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Evidence of SARS-CoV-2 symptomatic reinfection in four healthcare professionals from the same hospital despite the presence of antibodies

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ABSTRACT

Objectives: Since the onset of the COVID-19 pandemic, cases of reinfection with SARS-CoV-2 have been reported, raising additional public health concerns. SARS-CoV-2 reinfection was assessed in healthcare workers (HCWs) in Tunisia because they are at the greatest exposure to infection by different variants.

Methods: We conducted whole-genome sequencing of the viral RNA from clinical specimens collected during the initial infection and the suspected reinfection from 4 HCWs, who were working at the Habib Bourguiba University Hospital (Sfax, Tunisia) and retested positive for SARS-CoV-2 through reverse transcriptase–polymerase chain reaction (RT-PCR) after recovery from a first infection. A total of 8 viral RNAs from the patients' respiratory specimens were obtained, which allowed us to characterize the differences between viral genomes from initial infection and positive retest.

The serology status for total Ig, IgG, and IgM against SARS-CoV-2 was also determined and followed after the first infection.

Results: We confirmed through whole-genome sequencing of the viral samples that all 4 cases experienced a reinfection event. The interval between the 2 infection events ranged between 45 and 141 days, and symptoms were milder in the second infection for 2 patients and more severe for the remaining 2 patients. Reinfection occurred in all 4 patients despite the presence of antibodies in 3 of them.

Conclusion: This study adds to the rapidly growing evidence of COVID-19 reinfection, where viral sequences were used to confirm infection by distinct isolates of SARS-CoV-2 in HCWs. These findings suggest that individuals who are exposed to different SARS-CoV-2 variants might not acquire sufficiently protective immunity through natural infection and emphasize the necessity of their vaccination and the regular follow-up of their immune status both in quantitative and qualitative terms.

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Introduction

Since the outbreak of the COVID-19 pandemic, the duration of immunity against infection by SARS-CoV-2 has been the subject of hot debate (Rhee et al. 2021). Although being infected by SARS-CoV-2 several times is a rare event, recent studies have reported confirmed reinfection cases (Dhillon et al. 2021). A previous history of SARS-CoV-2 infection was associated with an 84% lower risk of

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infection with median protective effect observed 7 months after primary infection (Hall et al., 2021).

In addition, a large cohort study of antibody (Ab)-positive individuals in Qatar showed that patients developed robust immunity after primary infection, which lasted for at least 7 months; this immunity protects against reinfection with an efficacy of ~95% (Abu-Raddad et al. 2021). However, it has been reported that immunity from a mild infection does not last as long as a severe infection; a 6-month follow-up of Chinese patients showed that neutralizing Ab titers decrease a few weeks to a few months after the infection and that this decrease is significantly faster in patients with milder symptoms (Huang et al., 2021), suggesting that those who experience the mildest symptoms in their initial infection might have a higher likelihood of reinfection. Another study including 12,541 healthcare workers (HCWs) showed that the presence of anti-spike or anti-nucleocapsid IgG Abs was associated with a substantially reduced risk of SARS-CoV-2 reinfection in the ensuing 6 months (Lumley et al. 2021).

Tomassini and collaborators defined reinfection as reverse transcriptase–polymerase chain reaction (RT-PCR) positivity at least 28 days after a previous RT-PCR–positive COVID-19 episode (Tomassini et al. 2021), followed by clinical recovery and at least 1 negative RT-PCR. However, the confirmation of reinfection is only possible through whole-genome sequencing of viral variants of samples from the first and second infection, yielding 2 different mutation settings or lineages. Although reinfection remains a rare event, several reports of confirmed reinfection with variable symptom severity have been reported. As of November 26, 2021, the reinfection tracker website (<https://bnonews.com/index.php/2020/08/covid-19-reinfection-tracker/>) reported 544 confirmed cases of reinfection and over 190,000 suspected cases.

For confirmed cases, the average interval between the 2 infection events is 115 day, and 88% of the cases have shown mild to severe symptoms in the 2 infections.

As of June 5, 2021, more than 2 million people were registered in the national platform for vaccination in Tunisia; more than 1.6 million people have been vaccinated since March 13, 2021 (as of June 23, 2021); and the campaign has given priority to HCWs after older people. In fact, HCWs are on the frontline of the management of patients with COVID-19 and suspect cases, and this results in them being at a much greater risk of infection by different SARS-CoV-2 variants. Furthermore, HCWs, particularly if they are not vaccinated, may also increase the risk of infection in their patients through transmission of viral variants, particularly when the patients are not vaccinated. However, data on reinfection cases among HCWs are still very scarce.

In this work, we investigated the genomic features of SARS-CoV-2 variants from HCWs at the Habib Bourguiba University Hospital (Sfax, Tunisia) who had 2 demonstrated symptomatic COVID-19 episodes, suggesting possible reinfection despite the presence of anti-spike IgG Abs before the second suspected infection.

This article represents our ongoing effort to follow the evolution of SARS-CoV-2 in clinical setting (Wilkinson et al. 2021).

Materials and Methods

Evidence before this study

The PubMed database was searched with different combinations of keywords, including “COVID-19,” “healthcare workers,” “reinfection,” and “secondary infection.” We restricted our search to publications in English. As of June 5, 2021, only 6 publications confirmed the hypothesis of reinfection (Adrielle Dos Santos et al. 2021; Gupta et al. 2020; Selhorst et al. 2020; Loconsole et al. 2021; Brehm et al. 2021) through whole-genome

sequencing; however, not all showed distinct variants for both infection episodes (Table S1).

Description of cases

We report here 4 cases of reinfection, in which all are women and healthcare professionals (a medical doctor, a registrar agent working in emergency department, and 2 health technicians) aged between 32 and 46 years (Table 1). All patients provided oral consent to use their medical records for this publication. The ethical agreement was provided to the research project ADAGE (PRF-COVID19GP2) by the Committee of protection of persons (Tunisian Ministry of Health) under the reference CPP SUD N°0265/2020.

