Transmission dynamics of SARS-CoV-2 within-host diversity in two major hospital outbreaks in South Africa

James E. San,†,‡ Sinaye Ngcapu,‡,3 Aquillah M. Kanzi,1 Houriiyah Tegally,1 Vagner Fonseca,1,S Jennifer Giandhari,1 Eduan Wilkinson,1 Chase W. Nelson,4,5,** Werner Smidt,110 Anmol M. Kiran,6,7 Benjamin Chimukangara,1 Sureshnee Pillay,1 Lavanya Singh,1 Maryam Fish,1 Inbal Gazy,1 Darren P. Martin,8 Khulekani Khanyile,1 Richard Lessells1 and Tulio de Oliveira1,9,*

1KwaZulu-Natal Research Innovation and Sequencing Platform (KRISP), School of Laboratory Medicine & Medical Sciences, University of KwaZulu-Natal, Durban, South Africa, 2Centre for the AIDS Programme of Research in South Africa (CAPRISA), Durban, South Africa, 3Department of Medical Microbiology, University of KwaZulu-Natal, Durban, South Africa, 4Biodiversity Research Center, Academia Sinica, Taipei, Taiwan, 5Institute for Comparative Genomics, American Museum of Natural History, New York, NY, USA, 6Malawi-Liverpool-Wellcome Trust, Queen Elizabeth Central Hospital, College of Medicine, Blantyre, Malawi, 7Centre for Inflammation Research, Queens Research Institute, University of Edinburgh, Edinburgh, UK, 8Institute of Infectious Diseases and Molecular Medicine, Division of Computational Biology, Department of Integrative Biomedical Sciences, University of Cape Town, South Africa, 9Department of Global Health, University of Washington, Seattle, WA, USA and 10Africa Health Research Institute (AHRI), Durban, South Africa

*Corresponding author: E-mail: deoliveira@ukzn.ac.za and tuliodna@uw.edu
†San Emmanuel James and Sinaye Ngcapu contributed equally.
‡https://orcid.org/0000-0002-5736-664X
§https://orcid.org/0000-0001-5521-6448
**https://orcid.org/0000-0001-6287-1598

Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes acute, highly transmissible respiratory infection in humans and a wide range of animal species. Its rapid global spread has resulted in a major public health emergency, necessitating commensurately rapid research to improve control strategies. In particular, the ability to effectively retrace transmission chains in outbreaks remains a major challenge, partly due to our limited understanding of the virus’ underlying evolutionary dynamics within and between hosts. We used high-throughput sequencing whole-genome data coupled with
bottleneck analysis to retrace the pathways of viral transmission in two nosocomial outbreaks that were previously characterised by epidemiological and phylogenetic methods. Additionally, we assessed the mutational landscape, selection pressures, and diversity at the within-host level for both outbreaks. Our findings show evidence of within-host selection and transmission of variants between samples. Both bottleneck and diversity analyses highlight within-host and consensus-level variants shared by putative source-recipient pairs in both outbreaks, suggesting that certain within-host variants in these outbreaks may have been transmitted upon infection rather than arising de novo independently within multiple hosts. Overall, our findings demonstrate the utility of combining within-host diversity and bottleneck estimations for elucidating transmission events in SARS-CoV-2 outbreaks, provide insight into the maintenance of viral genetic diversity, provide a list of candidate targets of positive selection for further investigation, and demonstrate that within-host variants can be transferred between patients. Together these results will help in developing strategies to understand the nature of transmission events and curtail the spread of SARS-CoV-2.

Key words: SARS-CoV-2; transmission dynamics; bottleneck; within-host variants; selection; nonsynonymous; South Africa; NGS whole-genome sequencing.

1. Introduction

The emergence and spread of a novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in Wuhan, China, resulted in a public health emergency of international concern in just under two months (Zhu et al. 2020; WHO 2020). In South Africa, the first officially diagnosed case of SARS-CoV-2 was reported on 5 March 2020. Strict public health mitigation strategies and non-pharmaceutical interventions played a critical role in controlling the COVID-19 pandemic in South Africa, lowering the new cases reported each day to approximately 1,770 by 31 October 2020. Unfortunately, this brief lull was followed by the emergence of the 501Y.V2 variant and a resurgence in the pandemic (NICD 2020). Understanding the patterns of transmission and the selection pressures acting on viral populations leading up to that point in time could be critical to preventing a recurrence of the surge in infections and excess deaths such as that South Africa experienced between November 2020 and February 2021 (Tegally et al. 2020), which ultimately killed between 513 and 4027 (SAMRC 2020).

Phylogenetic inference can be used together with epidemiological investigations to elucidate transmission events and retrace transmission chains (He et al. 2020; Lauring 2020; Bajaj and Purohit 2020; Guo et al., 2020); however, phylogenetic reports based on consensus sequences (i.e. one sequence per case) only represent the dominant viral lineage in a host and thus provide limited resolution in transmission analyses (Mavian et al. 2020). Whole-genome analyses integrating within-host diversity have been proposed as a better alternative for capturing viral genetic diversity, including low-frequency variants in viral populations present within a given host (Sanjuan et al. 2004). Indeed, several studies have already revealed the existence of substantial genetic variation in within-host viral populations of SARS-CoV-2 (Wolfel et al. 2020; Shen et al. 2020; Lythgoe et al. 2020; Butler et al. 2020; Nelson et al. 2020a). For example, within-host analyses have revealed large numbers of within-host (intrahost) single nucleotide variants (iSNVs), including nonsynonymous (amino acid changing) iSNVs in SARS-CoV-2-positive nasopharyngeal and oropharyngeal swabs (Zhou et al. 2020; Siqueira et al. 2020) and bronchoalveolar lavage fluid samples (Shen et al. 2020). Importantly, Wang et al. (2020) have shown that different samples with matching consensus sequences can exhibit different iSNVs. Nevertheless, Shen et al. (2020) could not confirm the transmission of any iSNVs across two confirmed source-recipient pairs in the Wuhan area, suggesting either strong purifying selection or the stochastic occurrence and disappearance of within-host variants upon or following the transmission bottleneck (Shen et al. 2020). It therefore remains unclear whether iSNVs can be used to improve tracing of viral transmission and enhance our understanding of the evolutionary dynamics of SARS-CoV-2.

