Symptomatic SARS-CoV-2 reinfection of a health care worker in a Belgian nosocomial outbreak despite primary neutralizing antibody response

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A young, immunocompetent health care worker was symptomatically reinfected with SARS-CoV-2 in a transmission cluster with three patients, despite the development of an effective humoral immune response following symptomatic primary infection 185 days earlier.
Abstract

Background. It is currently unclear whether SARS-CoV-2 reinfection will remain a rare event, only occurring in individuals who fail to mount an effective immune response, or whether it will occur more frequently when humoral immunity wanes following primary infection.

Methods. A case of reinfection was observed in a Belgian nosocomial outbreak involving 3 patients and 2 health care workers. To distinguish reinfection from persistent infection and detect potential transmission clusters, whole genome sequencing was performed on nasopharyngeal swabs of all individuals including the reinfection case’s first episode. IgA, IgM, and IgG and neutralizing antibody responses were quantified in serum of all individuals, and viral infectiousness was measured in the swabs of the reinfection case.

Results. Reinfection was confirmed in a young, immunocompetent health care worker as viral genomes derived from the first and second episode belonged to different SARS-CoV-2 clades. The symptomatic reinfection occurred after an interval of 185 days, despite the development of an effective humoral immune response following symptomatic primary infection. The second episode, however, was milder and characterized by a fast rise in serum IgG and neutralizing antibodies. Although contact tracing and virus culture remained inconclusive, the health care worker formed a transmission cluster with 3 patients and showed evidence of virus replication but not of neutralizing antibodies in her nasopharyngeal swabs.

Conclusion. If this case is representative of most Covid-19 patients, long-lived protective immunity against SARS-CoV-2 after primary infection might not be likely.

Keywords

SARS-CoV-2, reinfection, humoral immunity, coronavirus, COVID-19, whole genome sequencing
Introduction

The mechanism, extent, and duration by which primary SARS-CoV-2 infection provides immunity against reinfection are currently unclear. For common cold coronaviruses, loss of immunity and reinfection with the same virus have been reported to occur frequently 12 months after primary infection [1]. For SARS-CoV-2, persistent viral shedding can occur over prolonged periods of time following clinical recovery [2]. To confirm genuine reinfection, whole-genome sequencing is required. Since the start of the SARS-CoV-2 pandemic 11 months ago, only 8 published and confirmed cases of reinfection were reported in Hong Kong [3], the USA [4,5], Belgium [6], the Netherlands [7], Ecuador [8], and India [9]. These cases likely are an underestimate due to the limited detection of asymptomatic cases. In fact many more cases were reported in the media and on preprint servers [2,10–12]. Hence, it remains to be seen whether these reinfection cases represent the tail end of the distribution with many more to come or whether SARS-CoV-2 reinfection remains a rare event.

One hypothesis could be that reinfections occurs as a result of immune evasion by another variant of SARS-CoV-2. However, the genomic variation seen across SARS-CoV-2 sequences are limited and likely the result of neutral evolution, rather than adaptive selection, and although the D614G mutation in spike has become consensus, there is no evidence that this mutation is linked to host immune pressure [13,14].

Another hypothesis could be that these reinfections only occur in individuals who do not develop an effective immune response during primary infection. Indeed, not all Covid-19 patients seroconvert [15,16] and not all who seroconvert develop neutralizing antibodies [17]. Furthermore, disease severity seems to correlate with higher IgG [18–20] and neutralizing antibody titers [19,20]. Unfortunately, in only two of all reported reinfection cases antibody testing was reported after the first episode. In the Hong Kong case, IgGs but no neutralizing antibodies were detected 10 and 43 days post-symptom onset (pso) [3,21] whereas in the Ecuador case, IgMs but no IgGs were detected
by rapid test 4 days pso [8]. Neutralizing antibodies were not measured. Hence, it is currently unclear whether these reinfection cases were able to mount an effective immune response after primary infection.

Finally, it is not known whether immunity prevents onward transmission from those who are re-infected. Most reported reinfection cases [3,4,6–8,11] showed nasopharyngeal samples with high RT-qPCR Ct values, from which virus is usually unculturable. Five Indian health care workers however, displayed high viral loads during their secondary infection but no viral culture was performed to determine the infectiousness of their virus [9,12].