Two of the cases have no history of clinically significant conditions; 1 case has hypothyroidism diagnosed in September 2020 (receiving treatment with Levothyrox 50 mg/day), and 1 case had a history of Behçet disease (receiving long-term treatment with colchicine and prednisone) (Table 1). All patients experienced mild to moderate symptoms during the first episode of SARS-CoV-2 infection, which occurred between August 15, 2020 and October 14, 2020. The time course between the 2 episodes of COVID-19 illnesses ranged between 45 and 141 days, with more aggressive clinical presentation during the second infection for 2 patients.

Patient #1, a 36-year-old health technician, showed symptoms consistent with a viral infection (sore throat, headache, fatigue, myalgia, cough, anosmia, dysgeusia, and diarrhea) on August 15, 2020. Seven days later (August 22, 2020), the patient tested positive for SARS-CoV-2 on RT-PCR (cycle threshold [Ct]=24.9) and was treated by inhaled corticosteroids. The patient's symptoms resolved, and evidence for recovery was provided by a negative RT-PCR on September 25, 2020. She continued to feel well until January 3, 2021. After which, she showed symptoms that included sore throat, cough, and diarrhea again. She tested positive for SARS-CoV-2 on RT-PCR (Ct=30.8) (January 7, 2021). She received no treatment and recovered after 10 days. The patient has been tested for total Ig, IgG, and IgM against SARS-CoV-2 since October 16, 2020, and positive results were obtained (Figure 1A).

Patient #2, a 32-year-old medical doctor, first tested positive for SARS-CoV-2 on RT-PCR (Ct=26.8) on September 21, 2020 after showing mild symptoms 1 week earlier. She received no treatment and recovered 5 days later, although a validation of this recovery by an RT-PCR test was not performed. The patient has been tested for total Ig, IgG, and IgM against SARS-CoV-2 since December 7, 2020 (Figure 1B). After suspected reinfection, she tested positive for SARS-CoV-2 again on December 19, 2020 (Ct=30.4) with similar mild symptoms as the first infection when RT-PCR test was conducted.

Patient #3, a 41-year-old health technician with hypothyroidism condition, had symptoms consistent with COVID-19 (fatigue, chills, anosmia, dysgeusia, diarrhea, and abdominal pain) that started on October 14, 2020. The patient tested positive for SARS-CoV-2 on October 19, 2020 (Ct=22.7). She recovered without treatment and continued to feel well until December 17, 2020, after which she experienced more severe symptoms (fatigue, headache, nasal congestion, chills, dyspnea, dizziness, and chest pain). The reinfection was confirmed on December 21, 2020 using RT-PCR (Ct=34.4). The symptoms were managed at home and she recovered 1 month later. The patient has been tested for total Ig, IgG, and IgM against SARS-CoV-2 since December 7, 2020 (Figure 1C).

Patient #4, a 46-year-old registrar agent with a history of Behçet disease, first tested positive for SARS-CoV-2 on September 21, 2020 after showing symptoms 1 week earlier (September 14, 2020). She recovered 10 days later (confirmed by negative RT-PCR on October 6, 2020) and continued to feel well for 3 weeks. On October 29, 2020, she showed very severe symptoms requiring hos-

Table 1
Clinical characteristics of patients during the 2 episodes of SARS-CoV-2 infection.

Characteristics of patients			First episode			Second episode		
Patient	Age	Comorbidities	Symptoms	Treatments	Outcome onset	Symptoms	Treatments	Outcome onset
1	36	None	August 15, 2020 Sore throat, headache, fatigue, myalgia, cough anosmia, dysgeusia, diarrhea	Inhaled corticosteroids	Cured (2 months)	January 03, 2021 Sore throat, cough, diarrhea	None	Cured (10 days)
2	32	None	September 13, 2020 Headache, fatigue, myalgia, fever	None	Cured (5 days)	December 19, 2020 Same symptoms	None	Cured (2 days)
3	41	Hypothyroidism (treatment with Levothyrox 50 mg/day since September 2020)	October 14, 2020 Fatigue, chills, anosmia, dysgeusia, diarrhea, abdominal pain	None	Cured (15 days)	December 17, 2020 Fatigue, headache, nasal congestion, chills, dyspnea, dizziness, chest pain	None	Cured (1 month)
4	46	Behçet disease	September 14, 2020	None	Cured (10 days)	October 29, 2020	Hospitalization	Cured (6 days)

Abbreviation: CT, computed tomography.

^aCT scan showed bilateral ground glass opacities, and the pulmonary involvement was estimated to be around 25%.

