Supplementary Appendix.

Detailed Methods.

Sample collection. Nasopharyngeal swabs were sampled from individuals suspected to have an infection with SARS-CoV-2 as part of clinical diagnostics or hospital admittance. Samples were collected in 3 mL viral transport medium (VTM; Copan universal transport medium or equivalent). Clinical testing was performed using the Cepheid Xpert SARS-CoV-2/Flu/RSV assays, for all samples described in this report, except for the initial positive test for Case 2, which was done on a Biofire instrument, that yields no Ct information.

RNA extraction, library preparation, sequencing and bioinformatic analysis. RNA was extracted from 400 μl of each nasopharyngeal swab specimen using the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit on the KingFisher flex system (Thermo Fisher Scientific) following the manufacturer's instructions. Total RNA (11 μl) was converted to first strand cDNA by random priming using the Superscript IV first-strand synthesis system (Invitrogen, ref# 180901050). Libraries were prepared using the XGen SARS-CoV-2 amplicon-based library prep method (IDT, Coralville, Iowa), following the manufacturer's instructions. Sequencing was performed on an Illumina NovaSeq 6000, 150PE, dual index run. Sequencing reads were demultiplexed using the Illumina bcl2fastq2 Conversion Software v2.20 and adapters and low-quality bases were trimmed with Trimmomatic v0.36. BWA v0.7.17 was utilized for mapping reads to the SARS-CoV-2 reference genome (NC_045512.2, wuhCor1). Panel-specific tiled primer sequences were removed using Primerclip v.0.3.8. BCFtools v1.9 was used to call mutations and assemble consensus sequences. Phylogenetic lineage was assigned using Pango nomenclature[1,2] (v.4.0.6 PLEARN-v1.8). Nextclade v2.0.0 and Auspice v2.37.3 phylogenomic visualization Nextstrain project) were used to examine viral genome clade assignment and mutation calling. Sequences that did not yield a near-complete viral genome (<23,000bp, <4000x coverage) were discarded from further analysis.

Structural analysis. The structure of the polymerase complex dimer is based on pdb 7egq with remdesivir added by structural overlay of a remdesivir-bound nsp12 complex (pdb 7l1f)[3,4]

References.

1. Rambaut A, Holmes EC, O'Toole A, et al. A dynamic nomenclature proposal for SARS-CoV-2 lineages to assist genomic epidemiology. Nat Microbiol 2020;5:1403-7.
2. Hadfield J, Megill C, Bell SM, et al. Nextstrain: real-time tracking of pathogen evolution. Bioinformatics 2018;34:4121-3.
3. Yan L, Yang Y, Li M, et al. Coupling of N7-methyltransferase and 3'-5' exoribonuclease with SARS-CoV-2 polymerase reveals mechanisms for capping and proofreading. Cell 2021;184:3474-85 e11.
4. Bravo JPK, Dangerfield TL, Taylor DW, Johnson KA. Remdesivir is a delayed translocation inhibitor of SARS-CoV-2 replication. Mol Cell 2021;81:1548-52 e4.

Supplemental Table 1. Complete list of all non-synonymous mutations in the longitudinal SARS-CoV-2 samples from Case 1 and Case 2