Although many (i)SNVs are unlikely to affect viral fitness, others can potentially result in viral genotypes with altered pathogenicity, improved host-specific adaptations (such as immune evasion phenotypes) or generally improved replication and/or transmission kinetics (Gojobori et al. 1990; Lucas et al. 2001). Examples of SNVs with such properties are those found in the recently emerged N501Y lineages, which putatively increases both the transmissibility of these viruses (Tegally et al. 2020; Rambaut et al. 2020; Faria et al. 2021; du Plessis et al. 2021) and their capacity to evade population-level immunity (Fontanet et al. 2021). The emergence of the 501Y lineages re-emphasizes the need to better understand how SARS-CoV-2 genomic diversity arises, the fitness costs and benefits of individual arising mutations, and the evolutionary pressures that ultimately drive some mutations to high frequencies in global populations.

Leveraging genomic and epidemiological data (Giandhari et al. 2020; Pillay et al. 2020) from two well-characterized nosocomial SARS-CoV-2 outbreaks with whole genome diversity analysis, we performed an in-depth analysis of multiple inferred SARS-CoV-2 transmission chains. Further, we analysed the frequency distribution of (i)SNVs within- and between-hosts to show how combining within-host diversity and bottleneck estimation can yield improved power to retrace transmission chains during SARS-CoV-2 outbreaks.

2. Methods

2.1 Two SARS-CoV-2 nosocomial outbreaks

This study analysed 109 SARS-CoV-2 positive cases from two nosocomial outbreaks in the Kwazulu-Natal province of South Africa. The first outbreak (CH1; thirty-five cases analysed) lasted four weeks, while the second outbreak (CH3; seventy-four cases analysed) lasted six weeks. Both outbreaks occurred at a time of relatively limited community transmission. Timelines for the two outbreaks are shown in Fig. 1.

2.2 Epidemiological investigation and identification of transmission chains

The investigation of transmission chains and clusters during the outbreaks was conducted by researchers at the Kwazulu
Natal Research and Innovation Sequencing Platform (KRISP) and the University of Kwazulu Natal. Investigation methods included medical record reviews, ward visits, and interviews with healthcare workers and hospital management. Detailed timelines of patient cases were constructed to generate hypotheses on the spread of infection among patients and healthcare workers within the two hospitals and inference of putative transmission pairs (KRISP 2020).

Figure 1. Phylogenetic analysis of the two outbreaks showing the clustering of sequences across hospital departments and associated Pangolin lineages to which the sequences belong. A) Phylogeny of samples from CH1 outbreak. B) Phylogeny of samples from the CH3 outbreak. Inset of each phylogeny is the TempEst plot showing showing the clocklike signal. Sample clustering was not consistent with the epidemiological settings in CH3.
2.3 Real time-polymerase chain reaction

All cases were confirmed using comparative real time (RT)-polymerase chain reaction (PCR). We used the TaqPath COVID-19 CE-IVD RT-PCR Kit (Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. The assays target genomic regions (ORF1ab, S protein and N protein) of the SARS-CoV-2 genome. RT-PCR was performed on a QuantStudio 7 RT-PCR instrument (Life Technologies, Carlsbad, CA).

2.4 Whole-genome sequencing and assembly

We performed cDNA synthesis from RNA using random primers followed by gene-specific multiplex PCR using the ARTIC protocol. Briefly, extracted RNA was converted to cDNA using the Superscript IV First Strand synthesis system (Life Technologies, Carlsbad, CA) and random hexamer primers. SARS-CoV-2 whole genome amplification by multiplex PCR was carried out using primers designed on Primal Scheme (http://primal.zibraproject.org/) to generate 400-bp amplicons with an overlap of seventy base pairs covering the thirty-base pair SARS-CoV-2 genome. PCR products were cleaned up using AmpureXP purification beads (Beckman Coulter, High Wycombe, UK) and quantified using the Qubit dsDNA High Sensitivity assay on the Qubit 4.0 instrument (Life Technologies, Carlsbad, CA).

The Illumina Nexterra Flex DNA Library Prep kit was used according to the manufacturer’s protocol to prepare uniquely indexed paired-end libraries of genomic DNA. Sequencing libraries were normalized to 4 nM, pooled and denatured with 0.2 N sodium acetate. Sample (12 pM) library was spiked with one per cent PhiX (PhiX Control v3 adapter-ligated library used as a control). Libraries were loaded onto a 500-cycle v2 MiSeq Reagent Kit and run on the Illumina MiSeq instrument (Illumina, San Diego, CA). Raw reads coming from Illumina sequencing were assembled using Genome Detective 1.126 (https://www.genomedetective.com/) and the Coronavirus Typing Tool (Vilsker et al. 2019; Cleemput et al. 2020a). The initial assembly obtained from Genome Detective was polished by aligning mapped reads to the references and filtering out low-quality variants using the bcftools 1.7-2 mpileup method. All variants were confirmed visually with bam files using Geneious (Biomatters Ltd, New Zealand). Indels resulting in mid-gene aligning mapped reads to the references and filtering out low-quality assembly obtained from Genome Detective was polished by SD: 0.5/C2 approach implemented in BEAST v 1.1.10 (Suchard et al. 2018). Runs were executed under a strict molecular clock assumption with a strong mutation rate prior \( (8 \times 10^{-3} \text{ substitution/site/year; SD: } 0.5 \times 10^{-5}) \) with a chain length of 100 million steps in the chain (sampling every 10,000 steps). Markov chain Monte Carlo runs were assessed in Tracer v 1.6.0 (Rambaut et al. 2018) for good convergence and proper mixing, that is for effective sample size values \( > 200 \) for each estimated parameter. The estimated root of each cluster was recorded and Maximum Clade Credibility (MCC) trees were constructed in TreeAnnotator, discarding the first ten per cent of sampled trees as burn-in (i.e. each MCC tree represented a sample of 9,000 similarly likely trees). Resulting trees were visualized using the R package, ggtree (Yu 2020).

2.6 Within-host variants identification

We used LoFreq v.2.1.5 (Wilm et al. 2012) to call iSNVs (intra-host SNVs), including low-frequency variants. Initial variants were called relative to the Wuhan-Hu-1 NC_045512.2 reference at sites with a minimum sequencing depth of \( \times 100 \) and employing a false discovery rate (FDR) cutoff of one per cent, as proposed by Costello et al. (2018) and Pightling et al. (2019) after thorough empirical evaluation of Illumina-based library preparation and sequencing errors. LoFreq automatically eliminates all variants that have a P-value below the FDR threshold and have \( \geq 85 \) per cent of reads mapping to just one strand to avoid strand bias. Additional filtering to further eliminate strand biases was performed using customized scripts to retain only minor alleles that were present at a frequency \( \geq 5 \) per cent, supported by at least two per cent of the total reads, and supported by a minimum of five reads on each strand, following Shen et al. (2020). Positions with more than one minor allele were filtered out to minimize false discovery, that is only biallelic sites were considered. Only variants in protein-coding regions were analyzed. Variants passing these criteria were then annotated using snpeff v. 4.5 (Cingolani et al. 2012b) and SnpSift v.4.3t (Cingolani et al. 2012a).