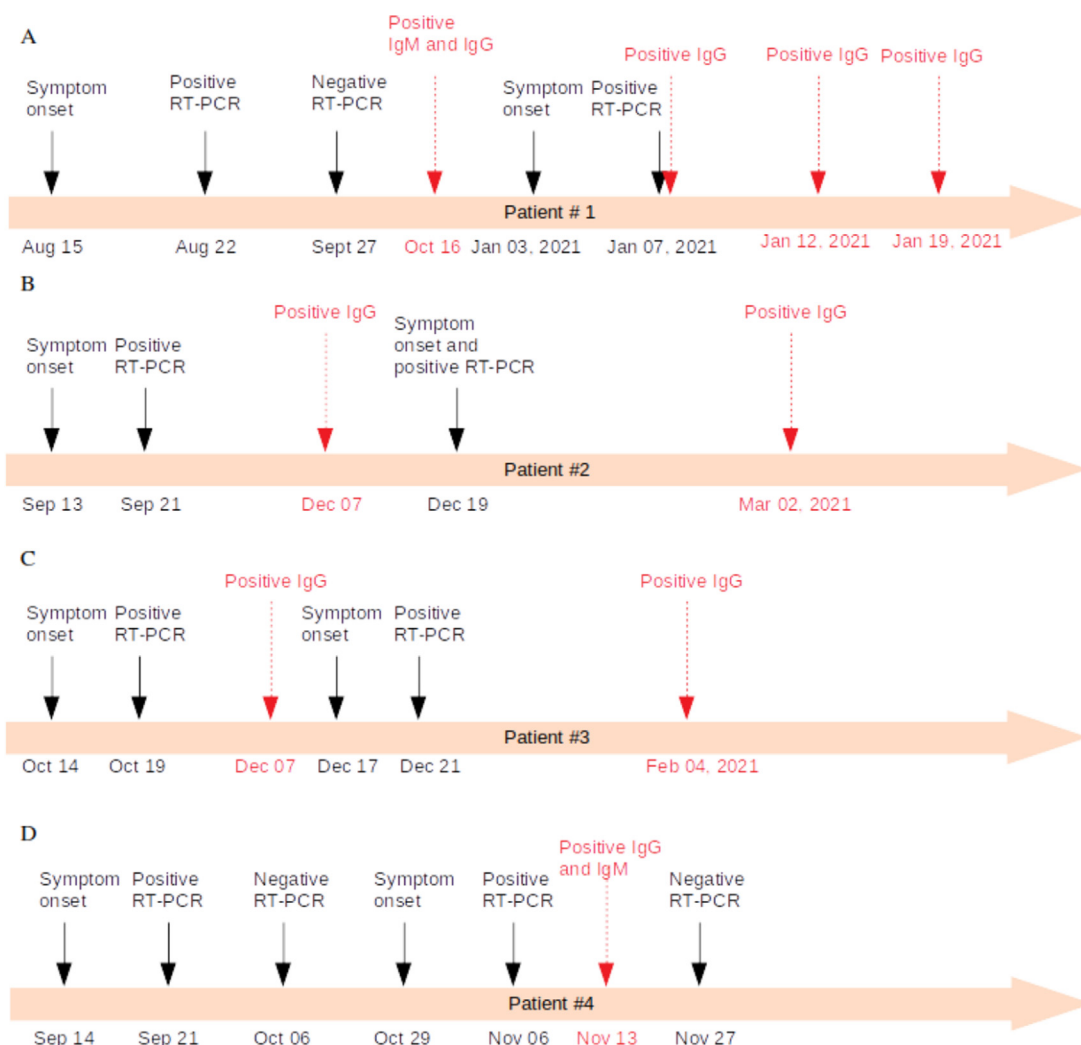


Figure 1. Timeline of symptom onset and molecular diagnosis of specimens.

Table 2

RT-PCR and serological testing for SARS-CoV-2 performed for the 4 patients during the 2 episodes of SARS-CoV-2 infection.

Patients	SARS-CoV-2 RT-PCR				SARS-CoV-2 serology			
	First episode		Second episode		Date	Result ^a		
	Date (days from onset)	Result (Ct)	Date (days from onset)	Result (Ct)		Anti-S1 IgG	Anti-S1 IgM	Anti-NP Total Ab
1	August 22, 2020 (7)	Positive (24.9)	January 07, 2021(4)	Positive (30.8)	October 16, 2020	Positive (10.04)	Positive (1.11)	Positive (49.05)
	September 25, 2020	Negative			January 07, 2021	Positive (3.46)	Negative	Positive (58.75)
					January 12, 2021	Positive (4.97)	Negative	
					January 19, 2021	Positive (10.2)	Negative	Positive (70)
2	September 21, 2020 (8)	Positive (26.8)	December 19, 2020 (0)	Positive (30.4)	February 20, 2021	Positive (11)	Negative	
					December 07, 2020	Positive (2.49)	Negative	Positive (83.08)
					March 02, 2021	Positive (26)	Negative	Positive (88.97)
3	October 19, 2020 (5)	Positive (22.7)	December 21, 2020 (4)	Positive (34.4)	December 07, 2020	Positive (3.75)	Negative	Negative
					February 04, 2021	Positive (1.34)	Negative	Positive (33)
					April 01, 2021	Positive (1.14)	Negative	Positive (15.75)
4	September 21, 2020 (7)	Positive ^b	November 06, 2020 (8)	Positive (30.9)	November 13, 2020	Positive (6.74)	Positive (2.54)	Positive (5.49)
	October 06, 2020	Negative	November 27, 2020	Negative	April 17, 2021	Positive (8.94)	Negative	Positive (25.62)

Abbreviations: Ab, antibody; anti-S1, anti-spike; CO, Cut off; Ct, cycle threshold; S, Sample.

^a Results are obtained for anti-S1 antibodies as index value (i)=Sample/S1 standard; a sample is considered as negative if (i)<1.00 and as positive if (i)≥1.00. Results for anti-NP Abs are obtained as ratio=S/CO (cut-off); a sample is considered as negative if S/CO ratio<1.00 and as positive if S/CO ratio ≥1.00.^b RT-PCR performed with ALLPLEX kit: E gene, 32.2; N gene, 28.7; and RdRP gene, 33.7.

pitalization and oxygen therapy combined with other treatments. The reinfection was confirmed by RT-PCR on November 6, 2020 (Ct=30.9). The recovery occurred after 6 days of treatments and was confirmed by a negative RT-PCR on November 27, 2020. The patient was tested for total Ig, IgG, and IgM against SARS-CoV-2 on November 13, 2020 (Figure 1D).

