

## **Omicron sub-lineages BA.4/BA.5 escape BA.1 infection elicited neutralizing immunity**

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**The SARS-CoV-2 Omicron (B.1.1.529) variant first emerged as the BA.1 sub-lineage, with extensive escape from neutralizing immunity elicited by previous infection with other variants, vaccines, or combinations of both<sup>1,2</sup>. Two new sub-lineages, BA.4 and BA.5, are now emerging in South Africa with changes relative to BA.1, including L452R and F486V mutations in the spike receptor binding domain. We isolated live BA.4 and BA.5 viruses and tested them against neutralizing immunity elicited to BA.1 infection in participants who were Omicron/BA.1 infected but unvaccinated (n=24) and participants vaccinated with Pfizer BNT162b2 or Johnson and Johnson Ad26.CoV.2S with breakthrough Omicron/BA.1 infection (n=15). In unvaccinated individuals, FRNT<sub>50</sub>, the inverse of the dilution for 50% neutralization, declined from 275 for BA.1 to 36 for BA.4 and 37 for BA.5, a 7.6 and 7.5-fold drop, respectively. In vaccinated BA.1 breakthroughs, FRNT<sub>50</sub> declined from 507 for BA.1 to 158 for BA.4 (3.2-fold) and 198 for BA.5 (2.6-fold). Absolute BA.4 and BA.5 neutralization levels were about 5-fold higher in this group versus unvaccinated BA.1 infected participants. The observed escape of BA.4 and BA.5 from BA.1 elicited immunity is more moderate than of BA.1 against previous immunity<sup>1,3</sup>. However, the low absolute neutralization levels for BA.4 and BA.5, particularly in the unvaccinated group, are unlikely to protect well against symptomatic infection<sup>4</sup>. This may indicate that, based on neutralization escape, BA.4 and BA.5 have potential to result in a new infection wave.**

We enrolled participants in November-December 2021 during the Omicron/BA.1 infection wave in South Africa (Table S1). We enrolled 24 SARS-CoV-2 infected unvaccinated participants and 15 vaccinated participants where eight were vaccinated with Pfizer BNT162b2 and seven with Johnson and Johnson Ad26.CoV.2S (Table S2). For this study we used blood samples collected a median of 23 days after symptoms onset (IQR 18-27 for vaccinated and 20-28 for unvaccinated participants), corresponding to a time post-symptoms when Omicron/BA.1 neutralizing immunity developed and plateaued<sup>3</sup>. As previously described (see also Table S2), infecting virus per participant was sequenced and all successfully sequenced viruses were Omicron/BA.1<sup>3</sup>.

We observed that FRNT<sub>50</sub> in unvaccinated participants declined from 275 for BA.1 to 36 for BA.4 (7.6-fold, 95% CI 4.9-12.0, Figure 1a) and 37 for BA.5 (7.5-fold, 95% CI 4.4-12.5, Figure 1b). In contrast, in vaccinated BA.1 infected participants, FRNT<sub>50</sub> declined from 507 to 158 for BA.4 (3.2-fold, 95% CI 2.3-4.4, Figure 1c) and 198 for BA.5 (2.6-fold, 95% CI 1.8-3.7, Figure 1d). Absolute BA.4 and BA.5 neutralization levels were about 5-fold higher in the vaccinated versus unvaccinated group (Figure 1d).

Neutralization capacity elicited to BA.1 infection in unvaccinated participants was low, accounting for the BA.4 and BA.5 fold-drops leading to very low residual neutralization levels. The low immunity to BA.1 is perhaps reflective of lower immunogenicity of this variant<sup>3,5</sup>. The vaccinated group showed about 5-fold higher neutralization capacity of BA.4 and BA.5 and should be better protected, although levels may decrease with waning. BA.4 and BA.5 show similar escape. This is not surprising given that sequence differences between BA.4 and BA.5 are outside spike.

The escape we observe is substantially higher than what we detected for BA.2, where it was slight and non-significant<sup>3</sup>. Given the higher escape and especially the low residual neutralization in the unvaccinated group, we speculate that a BA.4/BA.5 infection wave is a strong possibility. However, vaccination increase neutralization capacity against these emerging variants and would likely offer good protection against severe disease.