2.7 Mutational spectra

To characterize the mutational spectra, we considered only variants for which the reference allele matched the reference (Wuhan-Hu-1). For SNVs, the alternative allele was required to be the dominant allele (i.e. frequency \( > 50\% \)) and for iSNVs (within-host variants), only alleles with alternative allele frequency less than fifty per cent were considered. Reference minor alleles (i.e. alleles where the reference base was the minor allele) were not considered in this study.

2.8 Technical controls

To evaluate the efficiency and consistency of our sequencing process and variant identification protocols, we thoroughly assessed two representative biological replicates. The first replicate, CC0068518T6, was sequenced twice in separate runs to assess potential biases introduced by the sequencing step. The second replicate, EGO0465499T6, was taken from a new aliquot and sequenced to assess the possibility of contamination. Both replicates yielded similar results that were highly concordant in terms of variants identified and their associated frequencies (Fig. 2C, Supplementary Table S5). Supplementary Fig. S6 shows the correlation after positions that were not reproducible or occurred at an allele frequency less than five per cent in the second replicate had been filtered out. Furthermore, we sequenced two samples that had previously tested negative for SARS-CoV-2 and, for these, we did not obtain any viral reads on assembly, confirming the suitability of our DNA preprocessing, extraction, sequencing and analysis pipeline for the study.
Figure 2. Overview of general diversity of SARS-CoV-2 genomes from South African patients. (A) Nucleotide changes in SARS-CoV-2 genomes. (B) Distribution of variant frequencies across nucleotide changes. (C) Regression plot showing the correlation between frequencies of mutations in the two replicates. Outliers colored in red show variants that only occurred in a single replicate or at very low frequencies (<5%) in the second replicate and as such were filtered out. (D) The upset plot shows the distribution of iSNVs and SNVs across the outbreaks. The vertical bar chart shows the size of the intersection and the black dots and lines show the combination of iSNVs and SNVs. The horizontal bars show the unconditional frequency count of variants within each group. (E) Sequence variability detected in SARS-CoV-2 overlaid with major protein coding regions in the genome. Variants that only occurred as SNVs in more than ten samples are labelled in black while those that also occurred as iSNVs and in more than ten samples as SNVs are marked in red.
2.9 Nucleotide diversity and selection inference

To quantify within-host genetic diversity and infer selection pressures acting on SARS-CoV-2 protein-coding genes, we estimated nonsynonymous ($n_s$; amino acid changing) and synonymous ($n_\text{s}$; not amino acid changing) nucleotide diversity using the software SNPGenie (Nelson et al. 2015; https://github.com/chasewelson/SPNGenie), which implements the method of Nei and Gojobori (1986). The null hypothesis of neutrality ($n_s = n_\text{s}$) was evaluated with Z-tests using a bootstrap method (codon unit, 10,000 replicates for genes, 1,000 replicates for sliding windows), where $n_s > n_\text{s}$ is consistent with positive selection favoring nonsynonymous variants and $n_n < n_\text{s}$ with purifying (negative) selection favouring the elimination of nonsynonymous variants. Sites overlapping more than one protein-coding gene, including the entirety of ORF9b and ORF9c (both located in the +1 reading frame of $N$), were excluded from the analysis. Sliding windows of thirty codons were chosen based on the suggestion of (Harrison et al. 2014) and because this did not exceed the length of ORF10 (thirty-nine codons).

2.10 Transmission analyses

We hypothesized that direct or closely linked transmission pairs are likely to share a significant number of iSNVs. To determine the iSNVs shared between putatively related cases and the possibility of these iSNVs having been transmitted, we compared variant calling results of candidate source-recipient pairs of samples. For the CH1 outbreak, we leveraged the putative transmission source-recipient pairs that had previously been inferred by epidemiological investigation and supported by phylogenetic analysis.

Unlike CH1, no transmission pairs had been previously identified for the CH3 outbreak. To infer candidate pairs, we thus treated each sample both as a potential source and recipient for all possible pairs. We permuted the samples to generate source-recipient pairs as below;

$$P(n, 2) = n!/(n-2)!$$

where $n$ is the number of samples. This resulted in 5,402 pairs for seventy-four samples, that is each pair was considered twice, once with each member treated as the source or recipient. Pairs with negative sample date differences were eliminated, as that is a possible source was required to predate a possible recipient, while both pairs were retained if $n_\text{s} > n_\text{s}$ is consistent with positive selection favoring nonsynonymous variants and $n_n < n_\text{s}$ with purifying (negative) selection favouring the elimination of nonsynonymous variants. Sites overlapping more than one protein-coding gene, including the entirety of ORF9b and ORF9c (both located in the +1 reading frame of $N$), were excluded from the analysis. Sliding windows of thirty codons were chosen based on the suggestion of (Harrison et al. 2014) and because this did not exceed the length of ORF10 (thirty-nine codons).

3. Results

This study focused on the analysis of 109 SARS-CoV-2 cases from two different nosocomial outbreaks. Clinical characteristics of infected individuals are reported in Supplementary Table S1. The 109 cases were further categorized by outbreak as CH1 (35/109, 32%) and CH3 (74/109, 68%). Of the thirty-five samples collected from CH1 that were available for our analysis, 16 (45.7%) had putative transmission linkages that were supported by phylogenetic inference (Fig. 1A, Table 3). Samples from the beginning of the CH3 outbreak (24/74, 32.4%) were grouped by hospital department and reported social networks. Time-scaled Bayesian phylogenies were inferred for samples that yielded high quality genomes (coverage >90, $n = 21/24$). Phylogenetic analyses suggested multiple introductions of the virus, that is patients in the same epidemiological group such as the recovery room had different viral profiles (Fig. 1B), and therefore no putative transmission pairs were confirmed.

