and SARS-CoV-2
51 IgG seroconversion. Viral genomes from first and second episodes belong to different
52 clades/lineages. The genome from first episode contained a stop codon at position 64 of ORF8,
53 leading to a truncation of 58 amino acids. Another 23 nucleotide and 13 amino acid differences
54 located in 8 different proteins, including known B and T cell epitopes, were found between
55 viruses from the first and second episodes. Compared to viral genomes in GISAID, the first virus
56 genome was phylogenetically closely related to strains collected in March/April 2020, while the
57 second virus genome was closely related to strains collected in July/August 2020.

58 **Conclusions**

59 Epidemiological, clinical, serological and genomic analyses confirmed that the patient
60 had re-infection instead of persistent viral shedding from first infection. Our results suggest

61 SARS-CoV-2 may continue to circulate among humans despite herd immunity due to natural
62 infection. Further studies of patients with re-infection will shed light on protective correlates for
63 guiding vaccine design.

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64 **INTRODUCTION**

65 COVID-19 pandemic has affected over 23 million patients with more than 0.8 million
66 deaths in over 200 countries. The pandemic has severely disrupted the healthcare system and
67 halted socioeconomic activities. Household transmission has led to familial clusters [1,2]. The
68 high transmissibility of the etiological agent, severe acute respiratory syndrome coronavirus 2
69 (SARS-CoV-2), by airborne, droplet and contact routes has led to large outbreaks in eateries,
70 bars, cruise ships, workplaces, and healthcare institutions [3]. With the exception of few regions,
71 COVID-19 continues to circulate worldwide despite stringent control measures. Moreover,
72 resurgence of COVID-19 cases is seen in many areas after relaxation of social distancing policies
73 [4].

74 One of the key questions for COVID-19 is whether true re-infection occurs. Although
75 neutralizing antibody develops rapidly after infection [5,6], recent studies showed that antibody
76 titers start to decline as early as 1 to 2 months after the acute infection [7,8]. Due to prolonged
77 viral shedding at low levels near the detection limit of RT-PCR assays [5], patients tested
78 negative and discharged from hospitals are often having recurrence of positive results [9]. A case
79 report suggested that re-infection can occur, but viral genome analysis was not performed [10].
80 These reported cases have raised the controversy between persistent virus shedding and re-
81 infection.

82 We have encountered a patient with a second episode of infection which occurred 4.5
83 months after the first episode. Here, we differentiated re-infection from prolonged viral shedding
84 using whole genome analysis, which was also supported by epidemiological, clinical and
85 serological data.

86

87 **METHODS**

88 **RT-PCR and antibody testing**

89 SARS-CoV-2 RT-PCR was performed using the LightMix® E-gene kit as we described
90 previously [11]. Immunoglobulin G (IgG) against SARS-CoV-2 nucleoprotein was performed
91 using Abbott SARS-CoV-2 IgG assay according to manufacturer's instruction, or microsphere-
92 based antibody as we described previously [12].

94 **Viral whole genome sequencing**

95 RNA was extracted from posterior oropharyngeal saliva using Qiagen Viral RNA Mini
96 Kit as we described previously [4]. Reverse transcription was performed using SuperScript IV
97 reverse transcriptase (ThermoFisher Scientific, Waltham, MA, USA). The cDNA was then used
98 for SARS-CoV-2 tiling PCR and library preparation according to Nanopore protocol (Version:
99 PTC_9096_v109_revF_06Feb2020) with modifications [4]. End preparation and native barcode
100 ligation was performed using EXP-NBD196 (Oxford Nanopore Technologies). Barcoded and
101 pooled libraries were then ligated to sequencing adapter and was sequenced with the Oxford
102 Nanopore MinION device using R9.4.1 flow cell.

103 Bioinformatics analysis of nanopore sequencing data was performed using the workflow
104 from ARTIC network [13]. Minor modifications were made for converting raw data into the
105 consensus sequences using the Medaka pipeline, which include increasing the QC passing score
106 from 7 to 10, reducing the minimum length at the guppyplex step to 350 to allow potential
107 deletions to be detected, and increasing the "--normalise" value to 999999 to incorporate all the
108 sequenced reads.

109

110 **Phylogenetic analysis**

111 Multiple alignment was performed using MAFFT [14]. Maximum-likelihood whole
112 genome phylogenetic tree was constructed using IQ-TREE2 [15], with substitution model
113 TIM2+F as the best predicted model by BIC. The option -czb was used to mask unrelated
114 substructure of the tree with near zero branch length. The ultrafast bootstrap option was used
115 with 1000 replicates. We described the clade information using GISAID [16], Nextstrain [17] and
116 Pangolin [18] nomenclatures. Nucleotide position was numbered according to the reference
117 genome Wuhan-Hu-1 (GenBank accession number NC_045512.2).