Here we describe a case of symptomatic reinfection in a health care worker despite having developed a neutralizing antibody response following symptomatic primary infection. The reinfection occurred with an interval of 185 days during a nosocomial outbreak involving 5 individuals. Whole genome sequencing was performed, and humoral immune responses and viral infectiousness were quantified.

**Materials & Methods**

**Sample collection and diagnosis**

Sample collection and clinical evaluation were performed in view of diagnosis and standard of care and approved by the hospital’s ethical committee (EC/PM/nvb/2020.084). Oral consent was obtained from all patients before sampling followed by written consent prior to publication. Initial SARS-CoV-2 diagnosis was performed at the hospital on nasopharyngeal swabs using the Xpert Xpress SARS-CoV-2 test on the GeneXpert® Platform (Cepheid, USA) as per manufacturer’s instructions or by in-house PCR [22] with extraction on NucliSens EasyMag® (Biomerieux, France) and amplification on Cobas LightCycler® (Roche, Switzerland). Complete blood counts were performed on XN-9100® (Sysmex) and biochemistry parameters on Atellica® (Siemens, Germany).
**RNA extraction and RT-qPCR on nasopharyngeal samples**

At the Institute of Tropical Medicine Antwerp, RNA was extracted from UTM or eSwab medium after inactivation at 56°C with proteinase K using a Maxwell RSC Instrument. RNA from phocine distemper virus was added to all samples as an internal extraction and PCR inhibition control [23]. A SARS-CoV-2-specific RT-qPCR was then performed to amplify a 112 bp fragment of the E gene as previously described [22] with 5 µL RNA in a 25 µL reaction using the Bioline SensiFAST mix, Reverse Transcriptase and RiboSaf RNase inhibitor. To determine the presence of SARS-CoV-2 replicase activity, a negative strand RT-qPCR was performed as previously described [24] with FWD-Tag-primer-1 (catagcagggtagaa-GCAAGAGATGTTGTTCC), Tag-primer-1 (catagcagggtagaa), REV-primer-1 (GTAAATGTTGTACCATCAGCAG) and FAM-labeled negative-strand-probe-1 (CAGCAGCCAAACTAATGGTTGTCATA).

**Whole genome sequencing using MinION**

Whole genome sequencing was performed on an Oxford Nanopore MinION device using R9.4 flow cells (Oxford Nanopore Technologies, UK) after a multiplex PCR with an 800bp SARS-CoV-2 primer scheme as previously described [25]. Sequence reads were basecalled in high accuracy mode and demultiplexed using the Guppy algorithm v3.6. Reads were aligned to the reference genome Wuhan-Hu-1 (MN908947.3) with Burrows-Wheeler Aligner (BWA-MEM) and a majority rule consensus was produced for positions with ≥100x genome coverage, while regions with lower coverage, were masked with N characters.
Phylogenomic analysis

All SARS-CoV-2 genomes were compared at the nucleotide and amino acid level to the reference genome Wuhan-Hu-1. Clade assignment was performed using NextClade v0.7.2 [26]. BLAST+ was used to extract the top 15 matches for each of our sequences from the msa_0929.fasta file downloaded from GISAID (Global Initiative on Sharing All Influenza Data). In addition, we included the most recent (Aug 16-31, 2020) Belgian sequences and all Belgian L, O, V clade sequences collected between March 1-16, 2020. Sequence alignment was performed using MAFFT v7 and a maximum likelihood phylogenetic tree was inferred with IQ-TREE v1.6.12., using the TIM2+F+I model and 500 nonparametric bootstraps, and visualized in FigTree v1.4.4.

SARS-CoV-2 specific antibody detection tests

The Elecsys electrochemiluminescence immunoassay on the Cobas 8000® analyzer (Roche Diagnostics, Belgium) was used for the qualitative detection of total antibodies against the Nucleocapsid (N) antigen of SARS-CoV-2. A signal threshold ≥1 was defined as positive. For the separate quantification of IgM, IgG, and IgA antibodies, we used a Luminex bead-based assay [27]. In short, recombinant receptor binding domain (RBD) and N protein (BIOCONNECT, The Netherlands) were coupled to 1.25x10⁷ paramagnetic MAGPLEX COOH-microspheres from Luminex Corporation (Texas, USA). After incubation of beads and diluted sera (1/300 for IgG and IgM, 1/100 for IgA), a biotin-labeled anti-human IgG, IgA, and IgM (1:125) and streptavidin-R-phycoerythrin (1:1000) conjugate was added. Beads were read using a Luminex® 100/200 analyzer with 50 µL acquisition, DD gat 5000 - 25000 settings, and high PMT option. Results were expressed as crude median fluorescent intensities (MFI). Samples were considered positive if MFI >3x SD + mean of negative controls (n=16).
SARS-CoV-2 viral neutralization test and virus isolation