3.1 Allele frequencies and the mutational landscapes of SARS-CoV-2 genes

All 109 whole-genome sequences analyzed yielded near full-length genomes with coverage greater than ninety per cent and the average read depth ranging from 158.71 to 5046.56 (Supplementary Table S2). Sequencing depth was not detectably associated with the number of iSNVs recorded ($R = -0.13$, $P = 0.18$) (Supplementary Fig. S2). In total, 1,841 (1,232 unique) iSNVs were identified across coding regions of the 109 CH1 and CH3 samples at minor allele frequencies (MAFs) between five per cent and fifty per cent (Fig. 2A and B). Higher numbers of individual types of the nucleotide substitutions were observed in CH1 than CH3 samples, with the exception of C→A (45 vs 56) and C→U (147 vs 160). We also observed 820 SNVs, with different mutational patterns to iSNVs. These include C→U ($n = 284$), A→G ($n = 130$), G→U ($n = 63$), and G→A ($n = 62$) as the most prevalent. Overall, the frequency of the SNVs was higher in CH3 samples than in CH1 samples.

In terms of location in the SARS-CoV-2 genome, a large proportion of iSNVs (0.84) and SNVs (0.79) were found within the S and ORF1ab genes, with high concentrations specifically in the nsp3 protein of the ORF1ab gene (Supplementary Fig. S3F, Table S2). However, to objectively compare the accumulation of variants in different genes/ORFs, we normalized the variant counts to gene lengths. This revealed higher mutation loads in the N and S genes as well as ORF3a (Table 1). The SNVs identified were spread across 1,337 (4.5%) positions of the genomes of the 109 samples. Three positions (14,707, 1,637, 20,465) demonstrated fixation of the alternative allele in more than ten per cent of the samples in CH1 compared to seven positions (7,064, 16,376, 24,034, 28,628, and 28,881/2/3; Fig. 2E) in CH3.

The mutations could be further divided into 1,814 nonsynonymous, followed by 759 synonymous, and 88 nonsense (stop lost/gained) mutation categories (Table 1, Supplementary Table...
Table 1. Summary of iSNVs present at frequencies between 5% and 50% in the 109 SARS-CoV-2 genomes classified according to import on the genes and ORFs in which they occur.

| Gene     | Length | High (nonsense) | Moderate (non-synonymous) | Low (synonymous) | Total, N (v/kbgl) |
|----------|--------|-----------------|---------------------------|-----------------|-------------------|
| ORF1ab   | 21,393 | 41              | 1234                      | 466             | 1,741 (81.38)     |
| S        | 3,822  | 32              | 287                       | 141             | 460 (120.36)      |
| ORF3a    | 828    | 3               | 62                        | 32              | 97 (117.15)       |
| E        | 228    | 2               | 15                        | 5               | 22 (96.49)        |
| M        | 669    | 3               | 42                        | 13              | 58 (86.7)         |
| ORF6     | 186    | 0               | 2                         | 14              | 16 (86.62)        |
| ORF7a    | 366    | 0               | 11                        | 16              | 27 (73.77)        |
| ORF7b    | 132    | 1               | 1                         | 0               | 2 (15.15)         |
| ORF8     | 366    | 1               | 16                        | 9               | 26 (71.04)        |
| N        | 1260   | 5               | 139                       | 60              | 204 (161.9)       |
| ORF10    | 117    | 0               | 5                         | 3               | 8 (68.38)         |
| Total, N | 88     | 1,814           |                           |                 | 759               |

In the last column, total mutation counts are normalized to number of mutations per kilobase for easy comparison. Majority of the iSNVs detected were nonsynonymous.

Table 2. Common consensus mutations shared between putative source-recipient pairs in the CH1 outbreak.

| Source     | Recipient                                      |
|------------|------------------------------------------------|
| P3 (C241T, C3037T, C14408T, A23403G) | P7 (C241T, C3037T, C14408T, A23403G) |
|           | P10 (C241T, C3037T, C14408T, A23403G)         |
|           | HW4 (C241T, C3037T, C14408T, A23403G)         |
|           | P22 (C241T, C3037T, C14408T, C16376T, A23403G) |
|           | P5 (C241T, C3037T, C14408T, C16376T, A23403G)  |
|           | P20 (C241T, C3037T, C14408T, C16376T, A23403G) |
|           | P27 (C241T, C3037T, C14408T, C16376T, A23403G) |
|           | P29 (C241T, C3037T, C14408T, C16376T, A23403G) |
|           | P11 (C241T, C3037T, C14408T, A23403G)         |
|           | P15 (C241T, C3037T, C14408T, A23403G)         |
|           | P23 (C241T, C3037T, C14408T, C16376T, A23403G) |
|           | X1 (C241T, C2997T, C3037T, C14408T, A23403G)  |
|           | P26 (C241T, C3037T, C14408T, C16376T, A16561C, A23403G) |

Mutations in bold were present in the recipient but not in the source. SNP distances between the genomes were confirmed using snp-dists package. Mutations were called relative to the Wuhan-Hu-1 reference (NC044512.2).

S3). Of the observed iSNVs, the majority of nonsynonymous (757 vs 524), synonymous (282 vs 196) and nonsense (54 vs 28) were found in the CH1 samples compared to CH3 samples. In contrast, a larger fraction of nonsynonymous (380 vs 153) and synonymous (236 vs 45) SNVs were found in CH3 than in CH1 samples, unlike nonsense SNVs (4 vs 2).

The iSNVs were distributed in eleven protein-coding viral genes with variable frequencies. Individual iSNVs that were found in multiple different patients samples were most commonly found in genes encoding non-structural proteins, that is, nsp8 (A12240G in 24/109 samples), nsp14 (C18181T in 22/109 samples), nsp6 (A11556T in 20/109 samples), nsp9 (A13003G in 20/109 samples), nsp13 (A17929C and T17928G in 17/109 samples), S (T25312A in 17/109 samples), nsp2 (T1483C in 15/109 samples) and nsp15 (T20135A in 15/109 samples).

We also observed high-frequency SNVs A23403G (109/109 in S gene), C14408T (108/109 in nsp12), C3037T (95/109 in nsp3) in the analyzed samples (Fig. 2E). Other genes (E and M) and proteins (nsp5, nsp7, nsp9, nsp10, nsp11, ORF6, ORF7a, ORF7b, ORF8, and ORF10) were well conserved, with iSNVs and SNVs frequencies consistently less than ten per cent (Supplementary Fig. S3A and F).