118 To identify strains that are most closely related to those of the patient, strains in the
119 GISAID database deposited as of August 20, 2020 were analyzed. The file downloaded from
120 GISAID (msa_0820) has excluded duplicate and low-quality sequences with >5% NNNNs
121 (Supplementary Table S1). The following criteria were used for strain inclusion for the
122 phylogenetic analysis. We blast-searched whole viral genome against the GISAID database using
123 the two strains from the patient, and included the 10 top hits for each blast. BLAST+ toolkit was
124 used for the blast searches [19]. In addition to the 20 chosen strains from the BLAST results, we
125 have also included viruses from Hong Kong that were reported in our previous publication [4], 5
126 most recent strains from UK and Spain, and other strains reported in January 2020.

127

128 **Ethical approval**

129 The study protocol was approved by the Institutional Review Board of the University of
130 Hong Kong/Hospital Authority Hong Kong West Cluster UW 13-265. The patient has also
131 provided written informed consent for publication.

132

133 **RESULTS**

134 **Patient**

135 The patient was a 33-year old male residing in Hong Kong. He enjoyed a good past
136 health. During the first episode, he presented with cough and sputum, sore throat, fever and
137 headache for 3 days. The diagnosis was confirmed by a positive SARS-CoV-2 RT-PCR test from
138 his posterior oropharyngeal saliva specimen on March 26, 2020. He was hospitalized on March
139 29, 2020. By then, all his symptoms have subsided. The patient was discharged on April 14,
140 2020 upon two negative SARS-CoV-2 RT-PCR tests from nasopharyngeal and throat swabs
141 taken 24 hours apart.

142 During the second asymptomatic episode of COVID-19, the patient was returning to
143 Hong Kong from Spain via the United Kingdom, and was tested positive by SARS-CoV-2 RT-
144 PCR on the posterior oropharyngeal saliva taken for entry screening at the Hong Kong airport on
145 August 15, 2020. He was hospitalized again and remained asymptomatic all along. He was
146 afebrile with a temperature of 36.5 °C. His pulse rate was 86 beats per minute, his blood pressure
147 was 133/94 and his SaO₂ was 98% on room air. Physical examination was unremarkable. Ct
148 value of posterior oropharyngeal saliva was 26.69 upon hospitalization (Figure 1). On admission,
149 C-reactive protein (CRP) level was slightly elevated at 8.6 mg/L, but declined during
150 hospitalization (Figure 1). There was also hypokalemia, but other blood test results were normal
151 (Table 1). Serial chest radiographs did not reveal any abnormalities. No antiviral treatment was
152 given to the patient. Serial real-time RT-PCR Ct values in the posterior oropharyngeal saliva
153 gradually increased during hospitalization, indicating a reduction in viral load (Figure 1).

154

155 **SARS-CoV-2 IgG**

156 The serum specimens collected 10 days after symptom onset for the first episode and 1
157 day after hospitalization for the second episode tested negative for IgG against SARS-CoV-2
158 nucleoprotein. Serial serum specimens collected during the second episode were also tested for
159 SARS-CoV-2 IgG using Abbott assay, with the serum specimen collected from day 1 to 3 after
160 hospitalization tested negative but a subsequent serum specimen collected on day 5 after
161 hospitalization tested positive.

163 **Genome analysis**

164 Whole genome sequencing was performed from posterior oropharyngeal saliva
165 specimens collected during the first episode in March and from the second episode in August.
166 The sequenced genomes of both episodes encompass the entire genome, except for 54 bp from
167 the 5' end and 34 bp from the 3' end, excluding the polyA tail. The mean filtered coverage was
168 2579-fold and 2647-fold for the viral genome from the first infection (hCoV-19/Hong
169 Kong/HKU-200823-001/2020; GISAID accession number EPI_ISL_516798) and that of the
170 second infection (hCoV-19/Hong Kong/HKU-200823-002/2020; GISAID accession number
171 EPI_ISL_516799), respectively.

172 Genomic analysis showed that the first viral genome belongs to a different clade/lineage
173 from the second viral genome (Figure 2). The first viral genome belongs to GISAID clade V,
174 Nextstrain clade 19A, and Pangolin lineage B.2 with a probability of 0.99. The second viral
175 genome belongs to GISAID clade G, Nextstrain clade 20A, and Pangolin lineage B.1.79 with a
176 probability of 0.70. In addition to the presence of a stop codon at position 64 of ORF8 leading to
177 a truncation of 58 amino acids in the virus genome of the first episode of infection, the two virus
178 genomes also differ by another 23 nucleotides, in which 13 were non-synonymous mutations

179 resulting in amino acid changes (Figure 3 and Supplementary Table S2). The difference in the
180 amino acids between the two genomes are located in the spike protein (at the N-terminal domain,
181 subdomain 2 and upstream helix), nucleoprotein, non-structural proteins (NSP3, NSP5, NSP6,
182 NSP12), and accessory proteins (ORF3a, ORF8 and ORF10).