Serial dilutions (1/50 - 1/1600) of heat-inactivated (30 min at 56°C) serum or nasopharyngeal samples were incubated with 3x TCID100 of a SARS-CoV-2 primary isolate (2019-nCoV-Italy-INMI1) for 1h at 37°C / 7% CO2 and subsequently added to 18,000 Vero cells per well for a further 5 days incubation. Assay medium consisted of EMEM (Lonza, Belgium) supplemented with 2 mM L-glutamine, 2% fetal bovine serum, and penicillin - streptomycin (Lonza). After incubation, cytopathic effect caused by viral growth was scored microscopically. 50% (NT50) or 90% (NT90) neutralization titers were calculated using the Reed-Muench method. Similarly, virus isolation was attempted by incubating a serial dilution of nasopharyngeal samples on VeroE6-TMPRSS2 cells after 2 hours of spinoculation at 2500g and 25°C and following up cytopathic effect.

Results

Clinical evolution of the outbreak

In September 2020, a nosocomial outbreak occurred at an internal medicine ward in a Belgian hospital. A 75-year-old man (PAT1) developed influenza-like symptoms of cough, low grade fever and general malaise and tested positive for SARS-CoV-2 on September 13. Three days later a 77-year old woman (PAT2) tested positive after developing gastro-intestinal symptoms and general malaise (Table 1). In response to this outbreak all patients and health care workers in the ward were tested revealing 1 additional asymptomatic 87-year old man (PAT3) and 2 infected health care workers (HCW1 & HCW2) showing mild symptoms. All individuals recovered completely. HCW1, a 39-year old woman had already been infected with SARS-CoV-2 in March 2020. During this first episode she had a protracted mild illness with cough, dyspnea, headache, fever and general malaise. Her hematological and biochemical parameters were consistent with viral infection showing decreased white blood cell counts and mildly elevated CRP (Supplementary table 1). She was managed as an
outpatient and slowly resumed work after 1 month. During the second episode her clinical presentation was milder, and she resumed work 10 days after diagnosis although she experienced dyspneic spells for up to 3 weeks for which she sought emergency care. A blood sample and a second swab were taken 5 days after her second diagnosis. This time no laboratory abnormalities were found (Supplementary table 1).

**Whole genome sequencing revealing transmission cluster**

To distinguish reinfection from persistent infection and detect potential transmission clusters, whole genome sequencing was performed on all nasopharyngeal swabs taken from the 3 patients and 2 health care workers, including the swab taken from HCW1 during her first episode. 99.6% of the SARS-CoV-2 genome (nucleotide 55–29823) was recovered at an average mean depth of 771-fold (Supplementary table 2). Analysis of these sequences (EPI_ISL_582127–32) revealed that the virus which infected HCW1 in September belonged to a different SARS-CoV-2 clade (G clade) than the virus causing her first COVID-19 episode in March (V clade) (Figure 1). A total of 18 nucleotide differences, of which only one shared (i.e. G11083T, which defines clade V but is also known to occur in clade G), could be observed between the 2 strains (Figure 2). This confirms their distinct nature and is within the range of 9-24 nucleotide differences reported for other reinfections. Interestingly, the 3 infected patients shared the same virus and hence constitute a recent transmission cluster with HCW1. None of the amino acid mutations in the S gene of the reinfecting virus were previously reported to confer resistance to convalescent plasma or RBD-specific monoclonal antibodies [28].