3.2 Transmission of consensus mutations between source-recipient pairs in the CH1 outbreak

Here, we used consensus mutations (SNVs) to explore the transmission dynamics of SARS-CoV-2 within and across samples of sixteen in-hospital patients (P) and healthcare workers (HW). In our report into a nosocomial outbreak of SARS-CoV-2 infections at one of the private hospitals in Durban, South Africa, phylogenetic inferences showed that inpatient-3 (P3, source) infected by the index patient sustained the chains of transmission generating secondary clusters of recipients including HW4 and P7. Table 2 shows common consensus mutations (C241T, C3037T, C14408T, and A23403G) found in the viral consensus sequences of (Cluster1) and its putative recipients (P5, P7, P20, P27, and P29). In addition, P5, P20, P27, and P29 also developed an additional mutation C16376T. Similar mutations were found in secondary clusters 2 (HW4) and 3 (P7), with additional mutations found in consensus sequences of patient P26 (A16561C) and X1 (C2997T) in cluster 3. Development of the additional mutation could be attributed to multiple transmitted strains or selection pressure within the different hosts.

3.3 Transmission dynamics of shared within-host variants between samples

We investigated whether minor alleles with frequencies between five per cent and fifty per cent observed were shared between the epidemiologically inferred source and recipient pairs, and whether these could have been indicative of specific transmission events within the CH1 and CH3 hospital outbreaks.
From the sixteen CH1 samples analyzed, we observed 720 iSNVs. The CH1 iSNVs consisted of 412 nonsynonymous, 154 synonymous, and 29 nonsense variants (Supplementary Table S3). We assessed the evidence for the transmission of shared iSNVs between samples. We found that HW4 (source) was most likely to have transmitted eight iSNVs to the recipient P11 (Fig. 3F, Table 3). In addition, P7 potentially passed on nine iSNVs to P23 (Fig. 3B) and fourteen to X1. Of the fourteen, one (A20465G) later became established as a SNV (Fig. 3L). P3 shared seven iSNVs with P5 (Fig. 3A) and six with P7 (Fig. 3B). P3 was likely to have transmitted four iSNVs to P10, one of which later established as an SNV (Fig. 3G). These findings are consistent with the nine iSNVs were shared by two or more samples (Supplementary Table S4). Based on these findings, we see potentially linked transmission pairs to be considered. HW8 and HW9 shared seven iSNVs, we observed a number of positions including 6,763, 16,376, 22,675, 24,034, 26,530, and 28,881/2/3 that exhibited strong signals for shared variants (Fig. 5B). Of the 2,824 potential source-recipient pairs given seventy four patients, most (2,474/2,824) did not share any iSNVs, 154 pairs shared a single iSNV while 63 had 2 and 133 pairs shared 3 or more iSNVs (Fig. 5A). In terms of nucleotide positions where iSNVs were shared, we observed a number of positions including 6,763, 16,376, 22,675, 24,034, 26,530, and 28,881/2/3 that exhibited strong signals for shared variants (Fig. 5B).
The two were partners and shared up to fifteen iSNVs. Since the samples had been collected on the same day, we explored the bottleneck estimates in both directions. When HW19 was taken as the source, a bottleneck estimate of 85 was estimated and when HW20 was taken as the source, the estimate increased to 91, suggesting that HW19 was a more likely source of infection to HW20. HW25 also shared fifteen and twelve iSNVs with HW19 and HW20, respectively, suggesting possible transmission of the virus from either HW19 or HW20; however, upon comparing the SNP distance between the pairs, HW19 and HW25 were separated by an SNP distance of 3 while HW20 and HW25 were separated by an SNP distance of 2, as such, HW20 was considered the likely source of infection to HW25. Overall, these findings suggest that certain within-host variants in this study could have been transmitted to recipients; however, most within-host variants appear to have arisen independently after transmission.

### 3.4 Evaluation of the potential impact of false-positives

The presence of shared variants not related by transmission can bias conclusions from the results of transmission analyses and inflate the bottleneck estimates (Martin and Koelle 2021). We therefore explored the impact of masking suspicious variants by masking sixteen mutations that occurred in greater than ten samples (Supplementary Tables S8). First, we re-evaluated these variants against the criteria defined by Lythgoe et al. (2021) (i.e. consistent low-frequency, strand bias, or low reproducibility) and then following Sapoval et al. (2021), we confirmed that they did not occur near either end of the genome. For CH1, we observed an increase in some of the bottleneck estimates ($t$-test; $t = 1.6952$, $P > 0.9$), and sample pairs with iSNVs comprised mostly highly abundant variants were no longer supported. All shared variants between P7_P23 were lost. There was a drop in count of shared iSNVs between sample pairs HW4_P11 from 8 to 1, P3_10 dropped from 4 to 1 (Supplementary Tables S9). CH3, on the other hand, was barely affected as the number of shared variants and bottleneck estimates remained consistent ($t$-test; $t = 6.8493$, $P < 0.001$) (Supplementary Tables S10). The number of shared variants between HW19 and HW20 reduced from 15 to 13, and the bottleneck estimate from 85 to 77. Together these results highlight the potential of false-positive iSNVs to inflate bottleneck estimates.
3.5 Variant prioritisation and pervasion across outbreaks

Most of the variants expressed in viral sequences usually do not confer any advantage to the virus and therefore are lost; however, a small set of likely advantageous mutations can be positively selected for and fixed as the dominant variant in the population by selection pressure. Using the upset plot (Fig. 2D), we further captured the intersections between iSNVs and SNVs in the CH1 and CH3 datasets to identify frequently and universally occurring iSNVs that could be of potential significance.

Three convergent SNVs occurred in CH1 and CH3 independently. We also found eighteen iSNVs identified in the CH1 outbreak that occurred as SNVs in at least one CH3 sample and forty-four iSNVs in the CH3 outbreak that occurred as SNVs in at least one CH1 sample. Furthermore, seven iSNVs that were present in both outbreaks were also present as SNVs in CH3 while two that were present in both outbreaks occurred as SNVs in CH1. Figure 2E shows the dominant variants across outbreaks. Shared variants prevalent in both outbreaks could also be explained by potential spatio-temporal overlap of the outbreaks i.e. the time when they occurred (Fig. 1) and distance between the two hospitals (approx. 4KM apart).