## **Materials and methods**

### Informed consent and ethical statement

Blood samples were obtained after written informed consent from adults with PCR-confirmed SARS-CoV-2 infection who were enrolled in a prospective cohort study at the Africa Health Research Institute approved by the Biomedical Research Ethics Committee at the University of KwaZulu–Natal (reference BREC/00001275/2020). The Omicron/BA.1 and BA.4 was isolated from a residual swab sample with SARS-CoV-2 isolation from the sample approved by the University of the Witwatersrand Human Research Ethics Committee (HREC) (ref. M210752). The sample to isolate Omicron/BA.5 was collected after written informed consent as part of the COVID-19 transmission and natural history in KwaZulu-Natal, South Africa: Epidemiological Investigation to Guide Prevention and Clinical Care in the Centre for the AIDS Programme of Research in South Africa (CAPRISA) study and approved by the Biomedical Research Ethics Committee at the University of KwaZulu–Natal (reference BREC/00001195/2020, BREC/00003106/2021).

### Data availability statement

Sequences of outgrown Omicron sub-lineages have been deposited in GISAID with accession EPI\_ISL\_7886688 (Omicron/BA.1), EPI\_ISL\_12268495.2 (Omicron/BA.4),

EPI\_ISL\_12268493.2 (Omicron/BA.5). Raw images of the data are available upon reasonable request.

#### Reagent availability statement

Virus isolates and cell line are available from the corresponding author. A Biosafety Level 3 facility is required for laboratories receiving live SARS-CoV-2.

#### Competing interest statement

Salim S. Abdool Karim is a member in the COVID advisory panel for Emerging Markets at Pfizer. The authors declare no other competing interests.

#### Author contributions

AS and KK conceived the study and designed the study and experiments. AvG, QAK, SSKA, GL, ASi, and NS identified and provided virus samples. M-YSM, FK, BIG, MB, KK, TN, MM, NM, ZM, NN, YM, ZJ, KR, and YG set up and managed the cohort and cohort data. KK, ZJ, KR, SC, HT, JES, YG, JG, YR, AK, DA, and JB performed experiments and sequence analysis with input from AS, TdO, RJL, and JNB. AS, KK, FK, RM, and YR interpreted data with input from M-YSM, GG, SSKA, WH, TdO, RJL. AS and KK prepared the manuscript with input from all authors.

#### Whole-genome sequencing, genome assembly and phylogenetic analysis

RNA was extracted on an automated Chemagic 360 instrument, using the CMG-1049 kit (Perkin Elmer, Hamburg, Germany). The RNA was stored at  $-80^{\circ}\text{C}$  prior to use. Libraries for whole genome sequencing were prepared using either the Oxford Nanopore Midnight protocol with Rapid Barcoding or the Illumina COVIDseq Assay. For the Illumina COVIDseq assay, the libraries were prepared according to the manufacturer's protocol. Briefly, amplicons were tagged, followed by indexing using the Nextera UD Indexes Set A. Sequencing libraries were pooled, normalized to 4 nM and denatured with 0.2 N sodium acetate. An 8 pM sample library was spiked with 1% PhiX (PhiX Control v3 adaptor-ligated library used as a control). We sequenced libraries on a 500-cycle v2 MiSeq Reagent Kit on the Illumina MiSeq instrument (Illumina). On the Illumina NextSeq 550 instrument, sequencing was performed using the Illumina COVIDSeq protocol (Illumina Inc, USA), an amplicon-based next-generation sequencing approach. The first strand synthesis was carried using random hexamers primers from Illumina and the synthesized cDNA underwent two separate multiplex PCR reactions. The pooled PCR amplified products were processed for tagmentation and adapter ligation using IDT for Illumina Nextera UD Indexes. Further enrichment and cleanup was performed as per protocols provided by the manufacturer (Illumina Inc). Pooled samples were quantified using Qubit 3.0 or 4.0 fluorometer (Invitrogen Inc.) using the Qubit dsDNA High Sensitivity assay according to manufacturer's instructions. The fragment sizes were analyzed using TapeStation 4200 (Invitrogen). The pooled libraries were further normalized to 4nM concentration and 25  $\mu\text{L}$  of each normalized pool containing unique index adapter sets were combined in a new tube. The final library pool was denatured and neutralized with 0.2N sodium hydroxide and 200 mM Tris-HCL (pH7), respectively. 1.5 pM sample library was spiked with 2% PhiX. Libraries were loaded onto a 300-cycle NextSeq 500/550 HighOutput Kit v2 and run on the Illumina NextSeq 550 instrument (Illumina, San Diego, CA, USA). For Oxford Nanopore sequencing, the Midnight primer kit was used as described by Freed and Silander<sup>55</sup>. cDNA synthesis was performed on the extracted RNA using LunaScript RT mastermix (New England BioLabs) followed by gene-specific multiplex PCR using the Midnight Primer pools which produce 1200bp amplicons which overlap to cover the 30-kb SARS-CoV-2 genome. Amplicons from each pool were pooled and used neat for barcoding with the Oxford Nanopore Rapid Barcoding kit as per the manufacturer's protocol. Barcoded samples were pooled and