Finally, the viral sequence derived from HCW2 differed by 27 nucleotides from HCW1’s virus and belonged to GISAID’s GH clade (Figure 1 and 2). This suggests that HCW2 was infected by an external transmission source which corresponds with the fact that her partner also tested positive for SARS-CoV-2. As the phylogenetic analysis and visualization provided by Nextstrain [29] only uses a subsample of publicly available SARS-CoV-2 genomes, we performed a blast search to include the
most closely related known sequences in our analysis. As expected, the genomes from HCW1, PAT13, and HCW2 in September were closely related to Belgian sequences obtained in July-September whereas the virus from HCW1’s March episode was identical to Belgian sequences collected in that same month (Supplementary figure 1).

Assessment of SARS-CoV-2 specific humoral response and neutralizing antibodies

We then measured the presence of total SARS-CoV-2 specific antibodies as well as individual IgA, IgM, and IgG titers in the serum of the 4 individuals in the transmission cluster using all available samples. As antibody kinetics can depend on the antigen [20], we measured responses against the N protein and the RBD of the Spike protein. In addition, we performed in vitro viral neutralization tests on serum and swab samples to determine whether these antibodies have neutralizing capacity. HCW1 clearly seroconverted because 3 months after primary infection, she still displayed high serum IgG titers as well as neutralizing antibodies (200 NT50) at levels higher than those of the 3 patients 2 weeks post diagnosis (Table 1). As expected, serum IgM and IgA could not be detected at this timepoint. Since antibody testing was not available during the first wave of the pandemic in Belgium, and no baseline sample was taken upon reinfection, we could not evaluate whether her neutralization titers had been higher closer to primary infection or disappeared closer to reinfection. However, during reinfection, a rapid rise in neutralizing antibodies could be observed within 7 days pso (1309 NT50) which further increased 14 days later (>1600 NT50) in line with the high IgG titers and somewhat lower IgA and IgM titers at these timepoints (Table 1). PAT2 showed full seroconversion 13 days pso and displayed high IgA, IgM and IgG titers as well as a low neutralizing antibody response (75 NT50). For PAT1 however, who was first to develop symptoms, IgM levels were below the cut-off 17 days pso although IgA and IgG were clearly present as well as neutralizing antibodies (100 NT50). Finally, for PAT3 only low IgA titers and very low levels of neutralizing antibodies (<50 NT50) were detected 12 days post diagnosis which corresponds with his
asymptomatic disease and high Ct values upon diagnosis (Table 1 and Figure 3). No neutralizing antibodies were detected in any of the nasopharyngeal swabs including those of HCW1 taken at day 0 and day 7 pso.

**Potential onward transmission from the reinfection case**

How transmission exactly occurred within this cluster of 4 individuals as well as its origin remain unclear. The identical genomes and timelines would suggest one index patient who infected the others. All 3 patients tested negative for SARS-CoV-2 upon admission to the hospital with a diabetic foot problem 4 to 6 weeks prior to this outbreak (Table 1). They were put on compulsory bed rest, stayed in private rooms, and only received a maximum of 1 visitor a day as per hospital rules. Visitors, patients, and staff wore cloth/surgical masks as per hospital policy and aerosol generating procedures were performed in full PPE. It is unlikely that HCW1 contracted the virus and transmitted it to the 3 patients as HCW1 developed symptoms 4 days after PAT1 and did not nurse PAT1. One explanation could be that an asymptomatic visitor of PAT1 infected both PAT1 and HCW1 who then transmitted the virus while nursing PAT2 and PAT3 (Figure 3). However, none of the close contacts of HCW1, PAT1, and PAT3 tested positive for SARS-CoV-2, although cases could have been missed. PAT2 reported symptomatic SARS-CoV-2 infection in one of her daughters 2 days after her mother’s diagnosis, but is unlikely to be the index patient (and rather infected her daughter) as PAT2 developed symptoms 3 days after PAT1 who she did not have contact with. Finally, nasopharyngeal swabs taken from HCW1 at diagnosis and 6 days later showed high viral loads (Avg Ct 19 and 25 respectively), but lower than at diagnosis of primary infection (Avg Ct 13), and contained replicating virus as indicated by RT-qPCR for negative strand RNA (Avg Ct 25.5 and 28.5 respectively). Yet, we were unable to culture virus from these swabs, but this might be because we had to dilute the otherwise cytotoxic swab medium to a level where virus isolation can fail.
Discussion

One of the key questions in understanding SARS-CoV-2 immunity and predicting the course of the pandemic is for how long and how frequently primary infection protects against reinfection. Eight cases of reinfection have now been described showing intervals between episodes from 48 to 142 days. In this study, we describe another genuine case of SARS-CoV-2 reinfection with an interval of 185 days. The viral genomes from the first and second episode belonged to different SARS-CoV-2 clades and phylogenomic analysis showed that the closest relatives to these 2 genomes were strains collected mostly from Belgium around the same period.