3.6 Selection pressure in the SARS-CoV-2 genome

To assess within-host (intrahost) viral genetic diversity for all samples, we estimated nucleotide diversity ($\pi$) as the mean number of single nucleotide differences per site for all protein-coding regions (Nei and Li 1979). Mean $\pi$ was 2.88 $\times 10^{-4}$, ranging from 0 to 5.97 $\times 10^{-4}$ across samples and was significantly higher in CH1 (5.08 $\times 10^{-4}$) than in CH3 (1.84 $\times 10^{-4}$; $P = 2.21 \times 10^{-5}$; Mann–Whitney U test) (Supplementary Table S6). Diversity was also higher in CH1 than in CH3 samples at both non-synonymous ($\pi_{N} = 2.46 \times 10^{-4}$ vs 0.84 $\times 10^{-4}$; $P = 4.42 \times 10^{-6}$) and synonymous ($\pi_{S} = 2.62 \times 10^{-4}$ vs 0.99 $\times 10^{-4}$; $P = 2.79 \times 10^{-5}$) sites (Mann–Whitney U tests). This was true even when limiting to samples sequenced by the same laboratory (NHLS-IALCH, $P < 0.00268$; Mann–Whitney U test), suggesting this result is not a methodological artefact.

To infer selection pressures acting at the within-host level, we next compared $\pi_{N}$ to $\pi_{S}$, with $\pi_{N} > \pi_{S}$ ($\pi_{N}/\pi_{S} > 1$) being consistent with positive selection favoring amino acid changes, and $\pi_{N} < \pi_{S}$ ($\pi_{N}/\pi_{S} < 1$) with purifying selection eliminating amino acid changes. Despite the differences in overall diversity between the two outbreaks, their $\pi_{N}/\pi_{S}$ ratios were similar, with $\pi_{N}/\pi_{S} = 0.94$ for CH1 and 0.85 for CH3, both statistically indistinguishable from neutrality ($P > 0.345$; Wilcoxon signed rank tests) (Supplementary Table S6). This result is consistent with the documented preponderance among human viruses of purifying selection acting on viral genomes at the host population scale but the relaxation of selection within hosts (Holmes 2009).

Because disparate selection pressures are expected to act on different sites in a genome, we next computed $\pi_{N}$ and $\pi_{S}$ for individual genes and sliding windows within each gene to identify candidate targets of within-host positive selection. However,
To examine evidence for selection at the within-gene level, we analyzed sliding windows of thirty codons across each protein-coding gene to identify candidate targets of positive selection. Because of the limited number of variants, we took a conservative approach by combining all samples from both outbreaks, allowing us to identify sites undergoing consistent selection pressures in both outbreaks. This analysis yielded several windows for which \( n_S \) exceeded both whole gene \( n_S \) and the window’s \( n_S \) (i.e. \( n_S-SE(n_S) > n_S + SE(n_S) \)), including regions in \( nsp2, nsp3, nsp4, nsp6, nsp8, nsp13, nsp14, nsp15, nsp16, E, M, ORF3a, \) and \( ORF7b \) (Supplementary Fig. S3). The longest region was codons 21–190 of \( nsp8 \) (length 170 codons), which also had the highest \( n_S/n_S = 36.1 \) (Table 4). Specific codons exhibiting non-synonymous diversity within these regions are listed in Table 4 and serve as a list of candidates for further study.

4. Discussion

In the present study, we assessed the utility of studying within-host diversity to elucidate selection pressures within, and transmission events between, hosts. We confirmed that our method can improve the power of efforts to retrace transmission events during outbreaks. Specifically, the combination of within-host diversity and bottleneck estimation, SNP distance, and time series improved the resolution of transmission events between hosts in both outbreaks.

Of the fifteen putative source-recipient pairs from CH1, twelve shared more than three iSNVs, suggesting that transmission of iSNVs is indeed common with SARS-CoV-2. This was further supported by bottleneck analysis, which indicated transmission involving at least four virions. The three pairs CH1 putative source-recipient pairs sharing no iSNVs, may have either been incorrectly designated as such during that outbreak investigation (i.e. false categorization), or transmission events between these pairs may have involved only a single genetic variant (i.e. transmission monophyly (Leitner 2019)). However, given the fact that certain variants are shared by other recipients from the same source, we considered these to be incorrectly designated pairs.

Furthermore, using shared iSNVs and bottleneck estimates between CH3 source–recipient pairs, we brought additional support to the epidemiologically inferred transmission patterns that were originally not clear from phylogenetic analysis, including transmission events between HW7 and HWs 10 and 11. Samples were taken from these three HWs within a ten-day period and all were infected with a predominating genetic variant that was genetically identical, but had minor frequency variants carrying three or more iSNVs. Although HW8 and HW9 shared iSNVs with HW7, they also had an SNP distance of 3 indicating either a higher evolutionary rate of the virus within the new host or the infection of the host with genetically distinct viruses from a different source. We also show that HW20 most likely infected both HW19 and HW25, evidenced by shared iSNVs and estimates of >12 transmitted variants. Interestingly, HW19 and HW24 who were sampled three days apart had an SNP distance of 2 indicating that both HWs worked in the same recovery room, suggestive of unrelated transmission events and emphasizing that even healthcare workers are most often infected in the community rather than by patients (Braun et al. 2021).

The narrow transmission bottlenecks observed in these outbreaks may be attributed to a small number of variants that crossed the host cell barrier and established infection, or to deleterious stochastic dynamics via elimination within the...
respiratory tract (Wang et al. 2020). Low numbers of variants transmitted could also be attributed to the adaptive dynamics theory of evolution, which assumes very limited genetic variation in pathogen populations and that a single pathogen strain will reach equilibrium before a new strain arises by mutation (Berngruber et al. 2013). Ultimately, our bottleneck estimates are consistent with results from other studies (Ghafari et al. 2020; Martin and Koelle 2021; Lythgoe et al. 2021) showing that shared variants related by transmission are characterized by low bottleneck estimates. We also observed some large bottleneck estimates in sample pairs that did not share any iSNVs and in sample pairs where the iSNV frequency was nearly equal between the source and recipient, especially noticeable in samples sharing less than two variants. Similar results have been reported by Popa et al. (2020), which upon further evaluation by Martin and Koelle (2021), appeared to be more likely the results of variants not linked by transmission.

In this study, we set a threshold of at least three shared minor alleles required to support a putative transmission event. This, however, will only hold when the transmission bottleneck is high and not in the event that the bottleneck is low or only the dominant strain is transmitted. This challenge is further exacerbated by the stringent variant calling requirements to eliminate false-positive variants while retaining the true variants. As seen in this and other studies (Tonkin-Hill et al. 2020; Sapoval et al. 2021; Lythgoe et al. 2021; Martin and Koelle 2021), after application of quality control measures, most transmission pairs share only one to three minor alleles with many others sharing none. An alternative approach, stemming from the understanding that variants not linked by transmission, will likely inflate bottleneck sizes would be that a single variant passing all quality control criteria, shared at a low bottleneck estimate (e.g. 1–3) in the absence of fixed de novo variants in the recipient (Martin and Koelle 2021), together with strong epidemiological evidence for transmission could be considered linked by transmission. The main limitation to this approach is that the probability of a single variant being spurious is relatively high hence our choice of at least three. This, however, highlights the need for further research on robust techniques for inference of transmission events from shared minor variants under low transmission bottlenecks.