183 We have performed a blast search for the first and second genome. The first viral genome
184 is most closely related to strains from the USA or England collected in March and April 2020,
185 while the second viral genome is most closely related to strains from Switzerland and England
186 collected in July and August 2020. The second genome contains the mutation nsp6 L142F, which
187 is rarely found (0.009% [7/76828] genomes deposited into GISAID as of August 20, 2020).

188

189 **DISCUSSIONS**

190 We report the first case of re-infection of COVID-19. Several lines of evidence support
191 that the second episode is caused by re-infection instead of prolonged viral shedding. First,
192 whole genome analysis showed that the SARS-CoV-2 strains from the first and second episode
193 belong to different clades/lineages with 24 nucleotide differences, suggesting that the virus strain
194 detected in the second episode is completely different from the strain found in the first episode.
195 Second, the patient had elevated CRP, relatively high viral load with gradual decline, and
196 seroconversion of SARS-CoV-2 IgG during the second episode, suggesting that this is a genuine
197 episode of acute infection. Third, there was an interval of 142 days between the first and second
198 episode. Previous studies have shown that viral RNA is undetectable one month after symptom
199 onset for most patients [5,20,21]. Prolonged viral shedding for over one month has been reported
200 but rare [21,22]. In one report, a pregnant woman had virus detected for 104 days after her initial
201 positive test [23]. Fourth, the patient has recently traveled to Europe, where resurgence of

202 COVID-19 cases has occurred since late July, 2020. The viral genome obtained during the
203 second episode is phylogenetically closely related to strains collected from Europe in July and
204 August.

205 The confirmation of re-infection has several important implications. First, it is unlikely
206 that herd immunity can eliminate SARS-CoV-2, although it is possible that subsequent infections
207 may be milder than the first infection as for this patient. COVID-19 will likely continue to
208 circulate in the human population as in the case of other human coronaviruses. Re-infection is
209 common for “seasonal” coronaviruses 229E, OC43, NL63 and HKU1 [24]. In some instances,
210 re-infection occurs despite a static level of specific antibodies. Second, vaccines may not be able
211 to provide lifelong protection against COVID-19. Furthermore, vaccine studies should also
212 include patients who recovered from COVID-19.

213 Despite having an acute infection as evidenced by an elevated CRP and seroconversion,
214 the patient was asymptomatic during the second episode. A previous study of re-infection in
215 rhesus macaque also showed a milder illness during the re-infection [25]. This is likely related to
216 the priming of the patient’s adaptive immunity during the first infection. During SARS-CoV-2
217 infection, neutralizing antibody develops in most patients. In our patient, although anti-SARS-
218 CoV-2 antibody was not detected initially during the second episode, the residual low titer of
219 antibody may have partially controlled the virus. Since neutralizing antibodies target the spike
220 protein [26], variations in the spike protein may render the virus less susceptible to neutralizing
221 antibodies which were induced during the first infection. Several mutations in the spike protein
222 receptor binding domain and N-terminal domain have been shown to confer reduced
223 susceptibility to neutralizing antibodies [27]. For our patient, there are four amino acid residues
224 that differ in the spike protein between the first and second infection, including L18F, A222V,

225 D614G and Q780E. Amino acid residue 222 and 614 are located within the B cell
226 immunodominant epitopes which we have previously identified [28]. A222V and D614G may
227 affect the structure of these epitopes (Supplementary Figure S1). D614G, located at the
228 subdomain 2 of the spike protein, and is now found in most SARS-CoV-2 strains. Studies using
229 pseudovirus suggest that D614G enhances the replication of SARS-CoV-2 [29]. A recent study
230 using pseudovirus showed that 7% of convalescent sera from recovered COVID-19 patients had
231 reduced serum neutralizing activity against 614G than that of 614D [30]. Further serological
232 studies are required to determine whether these amino acid differences in the spike protein of the
233 SARS-CoV-2 strains between the first and second infection is responsible for the re-infection.