We then quantified the humoral immune response in HCW1 after her first and second SARS-CoV-2 episode as it was suggested that asymptomatic and mild primary infections might not protect against reinfection. The fact that HCW1 fully seroconverted and even had significant levels of serum neutralizing antibodies 3 months after primary infection, suggests that she wasn’t an exceptional patient unable to mount a humoral immune response. The re-infecting virus also didn’t harbor any known spike mutations that could have enabled escape from neutralizing antibodies induced during primary infection. The durability of the humoral immune response on the other hand, remains a debated issue. Although some early studies reported a rapid loss of humoral immunity [14,30] within 2-3 months in up to 40% of patients with mild disease [18], more recent evidence shows that neutralizing antibodies and IgGs actually reach a stable nadir after an initial decline [20,31], which persists for at least 5 to 7 months, presumably as short-lived plasma cells are replaced with long-lived antibody secreting cells [20]. However, as no blood sample was taken from HCW1 right before or at day 0 of the second episode, we could not reliably determine whether neutralizing antibodies persisted or whether a further loss allowed for the reinfection. Of note, neutralizing antibodies are only a marker of immunity and the antibody level needed to confer protection to SARS-CoV-2 is currently unknown.
The slightly milder clinical disease course of HCW1’s secondary infection with normal physiological parameters, is likely the result of the patient’s adaptive immunity being primed by the first infection. This corresponds with the lower viral loads and the strong and fast rise in serum IgG, and neutralizing antibody responses observed after reinfection while IgA and IgM levels remained rather low. Unfortunately, we were unable to compare these antibody levels to similar time points after the first episode.

It seems likely that HCW1 played a role in the spread of this outbreak as she provides the only link between some of the patients. Furthermore, although virus culture remained inconclusive, HCW1’s nasopharyngeal swabs contained replicating virus but no neutralizing antibodies which suggests the re-infecting virus was fully capable of onward transmission. However, none of her contacts tested positive, which might be reassuring.

In conclusion, we describe a case of SARS-CoV-2 reinfection in a young, immunocompetent patient who, in contrast to the Hong Kong case, developed an effective humoral response after primary infection. Although this implies that reinfection may be unavoidable due to waning antibody responses, particularly at the site of infection, secondary infection may result in less severe disease due to a primed immune response.
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Figure Captions

Figure 1. SARS-CoV-2 clade assignment of 6 genomes recovered from 3 patients (PAT) and 2 health care workers (HCW). Sequences were aligned to a representation of the global SARS-CoV-2 genetic diversity using a banded Smith-Waterman algorithm with an affine 160 gap-penalty and subsequently visualized using NextClade v0.7.2. Colors represent the different GSIAID clades. The 6 genomes and their clades are marked by respectively arrows and grey text.

Figure 2. Nucleotide and amino acid comparison of the nosocomial SARS-CoV-2 genomes to the reference genome Wuhan-Hu-1. * Mutations defining GSIAID’s G clade; + Mutations defining GSIAID’s V clade; † Mutation defining GSIAID’s GH clade; # Nt = number of nucleotide changes compared to Wuhan-Hu-1; # AA = number of amino acid changes compared to Wuhan-Hu-1; Clade = respectively Nextstrain (19A; 20A); GSIAID (V; G; GH); and Pangolin (B.1; B.2) nomenclature; PAT = patient; HCW = health care worker; ORF = open reading frame; M = membrane; S = spike; N = Nucleocapsid