MAF thresholds are an important driver of bottleneck estimates. While MAFs less than two per cent cannot be separated from noise and should be eliminated altogether, higher thresholds result in the loss of true variants. For example, at an allele frequency of two per cent, Popa et al. (2020) reported bottleneck estimates of >50 virions for each transmission pair. Raising the cutoff to three per cent significantly reduced the bottleneck estimates. A further re-analysis of the same data by Martin and Koelle (2021) with the MAF threshold raised to six per cent resulted in a drastic drop in the bottleneck estimates to under 3. Indeed, raising the frequency cutoff is a quick and efficient
Table 4. Candidate regions of positive selection within hosts.

| Gene product | Codonsb | Length (codons) | \(n_S (10^6)^c\) | \(n_S (10^6)^c\) | \(n_S/n_S\) (p-value)\[^d\] | Codons with nonsynonymous differences[^e,\[^f\] | \[^g\] |
|--------------|---------|----------------|----------------|----------------|-----------------|---------------------------------|-------|
| nsp2         | 331–369 | 39             | 2.98 (±1.14)   | 1.21 (±0.69)   | 2.46 (0.176)    | 332, 336, 338, 340+, 345, 355, 359, 360, 362, 365 |
| nsp3         | 103–155 | 53             | 1.60 (±0.75)   | 0.07 (±0.08)   | 22.92 (0.033)*  | 112±, 113, 126+, 132, 142, 143, 153 |
| nsp3         | 220–255 | 36             | 1.09 (±0.37)   | 0 (-)          | –               | 224, 230, 231+, 233, 236, 247, 249 |
| nsp3         | 419–457 | 39             | 1.68 (±0.60)   | 0 (-)          | –               | 422, 424+, 441, 442, 445, 448, 449, 457 |
| nsp3         | 511–540 | 30             | 2.60 (±1.19)   | 0.61 (±0.60)   | 4.27 (0.072)    | 511, 517, 520, 523, 528 |
| nsp3         | 962–1,007 | 46         | 2.55 (±1.65)   | 0.39 (±0.38)   | 6.56 (0.206)    | 966, 980, 981, 985+, 991 |
| nsp3         | 1,156–1,274 | 119     | 3.31 (±1.24)   | 0 (-)          | –               | 1,175, 1177, 1186, 1198, 1200, 1202, 1203, 1205, 1216, 1226, 1245, 1246, 1247 |
| nsp3         | 1,433–1,493 | 61         | 1.87 (±0.71)   | 0 (-)          | –               | 1,437, 1,449, 1,451, 1,462, 1,464, 1,475, 1,481, 1,482 |
| nsp3         | 1,589–1,644 | 56         | 1.17 (±0.41)   | 0.23 (±0.23)   | 5.03 (0.049)*   | 1,595, 1,597, 1,599, 1,615, 1,617, 1,618, 1,620, 1,641 |
| nsp3         | 1,733–1,765 | 33         | 1.98 (±1.14)   | 0.43 (±0.44)   | 4.58 (0.223)    | 1,738, 1,748, 1,760, 1,761 |
| nsp3         | 1,774–1,824 | 51         | 2.05 (±1.06)   | 0.47 (±0.46)   | 4.40 (0.186)    | 1,789, 1,795+, 1,796, 1,803, 1,804, 1,807 |
| nsp4         | 140–173 | 34             | 3.76 (±1.48)   | 0.61 (±0.61)   | 6.16 (0.059)    | 1,40, 1,444, 1,451, 1,521, 1,522, 1,61, 1,62, 1,70 |
| nsp6         | 65–127 | 63             | 5.88 (±2.66)   | 0.88 (±0.86)   | 6.71 (0.077)    | 74, 76, 83, 84, 86, 90, 91, 94, 98, 104, 106+, 112, 119 |
| nsp6         | 169–206 | 38             | 7.83 (±6.23)   | 0.86 (±0.84)   | 9.08 (0.266)    | 189, 190, 195, 197 |
| nsp8         | 21–190 | 170             | 3.51 (±2.03)   | 0.10 (±0.10)   | 36.12 (0.097)   | 50, 57, 59, 60, 85, 91, 92, 106, 107, 110, 112, 119, 129, 138, 141, 145, 159, 163, 174 |
| nsp13        | 565–594 | 30             | 17.55 (±11.66) | 0.83 (±0.87)   | 21.09 (0.158)   | 565, 566, 586, 588 |
| nsp14        | 248–289 | 42             | 7.09 (±4.54)   | 0.52 (±0.47)   | 13.71 (0.151)   | 255+, 267, 269, 272, 274, 276, 278, 286, 289 |
| nsp15        | 83–120 | 38             | 0.82 (±0.41)   | 0.08 (±0.08)   | 10.46 (0.044)*  | 92, 97, 107, 112+ |
| nsp16        | 236–337 | 102            | 5.10 (±3.35)   | 0 (-)          | –               | 250, 256, 267, 270+, 282, 287, 321, 324, 327, 336, 337 |
| ORF3a^[f]   | 97–136 | 40             | 5.99 (±3.11)   | 1.32 (±1.30)   | 4.55 (0.080)    | 100, 103, 117, 118, 121, 123, 125, 126, 128, 130 |
| E            | 44–76 | 33             | 4.51 (±2.00)   | 1.17 (±0.81)   | 3.86 (0.130)    | 50, 52, 58, 60, 68, 71, 72 |
| M            | 135–201 | 67             | 1.96 (±0.60)   | 0 (-)          | –               | 154, 158, 160, 161, 163, 164, 167, 187, 189, 193, 196, 198 |
| ORF7b       | 1–38   | 38             | 0.28 (±0.28)   | 0 (-)          | –               | 9 |

[^a]: Genes are ordered 5’ to 3’ by start site in the genome.
[^b]: Codons are numbered with respect to mature gene products, that is each nonstructural protein (nsp) is re-numbered starting at 1.
[^c]: Undefined values are indicated with a horizontal line (–).
[^d]: Codons with nonsynonymous differences[^e,\[^f\] | \[^g\] |

way to eliminate false-positive minor alleles; however, it also results in the loss of several potentially true minor alleles and also increases statistical uncertainty (Martin and Koelle, 2021). Instead the use of more effective filtration techniques such as position of the allele on the read, strand bias, number of reads supporting the allele may offer a more balanced criteria for identification and elimination of false positives while retaining true variants, and is encouraged.

