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SHORT COMMUNICATION

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Genomic evidence of SARS-CoV-2 reinfection in the Republic of Korea

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Abstract

As the coronavirus disease 2019 (COVID-19) pandemic continues, reinfection is likely to become increasingly common. However, confirming COVID-19 reinfection is difficult because it requires whole-genome sequencing of both infections to identify the degrees of genetic differences. Since the first reported case of re-infection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in the Republic of Korea in April 2020, four additional cases were classified as suspected reinfection cases. We performed whole-genome sequencing of viral RNA extracted from swabs obtained at the initial infection and reinfection stages of these four suspected cases. The interval between initial infection and reinfection of all four suspected cases was more than 3 months. All four patients were young (10–29 years), and they displayed mild symptoms or were asymptomatic during the initial infection and reinfection episodes. The analysis of genome sequences combined with the epidemiological results revealed that only two of the four cases were confirmed as reinfection, and both were reinfected with the Epsilon variant. Due to the prolonged COVID-19 pandemic, the possibility of reinfections with SARS-CoV-2 variants is increasing, as reported in our study. Therefore, continuous monitoring of cases is necessary.

KEYWORDS

Epsilon variant, reinfection, Republic of Korea, SARS-CoV-2, whole genome sequencing

Ae Kyung Park and Jee Eun Rhee contributed equally to this study.

1 | INTRODUCTION

Coronavirus disease 2019 (COVID-19) is continuing to spread worldwide, with around 219 million confirmed cases and more than 4.5 million deaths across almost 200 countries to date. In the Republic of Korea, the number of daily confirmed cases has been increasing in the face of a fourth wave of the pandemic. As severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an entirely new type of coronavirus, there are still many questions about immunity and the possibility of reinfections. It has been generally assumed that once infected, individuals mount an immune response that prevents the second infection in the same individual.[1] However, as the pandemic continues, cases of reinfection have been reported worldwide.[2,3] In particular, the possibility of reinfection was elevated after the emergence of variants with immune evasion capabilities.[4,5] In December 2020, herd immunity was attained in Manaus, Brazil, where more than 75% of the local population had been infected with COVID-19; however, there has been a recent surge in the number of COVID-19 cases, which may have been caused by the P.1 variant.[6] With the increasing number of suspected reinfection cases, the Centers for Disease Control and Prevention (CDC) in the United States and European CDC (ECDC) published criteria for the investigation of reinfection cases[7,8] and Korea Disease Control and Prevention Agency (KDCA) are also continuously monitoring for reinfection cases. However, it may be difficult to confirm the reinfection cases by real-time reverse-transcription polymerase chain reaction (RT-PCR)-based tests because a study reported that one of the recovered COVID-19 patients tested COVID-10 positive for a prolonged duration.[9] Whole-genome sequencing (WGS) may circumvent this limitation of RT-PCR-based tests and help identify cases of genuine reinfection by comparing the genetic differences in samples collected from patients with initial and subsequent infections.[10] In this report, four possible cases of reinfection in the Republic of Korea were analyzed using WGS of swab samples and the genetic difference(s) between the initial infection and reinfection episodes were compared.

2 | MATERIALS AND METHODS

2.1 | Real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Nasopharyngeal and oropharyngeal swabs were collected from four patients with SARS-CoV-2. RNA extraction and real-time RT-PCR were performed on the samples from the swab as per methods described in a previous report.[11] In brief, viral RNA extractions were prepared using QIAamp Viral RNA Mini Kit according to the manufacturers' instructions (Qiagen). For real-time RT-PCR, a 25-μl reaction mixture containing 5 μl of RNA, 12.5 μl of 2× reaction buffer provided with the Agpath IDTM 1 step RT-PCR system (Thermo Fisher Scientific), 1 μl of 25× enzyme

TABLE 1 Epidemiological and virological results of reinfection cases in Korea

	Case 1		Case 2		Case 3		Case 4	
	Initial infection	Reinfection[a]	Initial infection	Reinfection[a]	Initial infection[a]	Reinfection[a]	Initial infection[a]	Reinfection[a]
Gender/age group	Male/28		Female/25		Male/17		Male/17	
Confirmed date	Jun 18, 2020	Feb 15, 2021	Mar 4, 2020	Apr 28, 2021	Dec 21, 2020	May 2, 2021	Dec 25, 2020	May 8, 2021
Symptoms	Cough, phlegm, and loss of smell	Sore throat	Phlegm, muscle pain, and dizziness	Asymptomatic	Asymptomatic	Asymptomatic	Muscle pain, loss of smell, and snot	Asymptomatic
Time interval between infections	241 days		404 days		133 days		134 days	
Clade	–	GH	V (presumed)	GH	GH	GH	GH	ND
Lineage	–	B.1.497	–	B.1.429	B.1.497	B.1.429	B.1.497	–
Type of variant	–	–	–	ε	–	ε	–	–
Result	Suspected reinfection		Reinfection		Reinfection		Suspected reinfection	
Ct value in RdRp gene	27.3	–	21.6	24.5	24.3	23.8	19.0	30.0