bead-purified. After the bead clean-up, the library was loaded on a prepared R9.4.1 flow-cell. A GridION X5 or MinION sequencing run was initiated using MinKNOW software with the base-call setting switched off. We assembled paired-end and nanopore.fastq reads using Genome Detective 1.132 (<https://www.genomedetective.com>) which was updated for the accurate assembly and variant calling of tiled primer amplicon Illumina or Oxford Nanopore reads, and the Coronavirus Typing Tool<sup>56</sup>. For Illumina assembly, GATK HaploTypeCaller --min-pruning 0 argument was added to increase mutation calling sensitivity near sequencing gaps. For Nanopore, low coverage regions with poor alignment quality (<85% variant homogeneity) near sequencing/amplicon ends were masked to be robust against primer drop-out experienced in the Spike gene, and the sensitivity for detecting short inserts using a region-local global alignment of reads, was increased. In addition, we also used the wf\_artic (ARTIC SARS-CoV-2) pipeline as built using the nextflow workflow framework<sup>57</sup>. In some instances, mutations were confirmed visually with .bam files using Geneious software V2020.1.2 (Biomatters). The reference genome used throughout the assembly process was NC\_045512.2 (numbering equivalent to MN908947.3). For lineage classification, we used the widespread dynamic lineage classification method from the 'Phylogenetic Assignment of Named Global Outbreak Lineages' (PANGOLIN) software suite (<https://github.com/hCoV-2019/pangolin>).

### Cells

Vero E6 cells (originally ATCC CRL-1586, obtained from Cellnex in South Africa) were propagated in complete growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (Hyclone) containing 10mM of HEPES, 1mM sodium pyruvate, 2mM L-glutamine and 0.1mM nonessential amino acids (Sigma-Aldrich). Vero E6 cells were passaged every 3–4 days. The H1299-E3 cell line (H1299 originally from ATCC as CRL-5803) was propagated in growth medium consisting of complete Roswell Park Memorial Institute (RPMI) 1640 medium with 10% fetal bovine serum containing 10mM of HEPES, 1mM sodium pyruvate, 2mM L-glutamine and 0.1mM nonessential amino acids. Cells were passaged every second day. The H1299-E3 (H1299-ACE2, clone E3) cell line was derived from H1299 as described in our previous work<sup>1,6</sup>.

### Virus expansion

All work with live virus was performed in Biosafety Level 3 containment using protocols for SARS-CoV-2 approved by the Africa Health Research Institute Biosafety Committee. ACE2-expressing H1299-E3 cells were seeded at  $4.5 \times 10^5$  cells in a 6 well plate well and incubated for 18–20 h. After one DPBS wash, the sub-confluent cell monolayer was inoculated with 500  $\mu$ L universal transport medium diluted 1:1 with growth medium filtered through a 0.45- $\mu$ m filter. Cells were incubated for 1 h. Wells were then filled with 3 mL complete growth medium. After 4 days of infection (completion of passage 1 (P1)), cells were trypsinized, centrifuged at 300 rcf for 3 min and resuspended in 4 mL growth medium. Then all infected cells were added to Vero E6 cells that had been seeded at  $2 \times 10^5$  cells per mL, 20mL total, 18–20 h earlier in a T75 flask for cell-to-cell infection. The coculture of ACE2-expressing H1299-E3 and Vero E6 cells was incubated for 1 h and the flask was filled with 20 mL of complete growth medium and incubated for 4 days. The viral supernatant from this culture (passage 2 (P2) stock) was used for experiments.

### Live virus neutralization assay

H1299-E3 cells were plated in a 96-well plate (Corning) at 30,000 cells per well 1 day pre-infection. Plasma was separated from EDTA-anticoagulated blood by centrifugation at 500 rcf for 10 min and stored at  $-80^\circ\text{C}$ . Aliquots of plasma samples were heat-inactivated at  $56^\circ\text{C}$  for 30 min and clarified by centrifugation at 10,000 rcf for 5 min. Virus stocks were used at approximately 50-100 focus-forming units per microwell and added to diluted plasma.