Figure 3. Schematic of the most likely chain of transmission between the individuals involved in the nosocomial outbreak. Black and dashed arrows indicate potential viral transmission. Colored tick marks represent the day of symptom onset (PAT1, PAT2, HCW1, DAU) or diagnosis (PAT3) as well as the day of serological testing. VIS = visitor; PAT = patient; HCW = health care worker; DAU = daughter; Ig = immunoglobulin.
Table 1. Epidemiological and serological data on the 3 patients and 2 health care workers involved in the nosocomial outbreak

| Patient | Sex | Age | Condition | Covid-19 contacts (dd/mm) | Symptoms | Swab collection (dd/mm) | Avg Ct | Swab NT50 | Serum collection (dd/mm) | Serum total Ig (COI) | Serum IgA (MFI) RBD | Serum IgM (MFI) RBD | Serum IgG (MFI) RBD | NT50 |
|---------|-----|-----|-----------|--------------------------|----------|-------------------------|--------|-----------|-------------------------|---------------------|-------------------|-------------------|-------------------|------|
| HCW1    | F   | 39  | Healthy   | None                     | Cough; rhinitis; sore throat | D0 (16/03) D1 (17/09) D7 (23/09) | 13 19 25 | Neg Neg Neg | D94 (18/06) D105 (29/06) D7 (23/09) D21 (07/10) | 90.5 102.0 47.4 51.6 | Neg Neg - - | Neg Neg - - | 12774 24170 21274 12774 | 200 |
| PAT1    | M   | 76  | Diabetic foot | None                     | Cough; low fever; malaise | D1 (13/09) | 14  | Neg  | D17 (29/09) | 2774 20522 | Neg Neg 811 799 | - - | - - | 1882 1309 26301 1309 | 100 |
| PAT2    | F   | 77  | Diabetic foot | Daughter 18/09 | Gastrointestinal issues; malaise | D1 (16/09) | 21  | Neg  | D-13 (02/09) D13 (28/09) | 986 18216 | Neg Neg 535 4043 | 535 1016 | - - | 75 |
| PAT3    | M   | 87  | Diabetic foot | None                     | None | D0 (16/09) D12 (28/09) | 29 29 | Neg Neg | 768 292 | - - | Neg Neg Neg | 2023 2325 | - - | 2325 2325 |
| HCW2    | F   | 27  | Healthy | Partner | ? | 17/09 | 30 30 | Neg n/d | n/d n/d | n/d n/d | n/d n/d | n/d n/d | n/d n/d |

HCW = health care worker; PAT = patient; F = female; M = male; n/a = not applicable; Avg Ct = average cycle threshold; NT50 = 50% neutralization antibody titer; Ig = immunoglobuline; RBD = receptor binding domain; MFI = median fluorescent intensity; COI = cutoff index, >1 = reactive; n/d = not done; Neg = negative; N = nucleoprotein; D = days post symptom onset / diagnosis
Table 2. Nucleotide and amino acid comparison of the nosocomial SARS-CoV-2 genomes to the reference genome Wuhan-Hu-1.

| Sequence name         | Clade | #Nt | ORF1a | ORF1b | S   | ORF3a | M   | N   | ORF10 |
|-----------------------|-------|-----|-------|-------|-----|-------|-----|-----|-------|
| HCW1/16-03-2020/C5182 | 19A   | 4   | C     | C     | G   | C     | C   | G   | C     |
|                       | V     | 2   | C     | C     | G   | C     | C   | G   | C     |
| PAT1/13-09-2020/C5184 | 20A   | 16  | T     | T     | G   | T     | T   | G   | T     |
|                       | G     | 10  | T     | T     | G   | T     | T   | G   | T     |
| PAT2/16-09-2020/C5185 | 20A   | 21  | T     | T     | C   | T     | T   | C   | T     |
|                       | G     | 12  | T     | T     | C   | T     | T   | C   | T     |
| PAT3/16-09-2020/C5186 | 20A   |     | T     | T     | G   | T     | T   | C   | T     |
|                       | G     |     | T     | T     | G   | T     | T   | C   | T     |
| HCW2/17-09-2020/C5188 | 20A   |     | T     | T     | C   | T     | T   | C   | T     |
|                       | GH    |     | T     | T     | C   | T     | T   | C   | T     |

Amino acid change:
- H113Y
- M3087I
- L3606F
- A176S
- P34L
- V757L
- K141R
- E1184D
- S98F
- S477N
- D614G
- Q38R
- G172R
- V20L
- G21V
- P199L
- M734I
- A376T
- S23F
PAT1
HCW1
VIS
PAT2
PAT3
DAU
IgA+
IgG+
IgM+
DAU
IgA+
IgG+
IgM+