Abbreviations: ND, not determined; RdRp, RNA-dependent RNA polymerase; WGS, whole genome sequencing.
[a]Available for WGS.

mixture, 1 µl of forward and reverse primers (both 10 pM), and 0.5 µl of each probe (10 pM) was setup. Reverse transcription was performed at 50°C for 30 min followed by reverse transcriptase inactivation at 95°C for 10 min. PCR amplification was performed for 40 cycles at 95°C for 15 s and 60°C for 1 min.

2.2 | WGS

To perform WGS, libraries were prepared using the QIAseq SARS-CoV-2 Primer Panel and the QIAseq FC DNA Library Kit (Qiagen) according to the manufacturer's instructions and sequenced on MiSeq instrument (Illumina) with 2 × 150 base pairs using a MiSeq reagent kit V2 to obtain an average genome coverage greater than ×1000 for all the isolates.[12] For the analysis of sequence variants, reads were imported, trimmed, and mapped to the reference sequence MN908947.3, and variants were identified using the basic variant detection tool of CLC Genomics Workbench Version 20.0.3 (CLC Bio) by a minimum coverage of 500 reads. Viral lineages were identified with Phylogenetic Assignment of Named Global Outbreak Lineages (PANGOLIN).[13]

2.3 | Phylogenetic tree

For phylogenetic tree analysis, a total of 457 sequences isolated from the Republic of Korea uploaded to the global initiative on sharing avian influenza data (GISAID) were used to generate the tree. All the whole genomic sequences including three that were sequenced in this study were aligned with MAFFT v7.[14] Next, maximum likelihood phylogenetic trees were inferred with FastTree v2.1.9[15] and visualized using Interactive Tree of Life (iTOL) v5.[16]

3 | RESULTS

Since the first case of reinfection was reported in the Republic of Korea in April 2020,[17] an additional four suspected reinfection cases were reported based on epidemiological results. We tried to subject both the initial infection and reinfection swab samples of the four suspected cases to WGS for further analysis. However, swab samples of the initial infections for Cases 1 and 2 could not be obtained. Therefore, there were six samples that were available for WGS (Table 1). Among the six samples, the