Procedures

SARS-CoV-2 RT-PCR testing

Viral RNA was extracted from nasopharyngeal swab samples, which were collected between 0 and 8 days after the onset of symptoms (Table 2), using either QIAamp® Viral RNA Mini Kit (QIAGEN) on the QIAcube Connect instrument or the Chemagic Viral DNA/RNA kit special H96 (PerkinElmer, Inc.) on the Chemagic™ 360 instrument. The Real-Time RT-PCR for the detection of SARS-CoV-2 was performed using either Primerdesign Ltd COVID-19 Genesig® Real-Time PCR Assay (Primerdesign Ltd), targeting the ORF1ab region or Allplex™ 2019-nCoV Assay (Seegene Inc). The amplification reaction was carried out on the Applied Biosystems QuantStudio 5 instrument (Applied Biosystems™, ThermoFisher scientific). Ct values for our samples ranged from 22.7 to 34.4, falling within the recommended range (18–35) for sequencing.

SARS-CoV-2 Serology testing

Serological testing was performed for all patients using both enzyme-linked fluorescent assay (ELFA) for the detection of anti-spike (S1) (specifically, anti-receptor binding domain [anti-RBD]) IgM and IgG (VIDAS® SARS-CoV-2 IgG and IgM, Biomérieux SA) and Chemiluminescent Immunoassay (CLIA) for the detection of anti-nucleocapsid (NP) total Abs (Elecys Anti-SARS-CoV-2, on the Cobas e411, Roche Diagnostics).

Whole-genome sequencing of viral variants

Complementary DNA (cDNA) synthesis was performed using nasopharyngeal swab sample through a high-fidelity multiplex polymerase chain reaction (PCR) reaction for target enrichment using 2 pools of 400 base pair (bp) QIAseq SARS-CoV-2 primer panel amplicons, on the basis of a study from the ARTIC Network (Itokawa et al. 2020). The entire contents of both RT-PCR reaction pools per sample were combined into a single tube and then purified using 1X Agencourt AMPure XP Beads (Beckman Coulter, Inc).

After this step, the whole-genome high-throughput sequencing using QIAseq FX DNA Library CDI Kit (QIAGEN, catalog num-

ber [cat. no.] 180484) was performed according to manufacturer's standard protocol for Illumina Sequencing using MiSeq Reagent Kit v2, allowing for a read length of 2 × 150 bp. Therefore, enzymatic fragmentation time for the 100-ng input DNA was adjusted to 18 minutes resulting in a final fragment size of approximately 150 bp.

To make the fragments ready for the adapter ligation, the fragmented DNA was directly end-repaired by adding an “Adenine” to the 3' ends. Illumina-specific adapters were then ligated to the DNA fragments in both ends. The reaction was cleaned up to remove the adapter-dimers using 0.8X AMPure XP beads. Finally, we performed a high-fidelity amplification of sequencing libraries using the HiFi PCR Master Mix. The 6 amplification cycles were followed by a purification using 1X AMPure XP beads, and a final step of quality and quantity assessment was performed. All QIAseq FX DNA libraries were separately diluted to a concentration of 4 nM, pooled together in equimolar amounts and denatured with 0.2 N NaOH, and finally loaded at the recommended concentration (10 pM) onto a MiSeq sequencing System (Illumina).

The raw data quality was checked using MiSeq Sequencing Analysis Viewer software. We obtained cluster densities ranging from 900,000 to 1 million clusters/mm², and over 89% of generated bases were of Q30 value (with 85 % of clusters passing filter). After the quality check, the FASTQ files were imported and analyzed using CLC Genomics Workbench 20.0.3 (QIAGEN). Briefly, the workflow performed on CLC Genomics Workbench included raw reads previously trimmed on the basis of read quality and to remove adapters.

A reference-based mapping was performed to each trimmed read that were mapped to the Wuhan SARS-CoV-2 reference genome SARS-CoV-2/human/CHN/Wuhan-01/2019 (NCBI Accession: NC_045512). From each mapping, we collected a consensus sequence, sequence variants, and mapping coverage. Variant calling was performed, and on the basis of variant/amino acid change, resulted tables were obtained, in which we filtered the variants that met a minimum coverage of >30x, minimum coverage of 5 reads, minimum count of 5, and greater than 70% of total reads carrying the variation.

To ascertain the repeatability of results, a second bioinformatics analysis was performed using independent process and open-source tools. We assessed the quality of the Illumina paired-end reads using FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). We aligned the reads to the reference genome using the standalone Burrows-Wheeler Alignment tool (BWA) version (ver.) 0.7.17-r1188 (Li 2013). We have used paired-end mode for

mapping reads to the SARS-CoV-2 reference genome (NC_045512, version NC_045512.2). We used SAMtools ver. 1.7 (using htlib 1.7-2) to convert the output of BWA from SAM to BAM format and to sort and generate indices for the BAM files (Li et al. 2009). We carried out 2 rounds of filtration (by length cut-off 100 and quality cut-off 60), and the results were analyzed using Integrative Genomics Viewer (IGV) ver. 2.8.9 (Robinson et al. 2017). Bcftools (Tools for variant calling and manipulating VCFs and BCFs) ver. 1.7, as part of the SAMtools framework, was used to generate variant calling statistics. Finally, bcftools mpileup was used to generate the assembled sequences. Samtools depth tool was used for coverage calculation to further analyze mutations.

For phylogenetic analysis, the whole-genome sequences of the isolates (4 isolates) were compared with sequences showing S477N mutation and available in GISAID database as of March 21, 2021. Multiple sequence alignments (MSAs) were performed using MAFFT ver. 7.310 (Katoh et al. 2002). Gaps, incomplete sequences, and sequences showing low coverage were trimmed from the MSA. The duplicate sequences were removed using sRNAtoolbox (Rueda et al. 2015). We carried out phylogenetic analysis using maximum likelihood (ML) method, and the best tree was picked up according to their ML score using IQ-TREE multicore ver. 1.6.12 (Nguyen et al. 2015) with evolutionary model GTR+F+I+G4, showing lowest Bayesian information criterion (BIC) score. The branch

support was carried out using Shimodaira–Hasegawa–like approximate likelihood ratio test (SH-like aLRT) with 1,000 replicates. Finally, 836 full genome sequences were selected and annotated. The annotations of sequences include code, country, and date of collection. The annotations were manually adjusted later for expected errors, and the GISAID ID of sequences were replaced by their appropriate annotations using JavaScript.