Cautious application of masking can help eliminate false positive and strengthen the evidence for transmission events.
Low frequency variants can arise de novo within the host rather than through transmission and when selected for can be prevalent across multiple hosts resulting in false signals for transmission. Low-frequency variants can also arise at sites vulnerable to in vitro generation of variants (Lythgoe et al. 2021). In this study, we evaluated the impact of eliminating suspicious variants. Indeed, some sample pairs that were related by only highly abundant low-frequency alleles showed no relation by minor alleles in the masked dataset (Supplementary Table S9). The pairs affected were from the first outbreak. Our rationale for retaining these minor alleles was based on the fact that this outbreak was homogeneous (i.e. occurred among inpatients in a span of two weeks before it was controlled), implying that the infections were highly related and therefore it is not surprising that these variants are common to these patients either through direct or indirect transmission. Furthermore, the close proximity and similar time frame of the second outbreak suggest that some cases between the two outbreaks could be related. Overall, we show that the careful application of masking can help reduce bias in transmission analyses by eliminating false positives.

In order to understand the impact of selection pressures on the patterns of variation represented in the transmission events, we assessed both mutational patterns and the frequency and diversity of within-host variants and between-host variants. We found an excess of A→G, C→U, U→C, U→A and G→A nucleotide changes in both iSNVs and SNVs (Fig 2A), and, as expected based on its size, ORF1ab harboured most of the nonsynonymous and synonymous variants compared to other genes (Table 1). It also had the largest fraction of iSNV and SNV mutational patterns with higher numbers in the nsp3 and nsp12 encoding regions, followed by the S gene (Supplementary Figure S3F). However, adjusting for gene length, the highest concentration of variants occurred in the N, S and ORF3a (Table 1). In the S gene A→G, C→U and U→A mutations predominated, in nsp3 by C→U, A→G, and G→A mutations predominated while in nsp12 and nsp13 C→U mutations predominated, both for iSNVs and SNVs. These findings are consistent with previous studies that found an enrichment of C→U mutations in ORF1a (Di Giorgio et al. 2020; Saproval et al. 2020). It has been suggested that the C→U mutation enrichment in the SARS-CoV-2 genome is likely driven by host response to counter the virus through the APOBEC and ADAR deaminase activity (Di Giorgio et al. 2020; Simmonds and Schwemmle 2020). These studies also note that mutation changes in A→G and U→C in the SARS-CoV-2 genome were mediated by the actions of ADARs, while G→A mutations were derived from APOBEC-mediated C-to-U deamination (Porath et al. 2014; Roth et al. 2019; Di Giorgio et al. 2020; Saproval et al. 2020).

When assessing within-host nucleotide diversity (θ) of SARS-CoV-2 in our samples, we found no significant deviation from neutrality at the whole-genome level for either the CH1 or the CH3 outbreaks. However, at the per-gene level, the θS/θN ratios differed by gene and sometimes by outbreak, with nsp13, nsp15, ORF3a, M, and ORF7a showing mild evidence for positive selection in at least one outbreak. To increase the resolution of this analysis, we also generated a list of candidate regions undergoing positive selection by examining sliding windows across each gene. Of particular interest are nsp3 codon 424 and nsp6 codon 106, which (1) occur within our candidate regions of within-host positive selection; (2) have the highest within-host θS value in their region; and (3) also show evidence of between-host pervasive and episodic positive selection and an increasing frequency trend in the selection analysis of Pond (2020).

Although our dataset was underpowered to conduct a more fine-scale analyses, these results serve as an important starting point for further investigations into the possible targets of positive selection acting on the SARS-CoV-2 genome within and between hosts.

Our study is subject to several limitations. It is difficult to distinguish between the transmission of within-host variants and recurrent mutation of the same iSNV in independent hosts. Our quality control criteria yielded few iSNVs, severely limiting the power of our selection analysis; this could be ameliorated by increasing the number of samples in future studies, or by applying more powerful filtering criteria that allow more variants to be retained. Regions in which multiple genes overlap the same sites in different reading frames were excluded from analysis, because they can create artefactual signals of positive selection (Nelson, C. W. 2020b). Finally, our within-host sliding window analysis combined all variants from both the CH1 and CH3 outbreaks. While this allows the detection of selective pressures acting similarly in both outbreaks, it is possible that certain targets of selection experienced different pressures in each outbreak, for example, positive selection in CH1 but purifying selection in CH3.

In summary, we showed that integrating within-host diversity and bottleneck estimates in outbreak investigations can yield better resolution during transmission analyses by providing insights into both chains of infection and directions of transmission. We also showed a complex landscape of within-host diversity and evolution of SARS-CoV-2 during infection, with between-host purifying selection potentially explaining the small number of shared within-host variants (iSNVs) transmitted despite larger estimated viral founding populations. This study therefore enhanced our understanding of potential viral transmissions within and across SARS-CoV-2 cases and shed light on the use of within-host variants and bottleneck estimates to retrace the chains of viral transmission in a population. Results obtained from this study emphasize the need for additional research on the role of within-host variants in modulating antigenicity and pathogenicity to shed light on biological mechanisms driving the rapid spread and complex disease progression of SARS-CoV-2.

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Data and code availability

Viral consensus genomes reported in this article have been deposited at Global Initiative on Sharing All Influenza Data (GISAID) (all accessions in Supplementary Table S1)
(Shu and McCauley 2017) while the raw FastQ sequences have been deposited to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) (Leinonen et al. 2011) (Project Accession No. PRJNA636748). Analysis scripts are available at https://github.com/jsan4christ/within-host-diversity-manuscript-analysis-code

Authors contributions
SEJ, SN, RL, and TdO conceived and designed the analysis and SEJ, SN, AMK, EW, WS, CWN, RL, and TdO performed the analyses. SEJ, SN, AMK, HT, VF, JG, BC, SP, LS, MF, IG, EW, KK, CWN, AMK, WS, DPM, RL, and TdO have contributed to the interpretation and discussion of the results and writing of the article.

Supplementary data
Supplementary data are available at Virus Evolution online.

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