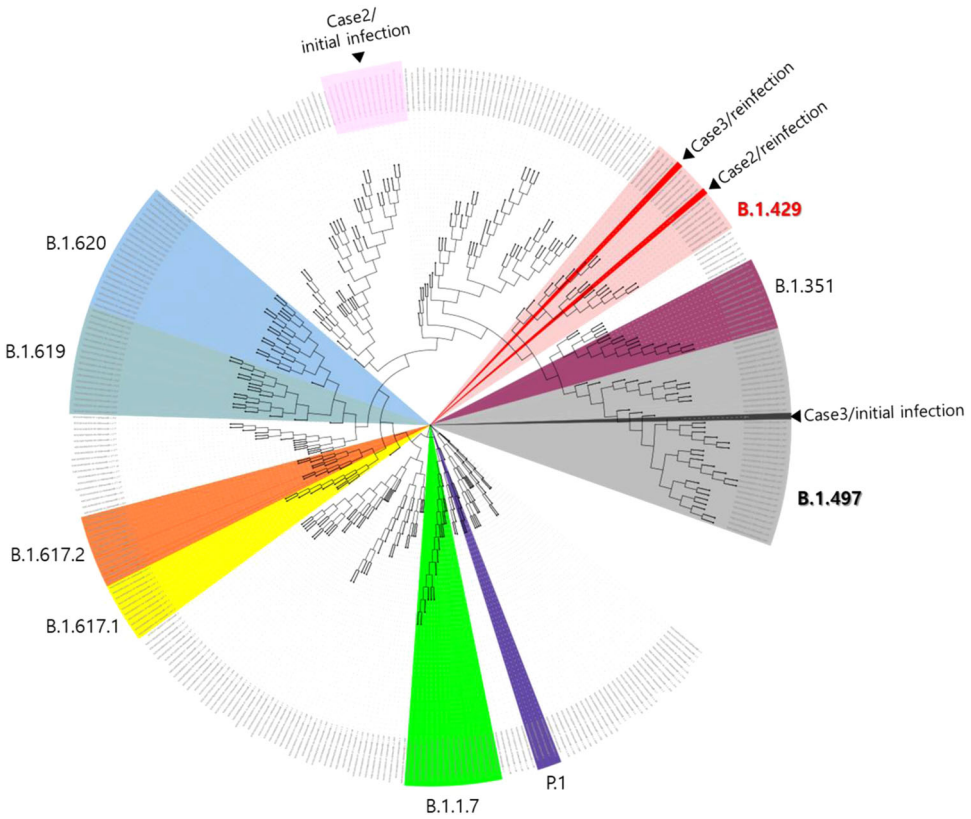

FIGURE 1 Phylogenetic analysis of reinfection cases in Korea. The sequences of initial and reinfection cases of Case 3 are highlighted by arrows. For Case 2, the sequence of the reinfection sample is highlighted by an arrow, and the V clades to which the initial sample may belong are highlighted pink

TABLE 2 Single nucleotide variation of initial and reinfection of Case 3 compared with the reference genome

			Case 3			
	Nucleotide position		Coverage (reads)	Allele frequency (%)	Forward/reverse balance[a]	Average quality[b]
Nucleotide in common for both initial and reinfection	C241T		2987	99.4	0.49	35.5
			2173	99.5	0.49	35.4
	C1059T		2606	93.6	0.43	35.5
			2574	99.9	0.43	35.5
	C3037T		881	93.9	0.47	34.5
			1269	93.9	0.48	34.6
	C14408T		4925	99.7	0.48	35.2
			4533	99.8	0.47	35.2
	A23403G		5051	99.9	0.45	36.3
			4070	100.0	0.44	36.2
	G25563T		2969	95.9	0.43	35.1
			2660	99.9	0.42	35.4
Specific nucleotide for each strain	G3395T	Initial infection	1761	99.8	0.44	35.4
	C11916T		5644	98.1	0.43	35.6
	C12084T		6534	94.8	0.44	36.1
	G18027T		1946	98.7	0.50	35.8
	A20675T		880	100.0	0.50	36.1
	G20679T		819	100.0	0.49	35.3
	G27065A		7589	99.9	0.43	35.7
	C28606T		1537	95.7	0.43	32.7
	G29179T		4163	95.8	0.50	35.3
	C29386T		1010	99.6	0.37	35.0
	G29745T		2785	87.5	0.41	36.1
	G29755T		2332	85.9	0.42	35.5
	G29779T		2220	86.8	0.44	35.4
	del505. .510 (TCATGG)	Reinfection	2962	73.9	0.49	34.1
	G805T		2593	100	0.42	36.0
	A6442G		1555	100	0.39	35.5
	C8947T		865	99.7	0.46	35.6
	C9286T		1321	99.9	0.42	35.4
	C10186T		2502	100.0	0.41	35.8
	C12100T		4539	99.7	0.44	35.1
	A12878G		5780	99.9	0.50	36.3
	G17014T		2166	99.9	0.45	34.8
	G21600T		2354	98.9	0.41	34.2
	G22317T		6204	99.9	0.45	35.2
	C22329T		6501	99.9	0.45	35.5
	T22917G		3613	99.7	0.42	34.5

TABLE 2 (Continued)

	Case 3			
Nucleotide position	Coverage (reads)	Allele frequency (%)	Forward/reverse balance[a]	Average quality[b]
T24349C	650	99.7	0.42	32.9
C26681T	3792	99.4	0.48	35.5
G27890T	787	100.0	0.42	36.0
G28191T	4093	99.8	0.42	33.7
A28272T	6412	99.8	0.47	34.8
C28887T	6104	99.2	0.48	33.4
G28975T	5975	99.7	0.42	35.0
C29362T	1205	83.9	0.36	36.0

[a]Ratio of forward to reverse reads covering the locus.

[b]Phred score.

whole genome sequence of the reinfection sample of Case 4 was incomplete because of low-quality data. Hence, we only obtained five complete whole-genome sequences. Notably, Case 3 was the only case with complete whole-genome sequences for both the initial infection and reinfection episodes.