Newick trees were visualized using Interactive Tree Of Live ver. 4 (Letunic et Bork 2019). Major SARS-CoV-2 clade memberships and the worldwide distribution of S477 mutation were predicted using Nextclade (Hadfield et al. 2018).

Results

The time course between the 2 episodes of COVID-19 illnesses of Tunisian HCW patients ranged between 45 and 141 days, with more aggressive clinical presentation during the second infection in 2 patients. The whole-genome sequences of viral variants of the 8 samples (2 samples from each case of reinfection) were deposited in the GISAID database (EPI_ISL_1116468 and EPI_ISL_1116469—patient #1; EPI_ISL_1118675 and EPI_ISL_1118884—patient #2; EPI_ISL_1116467 and EPI_ISL_1116464—patient #3; EPI_ISL_712068 and EPI_ISL_1118926—patient #4) (Table 3).

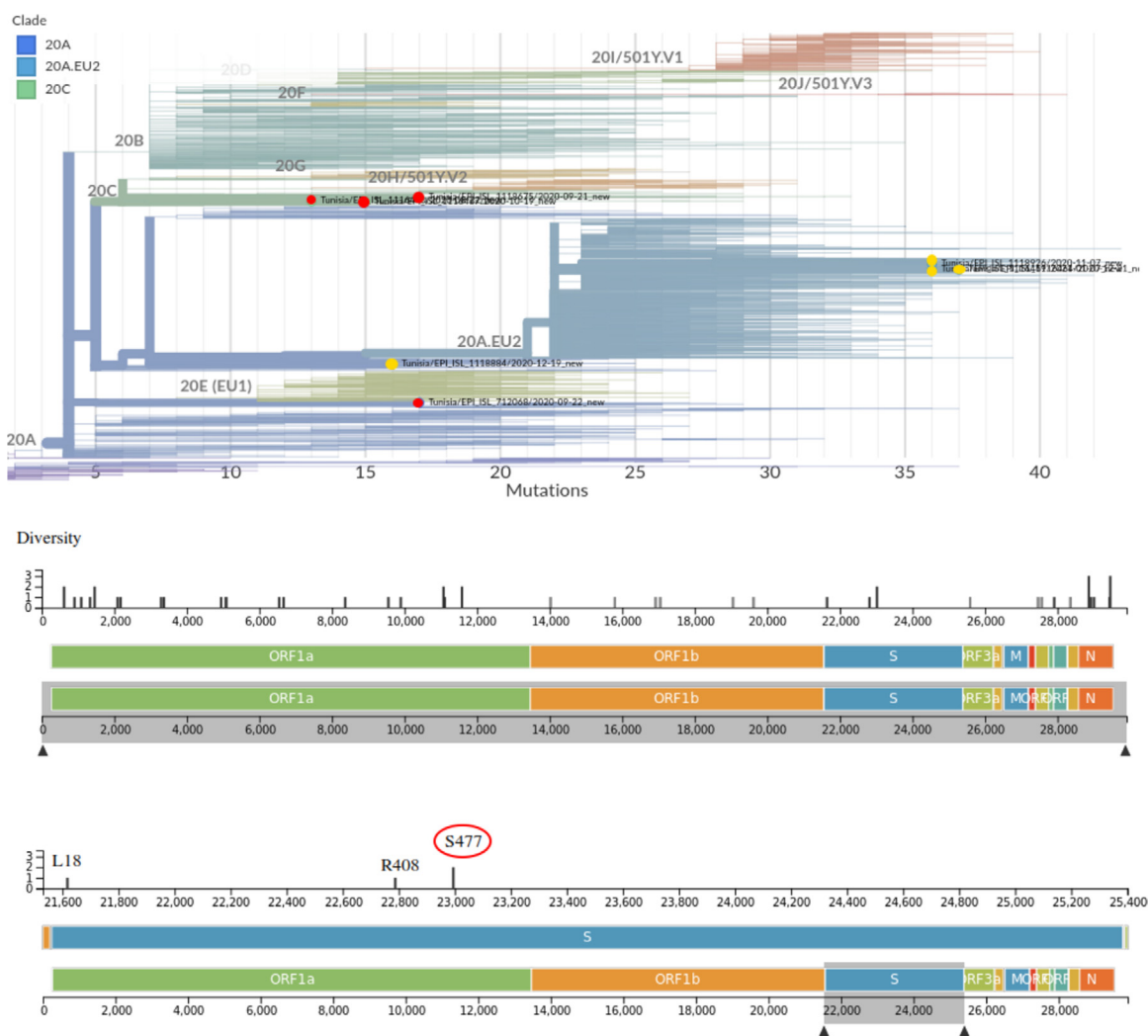


Figure 2. Nextclade analysis of the 8 SARS-CoV-2 isolates from the 4 reinfection cases. Other genomes were retrieved from GISAID (<https://www.gisaid.org>, accessed on March 21, 2021). Clade information using the GISAID and Nextstrain nomenclatures is shown. The strains of the first infection and reinfection episode are shown in red and yellow circles, respectively. The S477N mutation in the S glycoprotein is indicated.

Table 3
Variants of SARS-CoV-2 from samples of reinfection cases.