234 T cell immunity may also play a role in ameliorating the severity during re-infection.
235 Studies on COVID-19 and other coronaviruses showed that coronaviruses can induce long-
236 lasting T cell immunity [31,32]. T cell mainly targets the structural proteins, although CD4 or
237 CD8+ T cell response against other viral proteins can be detected [31,33-35]. Spike protein
238 A222V is a potential site eliciting CD4+ T cell responses [36]. CD4+ T cells also targets the
239 nsp3, nsp4 and ORF8, while the CD8+ T cells target the nsp6, ORF3a and ORF8 as reported up
240 to this stage [34].

241 IgG against SARS-CoV-2 was undetectable in the blood collected shortly after the
242 diagnosis during the second episode. The low antibody level may be related to his mild illness
243 during the first episode. We and others have shown that patients with milder disease had lower
244 antibody titers than those with more severe disease [6,7]. During the second episode of infection
245 in our patient, IgG against SARS-CoV-2 was not detected until 5 days after hospitalization. One
246 possibility is that he did not mount an antibody response after the first infection, but this cannot
247 be ascertained as we only had the archived serum collected 10 days after the onset of symptoms

248 for the first episode. Previous studies have shown that antibody response was not detectable in
249 some patients until 2-3 weeks after onset of symptoms. Another possibility is the he indeed
250 mount an antibody response after the first infection, but the antibody titer decreases below the
251 detection limit of the assays. This waning of antibody has been well described. In one study,
252 33% of recovered COVID-19 patients were negative for neutralizing antibodies during the
253 convalescent phase (average 39 days after symptom onset) [8]. Another study showed that 40%
254 of asymptomatic individuals are seronegative within 8 weeks after the onset of symptoms [7].
255 Besides the lack of protection against re-infection, another implication of rapid decline in
256 antibody titers is that seroprevalence studies may underestimate the true prevalence of infection.

257 The lack of antibody response after COVID-19 can have implications on both the
258 susceptibility to re-infection and the severity of infection. Although our patient is asymptomatic
259 during the second infection, it is possible that re-infection in other patients may result in more
260 severe infection. Our previous study on SARS-CoV showed that antibodies against the spike
261 protein can be associated with more severe acute lung injury [37].

262 There are several limitations in this study. First, only one archived serum specimen
263 collected from the first episode was available for serology testing. Since patients may not mount
264 antibody response within 10 days, the negative antibody test does not exclude the possibility that
265 the patient indeed developed antibody response during the early convalescent phase for the first
266 episode. Antibody avidity study was not performed. Second, the virus culture using upper
267 respiratory tract specimens from both episodes are still ongoing, and therefore the neutralizing
268 antibody titer against the virus from the first and second episode cannot be compared.

269 This case illustrates that re-infection can occur even just after a few months of recovery
270 from the first infection. Our findings suggest that SARS-CoV-2 may persist in humans as is the

271 case for other common-cold associated human coronaviruses, even if patients have acquired
272 immunity via natural infection or via vaccination. In rhesus macaques that have recovered from
273 SARS-CoV-2 infection and re-challenged with the same virus, the peak viral load during re-
274 challenge was $>5 \log_{10}$ lower in the BAL but only $\sim 2 \log_{10}$ lower in the nasal swab when
275 compared with those during the first challenge [25]. Similarly, in vaccine studies, viral RNA
276 could still be detected in the upper respiratory tract for vaccinated animals [38]. Further studies
277 on re-infection, which will be vital for the research and development of more effective vaccines,
278 are warranted. In summary, reinfection is possible 4.5 months after a first episode of
279 symptomatic infection. Vaccination should also be considered for persons with known history of
280 COVID-19. Patients with previous COVID-19 infection should also comply with
281 epidemiological control measures such as universal masking and social distancing.

282

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294

295 **CONFLICT OF INTEREST**

296 All authors declare no conflict of interest.

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408 **FIGURE LEGEND**

409 **Figure 1.** Serial C-reactive protein level, viral load (Ct value) and SARS-CoV-2 IgG results
410 during the second episode. Anti-SARS-CoV-2 IgG was performed with Abbott SARS-CoV-2
411 antibody assay.

412
413 **Figure 2.** Phylogenetic analysis of whole SARS-CoV-2 genomes showing the relationship
414 between the viruses collected from first (March 2020) and second infection (August 2020). The
415 tree was constructed by maximum likelihood method. Clade information as inferred by GISAID,
416 Nextstrain and Pangolin nomenclatures, are shown. The reference genome Wuhan-Hu-1
417 (GenBank accession number NC_045512.2) is used as the root of the tree.

418
419 **Figure 3.** Schematic diagram showing differences in amino acids between the first and second
420 episode. *Stop codon at amino acid position 64 of ORF8 leading to a truncation of 58 amino
421 acids in the virus genome of the first episode of infection.

422