Antibody–virus mixtures were incubated for 1 h at 37 °C, 5% CO<sub>2</sub>. Cells were infected with 100 µL of the virus–antibody mixtures for 1 h, then 100 µL of a 1X RPMI 1640 (Sigma-Aldrich, R6504), 1.5% carboxymethylcellulose (Sigma-Aldrich, C4888) overlay was added without removing the inoculum. Cells were fixed 18 h post-infection using 4% PFA (Sigma-Aldrich) for 20 min. Foci were stained with a rabbit anti-spike monoclonal antibody (BS-R2B12, GenScript A02058) at 0.5 µg/mL in a permeabilization buffer containing 0.1% saponin (Sigma-Aldrich), 0.1% BSA (Sigma-Aldrich) and 0.05% Tween-20 (Sigma-Aldrich) in PBS. Plates were incubated with primary antibody overnight at 4 °C, then washed with wash buffer containing 0.05% Tween-20 in PBS. Secondary goat anti-rabbit HRP conjugated antibody (Abcam ab205718) was added at 1 µg/mL and incubated for 2 h at room temperature with shaking. TrueBlue peroxidase substrate (SeraCare 5510-0030) was then added at 50 µL per well and incubated for 20 min at room temperature. Plates were imaged in an ImmunoSpot Ultra-V S6-02-6140 Analyzer ELISPOT instrument with BioSpot Professional built-in image analysis (C.T.L).

### Statistics and fitting

All statistics and fitting were performed using custom code in MATLAB v.2019b. Neutralization data were fit to:

$$Tx=1/1+(D/ID_{50}).$$

Here Tx is the number of foci normalized to the number of foci in the absence of plasma on the same plate at dilution D and ID<sub>50</sub> is the plasma dilution giving 50% neutralization. FRNT<sub>50</sub> = 1/ID<sub>50</sub>. Values of FRNT<sub>50</sub> <1 are set to 1 (undiluted), the lowest measurable value. We note that the most concentrated plasma dilution was 1:25 and therefore FRNT<sub>50</sub> < 25 were extrapolated. To calculate confidence intervals, FRNT<sub>50</sub> or fold-change in FRNT<sub>50</sub> per participant was log transformed and arithmetic mean plus 2 std and arithmetic mean minus 2 std were calculated for the log transformed values. These were exponentiated to obtain the upper and lower 95% confidence intervals on the geometric mean FRNT<sub>50</sub> or the fold-change in FRNT<sub>50</sub> geometric means.

### Acknowledgements

This study was supported by the Bill and Melinda Gates award INV-018944 (AS), National Institutes of Health award R01 AI138546 (AS), and South African Medical Research Council Award 6084COAP2020 (AS). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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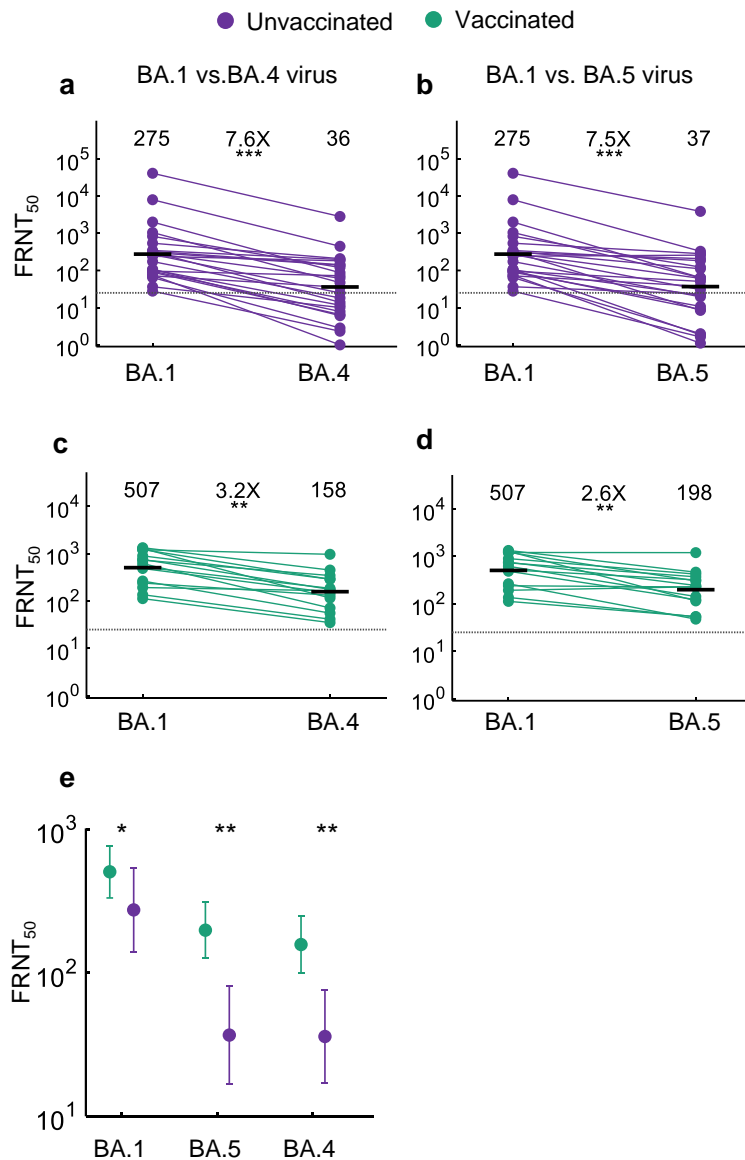
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All participants infected in BA.1 infection wave in South Africa