Case number	Age	First infection			Second infection		
		GISAID code	Lineage	Mutations in Spike	GISAID code	Lineage	Mutations in Spike
1	36	EPI_ISL_1116468	B.1.367	D614G	EPI_ISL_1116469	B.1.160	L18F/S477N/D614G
2	32	EPI_ISL_1118675	B.1	D614G	EPI_ISL_1118884	B.1.367	S477N/D614G
3	41	EPI_ISL_1116467	B.1.367	D614G	EPI_ISL_1116464	B.1.160	S477N/D614G
4	46	EPI_ISL_712068	B.1	R408K/D614G	EPI_ISL_1118926	B.1.160	R21S/ S477N/D614G

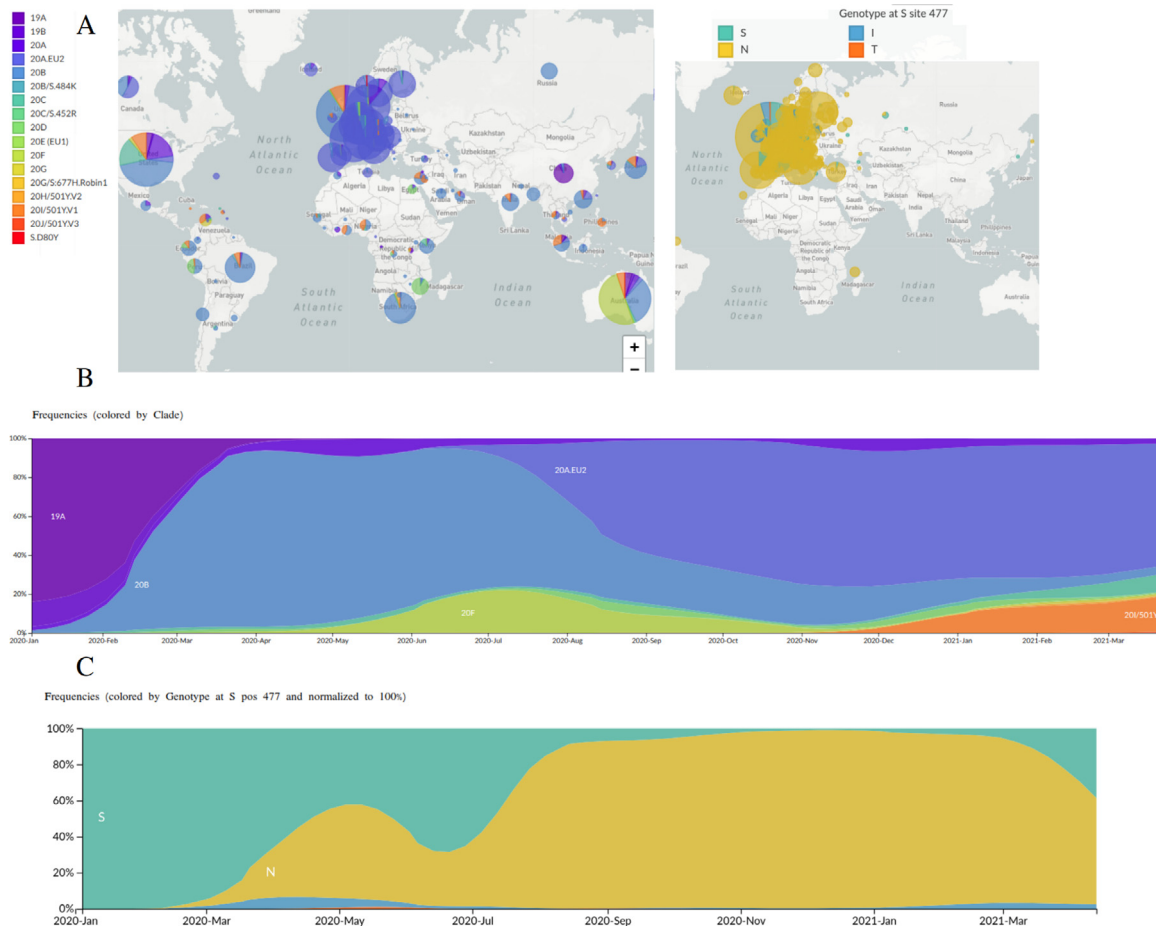


Figure 3. Clade distribution in the World. (A) Geographic distribution of SARS-CoV-2 clades (Nextclade, accessed on March 21, 2021); (B) Time evolution of clades; (C) Time evolution of S477 frequency.

The results show that the first infection was caused by clades 20C (3 cases; 2 lineages B.1.597 and 1 lineage B.1.428.2) and 20A (1 case; lineage B.1), whereas the second infection was caused by clades 20A.EU2 (3 cases; 2 lineages B.1.160 and lineage B.1) and 20A (1 case; lineage B.1) (Figure 2, Table S2).

The clade 20A.EU2 emerged in the beginning of the pandemic, but by July 2020 it became the dominant form of clades, circulating globally and mainly in Europe (Figure 3A and 3B). It is worth noting that all variants from second infection carry the Spike S477N and NSP1 L107I mutations, showing high reads coverage (Table S2) compared with the first infection.

The combination S477N/L107I is found in only 5 Tunisian SARS-CoV-2 isolates (GISAID, as of June 24, 2021), among which 4 isolates are those of the current study (EPI_ISL_1116464, EPI_ISL_1116469, EPI_ISL_1118884, and EPI_ISL_1118926), whereas the fifth isolate (EPI_ISL_1137609) was detected in another study (Sousse, Tunisia).

NSP1 L107I was detected in other 14 SARS-CoV-2 isolates (GISAID by June 24, 2021) from Germany (2 isolates), the United

States (7 isolates), India (4 isolates), and Nigeria (1 isolate); none of them was in association with S477N mutation.

Interestingly, 3 of the 4 reinfection cases have an additional mutation in NSP4 gene (M324I). The combination L107I/M324I is found only in these Tunisian SARS-CoV-2 isolates and, in another isolate, detected in a 3-month Tunisian newborn (EPI_ISL_1138747) (GISAID as of June 24, 2021); all of these isolates are associated with S477N mutation.