In Case 1, the first infection was diagnosed during the quarantine process (patient returned from Bangladesh), and reinfection occurred at his workplace from an outbreak after 241 days of his first infection. In Case 4, the initial infection and reinfection episodes occurred due to different outbreaks and had an interval of 134 days. The infection episodes in these two patients were separated by intervals that were longer than 3 months and most likely caused by different origins of the virus based on the epidemiological results. However, due to the lack of sequence analysis that can support the reinfection, these two cases remained suspected cases.

The initial infection in Case 2 occurred in March 2020, and reinfection occurred in April 2021. For this case, we only obtained the reinfection swab sample, and the sequencing results indicated that the reinfection was caused by the Epsilon variant (Table 1). In the Republic of Korea, the Epsilon variant was first identified in December 2020; thus, there was no chance of infection with this variant during the initial infection in Case 2. During the initial infection, the prevalent clades in the Republic of Korea were S and V,[12] and we assumed that this patient's initial infection might have been with the V clade based on the outbreak at the time of initial infection. Case 3 is the only case in which complete sequences were obtained for both initial infection and reinfection. The analysis of sequences clearly showed that the two episodes of COVID-19 were caused by different SARS-CoV-2 lineages. As determined by the Phylogenetic Assignment of Named Global Outbreak Lineages, the B.1.497 and B.1.429 lineages were responsible for the initial infection and reinfection, respectively, and a phylogenetic tree clearly showed that they belonged to distinct clusters (Figure 1). The detailed sequence analysis of initial infection and reinfection in Case 3 indicated that they shared six single-nucleotide variants (SNVs), including C241T, C1059T, C3037T, C14408T, A23403G, and G25563T. In contrast, they showed an additional 13 and 21 SNVs compared with the reference genome, respectively (Table 2).

4 | DISCUSSION

According to the previous report and guidance of reinfection based on the CDC and ECDC, true reinfection must fulfill certain criteria, including isolation of the complete genome of the virus (and not just genomic fragments) from the first and second confirmed specimens, detection of noncirculating variant in the first infection episode, epidemiologic data, such as the history of re-exposure to patients with COVID-19 in the second event and timing between episodes, with a longer time interval between the two events favoring the reinfection hypothesis.[18] Considering the criteria for true reinfection as stated above, among the four suspected reinfection cases, which were studied here, two of these were confirmed as reinfection based on epidemiological and virological data. Specifically, these two confirmed patients were reinfected with the Epsilon variant (B.1.429) which has been classified as a variant that was first identified in the United States.[19] As the Epsilon variant did not circulate during the initial infection period in Case 2 (March 2020) in the Republic of Korea, we can hypothesize that the patient was reinfected, even in the absence of a genomic sequence for the initial infection sample. The analysis of sequencing results of the viruses from the two samples (initial infection and reinfection samples) in Case 3 indicated that they were totally clustered differently; thus, this case was reinfected. In both of the confirmed reinfection cases, the patients had no symptoms during their reinfection; of note, Patient 4 was also asymptomatic during the initial infection. We assume that a second infection presents with milder symptoms or no symptoms at all. This is consistent with the previous reports that COVID-19 reinfections are milder than initial infections.[20] Therefore, these results emphasize the possibility of undetected SARS-CoV-2 reinfections and the need for surveillance of suspected SARS-CoV-2 reinfections.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

ETHICS STATEMENT

The study was approved by the KDCA Institutional Review Board (2020-03-01-P-A). The board waived the requirement for written informed consent.

AUTHOR CONTRIBUTIONS

Sang-Eun Lee, Young Joon Park, Jung Yeon Kim, Jin Gwack, Gi-Eun-Rhie, Cheon-Kwon Yoo, and Eun-Jin Kim conceived and planned the experiments. Ae Kyung Park, Il-Hwan Kim, Heui Man Kim, Hyeokjin Lee, Jeong-Ah Kim, Chae Young Lee, Nam-Joo Lee, SangHee Woo, Jin Sun No, and Jaehee Lee contributed to sample preparation and carried out the experiments. Seong Jin Wang and Gemma Park analyzed the epidemiological data. Ae Kyung Park, Jee Eun Rhee, Il-Hwan Kim, and Eun-Jin Kim contributed to the interpretation of the results. Ae Kyung Park and Jee Eun Rhee took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

DATA AVAILABILITY STATEMENT

Sequences are available at GISAID: EPI_ISL_1675277, EPI_ISL_4570332, EPI_ISL_342, EPI_ISL_343, EPI_ISL_344.

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