Case 1	d0 EPI_ISL_8715853	d24 EPI_ISL_9440988	d32 EPI_ISL_10650972	d38 EPI_ISL_10651032	d58 EPI_ISL_11624936	d109 EPI_ISL_12511256	d116 EPI_ISL_12972268	Case 2	d18 EPI_ISL_9440904	d25 EPI_ISL_9710398	d32 EPI_ISL_10650964
											NSP2:V485I
	NSP3:K38R	NSP3:K38R	NSP3:K38R	NSP3:K38R	NSP3:K38R	NSP3:K38R	NSP3:K38R		NSP3:K38R	NSP3:K38R	NSP3:K38R
							NSP3:G301S				
						NSP3:S702P	NSP3:S702P				
									NSP3:V1069I	NSP3:V1069I	NSP3:V1069I
	NSP3:SL1265-1266I deletion	NSP3:SL1265-1266I deletion	NSP3:SL1265-1266I deletion	NSP3:SL1265-1266I deletion	NSP3:SL1265-1266I deletion	NSP3:SL1265-1266I deletion	NSP3:SL1265-1266I deletion		NSP3:SL1265-1266I deletion	NSP3:SL1265-1266I deletion	NSP3:SL1265-1266I deletion
	NSP3:A1892T	NSP3:A1892T	NSP3:A1892T	NSP3:A1892T	NSP3:A1892T	NSP3:A1892T	NSP3:A1892T		NSP3:A1892T	NSP3:A1892T	NSP3:A1892T
	NSP4:T492I	NSP4:T492I	NSP4:T492I	NSP4:T492I	NSP4:T492I	NSP4:T492I	NSP4:T492I		NSP4:T492I	NSP4:T492I	NSP4:T492I
	NSP5:P132H	NSP5:P132H	NSP5:P132H	NSP5:P132H	NSP5:P132H	NSP5:P132H	NSP5:P132H		NSP5:P132H	NSP5:P132H	NSP5:P132H
	NSP6:LSG105-107 deletion	NSP6:LSG105-107 deletion	NSP6:LSG105-107 deletion	NSP6:LSG105-107 deletion	NSP6:LSG105-107 deletion	NSP6:LSG105-107 deletion	NSP6:LSG105-107 deletion		NSP6:LSG105-107 deletion	NSP6:LSG105-107 deletion	NSP6:LSG105-107 deletion
	NSP6:I189V	NSP6:I189V	NSP6:I189V	NSP6:I189V	NSP6:I189V	NSP6:I189V	NSP6:I189V		NSP6:I189V	NSP6:I189V	NSP6:I189V
		NSP6:I210V	NSP6:I210V	NSP6:I210V	NSP6:I210V	NSP6:I210V	NSP6:I210V				
						NSP6:R233C	**NSP6:R233C**				
	NSP12:P323L	NSP12:P323L	NSP12:P323L	NSP12:P323L	NSP12:P323L	NSP12:P323L	NSP12:P323L		NSP12:P323L	NSP12:P323L	NSP12:P323L
				NSP12:V792I	**NSP12:V792I**	**NSP12:V792I**	**NSP12:V792I**			**NSP12:V792I**	NSP12:V792I
	NSP14:I42V	NSP14:I42V	NSP14:I42V	NSP14:I42V	NSP14:I42V	NSP14:I42V	NSP14:I42V		NSP14:I42V	NSP14:I42V	NSP14:I42V
	S:A67V	S:A67V	S:A67V	S:A67V	S:A67V	S:A67V	S:A67V				**NSP14:V381I**
									NSP15:A171V	NSP15:A171V	NSP15:A171V
									S:A67V	S:A67V	S:A67V
	S:HV69-70 deletion	S:HV69-70 deletion	S:HV69-70 deletion	S:HV69-70 deletion	S:HV69-70 deletion	S:HV69-70 deletion	S:HV69-70 deletion		S:HV69-70 deletion	S:HV69-70 deletion	S:HV69-70 deletion
	S:T95I	S:T95I	S:T95I	S:T95I	S:T95I	S:T95I	S:T95I		S:T95I	S:T95I	S:T95I
	S:GVYY142-145D deletion	S:GVYY142-145D deletion	S:GVYY142-145D deletion	S:GVYY142-145D deletion	S:GVYY142-145D deletion	S:GVYY142-145D deletion	S:GVYY142-145D deletion		S:GVYY142-145D deletion	S:GVYY142-145D deletion	S:GVYY142-145D deletion
	S:G339D	S:G339D	S:G339D	S:G339D	S:G339D	S:G339D	S:G339D		S:G339D*	S:G339D	S:G339D
	S:R346K	S:R346K	S:R346K	S:R346K	S:R346K	S:R346K	S:R346K				
	S:S371L	S:S371L	S:S371F	S:S371L	S:S371L	S:S371L	S:S371L		S:S371L	S:S371L	S:S371L
	S:S373P	S:S373P	S:S373P	S:S373P	S:S373P	S:S373P	S:S373P		S:373P	S:373P	S:373P
	S:S375F	S:S375F	S:S375F	S:S375F	S:S375F	S:S375F	S:S375F		S:375F	S:375F	S:375F