Phylogenetic analysis showed that 3 of the 4 reinfection sequences are clustered with the US strain (hCoV-19/USA/NY-NYCPHL-003747/2021) collected on March 02, 2021. The remaining reinfection case is clustered with strains from Mayotte, France, and Hungary collected on January 06, 2021; February 02, 2021; and October 10, 2020, respectively (hCoV-19/Mayotte/IPP00202/2021, hCoV-19/France/BRE-IPPO2818/2021, and hCoV-19/Hungary/UD-94387/2020) (Figure 4).

In addition, if we examine the frequency trend of the S477N mutation on the basis of data in GISAID, we see a clear increase corresponding to the second wave of COVID-19 in Europe

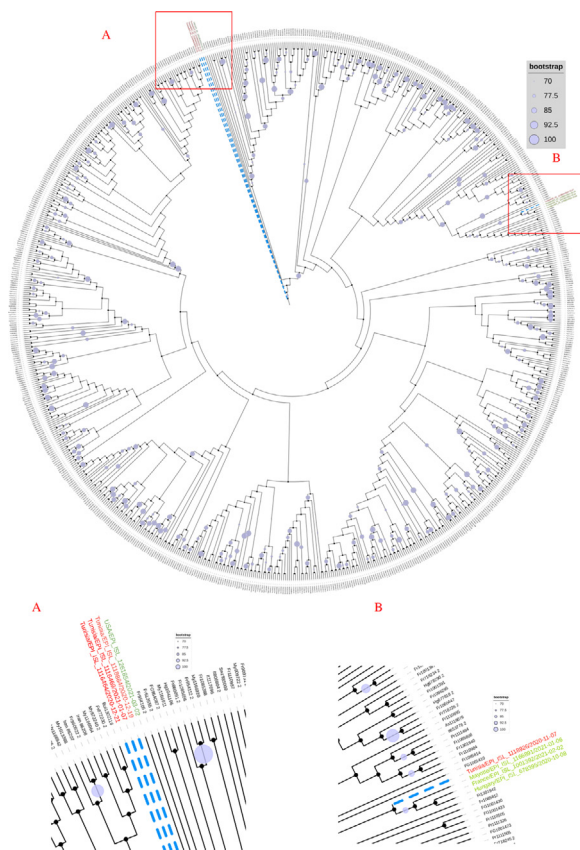


Figure 4. Phylogenetic analysis of the 4 SARS-CoV-2 isolates from the reinfection episodes. Other 836 sequences undergoing the S477N mutation were retrieved from GISAID (<https://www.gisaid.org>, accessed on March 21, 2021). The strains of the reinfection are highlighted in red color.

during the summer period of 2020 (Figure 3C) (Hodcroft et al. 2020).

All patients tested before the second infection (patients #1, #2, and #3) (Table 2) were positive for anti-S1 IgG and negative for anti-S1 IgM, suggesting the presence of residual immunity at the time of reinfection. Anti-NP total Ab were detected only in 2 cases (patients #1 and #2).

For patient #1, further serological testing, after 12 days, showed a significant increase in IgG concentration, which were maintained for up to 1 month. Similarly, a sustainable response for anti-S1 IgG was observed for patient #2, approximately 3 months after the first testing. Surprisingly, for patient #3, a progressive decline in IgG response seemed to occur despite the second episode of infection.

Unfortunately, no serum was available for patient #4 before the reinfection. This patient was first tested 15 days after the onset of symptoms during the second episode. At this time, both anti-S1 IgM and IgG were positive along with a weakly positive anti-NP total Ab. Further testing performed 5 months later showed the absence of IgM with detectable IgG.

In summary, our results show that the variants' features of first and second infections are different in all 4 cases, providing irrefutable evidence for reinfection despite the presence of anti-S1 IgG.

Discussion

The diagnosis of SARS-CoV-2 reinfection is currently based on the evidence of a new infection by a phylogenetically distinct form of SARS-CoV-2 after the elimination of the previous one. In our

case series of 4 HCWs who have experienced 2 separate symptomatic episodes of COVID-19, distinct viral lineages were documented by comparative genome analysis. The presence of presumed residual immunity at the time of reinfection for 3 patients raises the concern about the protective role of immunity after COVID-19.

It has been reported in the literature that SARS-CoV-2 infection results in protection against symptomatic illness in HCWs at least in the short term (Lumley et al. 2021). Furthermore, in the same study by Lumley et al., no symptomatic reinfections in HCWs with anti-Spike Abs were observed suggesting that previous infection resulting in Abs to SARS-CoV-2 is associated with protection from reinfection for most people for at least 6 months (Lumley et al. 2021). For our cases, although anti-S1 IgG were detectable before reinfection in 3 patients, all of them developed mild to severe clinical presentation after a symptom-free interval ranging between 45 and 141 days. The possible explanations of these results could be the decreasing levels of neutralizing Abs, which may predispose patients to reinfections, or a second episode of SARS-CoV-2 infection caused by a viral mutant showing higher affinity for the receptor, and this interaction cannot be prevented by Abs of suboptimal avidity/affinity.

In our cohort of 4 HCWs, it is appealing that all reinfections have occurred with viral variants carrying the D614G/S477N mutations in the Spike protein, although belonging to different lineages. None of the variants of concern (VOC) according to the Centers for Disease Control and Prevention (CDC) <https://www.cdc.gov/coronavirus/2019-ncov/variants/variant-info.html> carry the D614G/S477N mutations, and a single variant of interest carries S477N (B.1.526, detected in the Iota, United States in November 2020) that has reduced neutralization by convalescent and post-vaccination sera (Annavejhal et al. 2021). The S477N/D614G double mutation appeared in Australia during July and August 2020 and was traced to a single event from Australian hotel quarantine (Chen et al. 2020); it currently represents 3% of the submitted sequences in GISAID (as of June 5, 2021).