	S:K417N*	S:K417N	S:K417N*	S:K417N*	S:K417T*	S:K417T*	S:K417T		S:K417N*	S:K417N*	S:K417N*
	S:N440K	S:N440K	S:N440K	S:N440K	S:N440K	S:N440K	S:N440K		S:N440K	S:N440K	S:N440K
	S:G446S	S:G446S	S:G446S	S:G446S	S:G446S	S:G446S	S:G446S		S:G446S	S:G446S	S:G446S
	S:S477N	S:S477N	S:S477N	S:S477N	S:S477N	S:S477N	S:S477N		S:S477N	S:S477N	S:S477N
	S:T478K	S:T478K	S:T478K	S:T478K	S:T478K	S:T478K	S:T478K		S:T478K	S:T478K	S:T478K
	S:E484A	S:E484A	S:E484A	S:E484A	S:E484A	S:E484A	S:E484A		S:E484A	S:E484A	S:E484A
	S:Q493R	S:Q493R	S:Q493R	S:Q493R	S:Q493R		S:Q493R		S:Q493R	S:Q493R	S:Q493R
	S:G496S	S:G496S	S:G496S	S:G496S	S:G496S	S:G496S	S:G496S		S:Q496S	S:Q496S	S:Q496S
	S:Q498R	S:Q498R	S:Q498R	S:Q498R	S:Q498R	S:Q498R	S:Q498R		S:Q498R	S:Q498R	S:Q498R
	S:N501Y	S:N501Y	S:N501Y	S:N501Y	S:N501Y	S:N501Y	S:N501Y		S:N501Y	S:N501Y	S:N501Y
	S:Y505H	S:Y505H	S:Y505H	S:Y505H	S:Y505H	S:Y505H	S:Y505H		S:Y505H	S:Y505H	S:Y505H
	S:T547K	S:T547K	S:T547K	S:T547K	S:T547K	S:T547K	S:T547K		S:T547K	S:T547K	S:T547K
	S:D614G	S:D614G	S:D614G	S:D614G	S:D614G	S:D614G	S:D614G		S:D614G	S:D614G	S:D614G
											S:H625Q
	S:H655Y	S:H655Y	S:H655Y	S:H655Y	S:H655Y	S:H655Y	S:H655Y		S:H655Y	S:H655Y	S:H655Y
	S:N679K	S:N679K	S:N679K	S:N679K	S:N679K	S:N679K	S:N679K		S:N679K	S:N679K	S:N679K
	S:P681H	S:P681H	S:P681H	S:P681H	S:P681H	S:P681H	S:P681H		S:P681H	S:P681H	S:P681H
									S:A701V	S:A701V	S:A701V
	S:N764K	S:N764K	S:N764K	S:N764K	S:N764K	S:N764K	S:N764K		S:N764K*	S:N764K	S:N764K
	S:D796Y	S:D796Y	S:D796Y	S:D796Y	S:D796Y	S:D796Y	S:D796Y		S:D796Y*	S:D796Y	S:D796Y
	S:N856K	S:N856K	S:N856K	S:N856K	S:N856K	S:N856K	S:N856K		S:N856K	S:N856K	S:N856K
	S:Q954H	S:Q954H	S:Q954H	S:Q954H	S:Q954H	S:Q954H	S:Q954H		S:Q954H	S:Q954H	S:Q954H
	S:N969K	S:N969K	S:N969K	S:N969K	S:N969K	S:N969K	S:N969K		S:N969K	S:N969K	S:N969K
	S:L981F	S:L981F	S:L981F	S:L981F	S:L981F	S:L981F	S:L981F		S:L981F	S:L981F	S:L981F
	S:K1266R	S:K1266R	S:K1266R	S:K1266R	S:K1266R	S:K1266R	S:K1266R				
						ORF3a:T34M	**ORF3a:T34M**				
	E:T9I	E:T9I	E:T9I	E:T9I	E:T9I	E:T9I	E:T9I		E:T9I	E:T9I	E:T9I
	M:D3G	M:D3G	M:D3G	M:D3G	M:D3G	M:D3G	M:D3G		M:D3G	M:D3G	M:D3G
	M:Q19E	M:Q19E	M:Q19E	M:Q19E	M:Q19E	M:Q19E	M:Q19E		M:Q19E*	M:Q19E	M:Q19E
	M:A63T	M:A63T	M:A63T	M:A63T	M:A63T	M:A63T	M:A63T		M:A63T	M:A63T	M:A63T
	N:P13L	N:P13L	N:P13L	N:P13L	N:P13L	N:P13L	N:P13L		N:P13L	N:P13L	N:P13L
	N:ERS31-33 deletion	N:ERS31-33 deletion	N:ERS31-33 deletion	N:ERS31-33 deletion	N:ERS31-33 deletion	N:ERS31-33 deletion	N:ERS31-33 deletion		N:ERS31-33 deletion	N:ERS31-33 deletion	N:ERS31-33 deletion
	N:R203K	N:R203K	N:R203K	N:R203K	N:R203K	N:R203K	N:R203K		N:R203K	N:R203K	N:R203K
	N:G204R	N:G204R	N:G204R	N:G204R	N:G204R	N:G204R	N:G204R		N:G204R	N:G204R	N:G204R

Footnotes:

*coverage below 1000x

Timepoints refer to RT-PCR positive tests, with d0 (Day 0) being the first positive test for the patient

EPI_ISL_ numbers are the GISAID numbers for our deposited sequences

Longitudinaly newly acquired mutations are in bold