According to recent studies, variants with the S477N mutation have stronger binding of the Spike protein to the human receptor ACE2 (Singh et al. 2021) and were resistant to neutralization by multiple monoclonal Abs (mAbs) (Z. Liu et al. 2021). Therefore, the emergence of S477N/D614G could result in an immune evasive variant leading to less Ab binding and a resistance to virus neutralization. In addition, it was shown that S477N/D614G is broadly resistant to many neutralizing Ab (Tea et al. 2021). Liu and collaborators also reported that some variants, including those with S477N and E484K mutations, are resistant to or escape neutralization by multiple mAbs, suggesting a possible reinfection by these viral variants of previously infected patients (L. Liu et al. 2020).

The presence of S477N mutation in the Spike protein may explain the occurrence of symptomatic reinfection in our 4 cases. Indeed, higher affinity of S1 toward the cellular receptor ACE2 is seen in this mutated virus than in the wildtype virus, suggesting that interference with such binding between S1 and ACE2 through neutralizing Abs will require higher affinity of these Abs. Nevertheless, the factors that determine the outcome of reinfection remain unclear. Although the S477N mutation was uniformly carried in all subvariants isolated after secondary infection, the clinical presentation in our cases was heterogeneous, ranging from mild to severe forms. The quality of humoral and cell-mediated immunity responses against the virus might play a major role in determining the clinical outcome of COVID-19 reinfection.

Interestingly, in this report, although patients #1 and #2 experienced a milder course of infection during the second episode with a faster recovery, patients #3 and #4 showed a more severe secondary infection, requiring oxygen support with indication of hospitalization in 1 case (patient #4). The analysis of serum Ab

profile of patient #1 clearly showed the decrease in the anti-S1 IgG concentration between October 16 (10.04) and January 07 (3.46), which corresponds to the day of re-positive RT-PCR. This finding is in line that a decrease in anti-S1 IgG precedes secondary infection (To et al. 2020, 1). Furthermore, as a consequence of secondary infection, in patients #1 and #2, the IgG concentration is increasing to substantially higher levels. This indicates that secondary infection enhances IgG production and also might improve the quality of the response, specifically its affinity/avidity. This adequate and more robust Ab response may be the cause of the less intense and rapidly resolved reinfection (To et al. 2020, 1).

Recently, it has been shown that in SARS-CoV-2 infected individuals, the IgG response is characterized by incomplete avidity maturation in contrast to 2 rounds of vaccination (Struck et al. 2021). Incomplete avidity maturation might facilitate secondary SARS-CoV-2 infections and thus prevent the establishment of herd immunity (Bauer 2021; Struck et al. 2021). Interestingly, Struck et al. also show that the combination of natural infection with 1 step of vaccination leads to the same degree of high avidity of IgG as 2 rounds of vaccination, hence improving the protective immunity status.

In contrast to patients #1 and #2, patient #3 who has much more severe symptoms, did not show an increase in anti-S1 IgG responses after secondary infection. This finding suggests that the more severe clinical outcome observed in this patient may be in relation, at least in part, with inadequate Abs response during reinfection. For patient #4, the lack of a serological test before the second episode does not allow us to conclude the presence or absence of a residual immunity. However, the presence of both anti-S1 IgG and IgM 15 days after the onset of symptoms during reinfection suggests the absence of Abs before reinfection, especially in this patient's context. Indeed, the underlying condition along with immunosuppressive drugs might have compromised the response of Abs, leading to a more severe clinical presentation during the second infection. Of note, for patients #3 and #4, the interval between the 2 episodes of SARS-CoV-2 infection was 64 and 45 days, respectively. This is in line with a recent study showing that the second episode of SARS-CoV-2 positivity is more severe if it happens within 60 days after the first positive PCR (Vánca et al. 2021, 2). Altogether, our results support the close association between the outcome of reinfection and the immunity response of the host; an inadequate immunity developed after the first infection may predispose to an early reinfection with more severe clinical presentation, especially in case of HCWs who are frequently exposed to SARS-CoV-2.

This case study brings new evidence for the possibility of reinfection with COVID-19 and highlights the different clinical pictures that may occur during this reinfection. Nevertheless, in our study, it was not possible to fully assess the effectiveness of the immune response during the 2 episodes of SARS-CoV-2 infection because neutralizing Ab titer against viral strains was not measured.

In summary, our findings highlight the importance of measures to protect HCWs during the pandemic, such as providing suitable protection devices and giving them priority for vaccination, even for those who have recovered from COVID-19. Our analyses also suggest that HCWs who have had the disease should still be vaccinated. Whether the risk of reinfection in HCWs is higher than in the general population is still an open question; further investigations are still needed to address this issue, especially under the moving landscape of viral variants.

Declaration of Competing Interest

All authors declare no competing interest.

Availability of data and material

FASTA-format sequences of complete viral genomes and meta-data are available online through GISAID (<http://gisaid.org>). The datasets generated and analyzed during the current study are also available from the corresponding author on request.

Ethics approval and consent to participate

The study protocol adhered to the declaration of Helsinki and was approved by the ethic Committee of protection of persons (Tunisian Ministry of Health) under the reference Comité de Protection des Personnes(CPP SUD) N°0265/2020.

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Authors contributions

SG contributed to the design of the study, sample processing, immunological data analyses, and writing of the paper. AS contributed to genome sequencing, sequence assembly, and writing of the paper. NA contributed to sequence assembly, sequence analyses, phylogeny, figures' and tables' production, and writing and editing of the paper. AC contributed to samples processing and analyses. LFB contributed to samples processing and analyses and manuscript editing. RK, HK, OK, and AN contributed to case identification, clinical care, and collection of clinical data. AH, NR, HKH, and SM supervised the work. AR contributed to the study design, data analyses, and writing/editing of the paper and is a coordinator of the ADAGE consortium.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ijid.2022.01.006](https://doi.org/10.1016/j.ijid.2022.01.006).

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