**Figure 1: Escape of BA.4 and BA.5 from Omicron/BA.1 elicited immunity.** Neutralization of BA.4 (a) or BA.5 (b) compared to BA.1 virus by immunity elicited through BA.1 infection in n=24 unvaccinated participants. Neutralization of BA.4 (c) or BA.5 (d) compared to BA.1 virus by immunity elicited through BA.1 infection in n=15 participants vaccinated either with Pfizer BNT162b2 (n=8) or Johnson and Johnson Ad26.CoV2.S (n=7). Numbers are geometric mean titer (GMT) FRNT<sub>50</sub>. Dashed line is most concentrated plasma tested. (e) Neutralization of BA.1, BA.4, and BA.5 in the n=15 Omicron/BA.1 infected vaccinated compared to the n=24 Omicron/BA.1 infected unvaccinated participants. Numbers are GMT FRNT<sub>50</sub> and error bars are GMT 95% confidence intervals. Data is from 2 (BA.4, BA.5) or 3 (BA.1) independent experiments done on different days. p-values were determined by a two-sided Wilcoxon rank sum test and represented as \*0.05-0.01, \*\*0.01-0.001.

**Table S1: Summary of participant details**

	All 39	Vaccinated 15 (38%)	Unvaccinated 24 (62%)
Age	35 (27-55)	37 (32-60)	31.5 (26-49)
Female	25 (64%)	9 (60%)	16 (67%)
Vaccination to symptom onset (days)	-	135 (111-195)	-
Symptom onset to collection (days)	23 (19-27)	23 (18-27)	23 (20-28)
Required supp. O <sub>2</sub>	7 (18%)	2 (13%)	5 (21%)
Hospitalized	27 (69%)	8 (53%)	19 (79%)
Duration of hospitalization (days)	7 (3-11)	3.5 (2.5-14.5)	8 (3-11)

**Table S2: Participant characteristics and vaccination status**

#	Age	Sex	Vacc. type	Date of vacc.	Date symp. onset	Symp. to sample (days)	Sub-lineage	Seq. GISAID ID	Supp. O <sub>2</sub>	Hosp.
1	30-39	M	AD26.COV2	MAR-2021	DEC-2021	23	BA.1	EPI_ISL_9967759	No	No
2	30-39	M	AD26.COV2*	NOV-2021	NOV-2021	22	BA.1	EPI_ISL_9967761	No	No
3	50-59	F	BNT162b2	JUL-2021	DEC-2021	27	BA.1	EPI_ISL_8604915	No	No
4	30-39	F	AD26.COV2	MAY-2021	DEC-2021	13	BA.1	EPI_ISL_8604910	No	No
5	20-29	F	AD26.COV2	SEP-2021	DEC-2021	27	BA.1	EPI_ISL_9967760	No	Yes
6	10-19	F	BNT162b2	JUL-2021	DEC-2021	12	BA.1	EPI_ISL_8604906	No	Yes
7	20-29	F			NOV-2021	24	N/A	N/A	No	Yes
8	30-39	M			DEC-2021	23	BA.1	EPI_ISL_8604919	No	Yes
9	40-49	F			DEC-2021	28	BA.1	EPI_ISL_8604901	No	Yes
10	20-29	M			DEC-2021	13	BA.1	EPI_ISL_8604908	No	Yes
11	20-29	F			DEC-2021	22	BA.1	EPI_ISL_8604913	No	No
12	20-29	F			DEC-2021	22	BA.1	EPI_ISL_8604912	No	Yes
13	30-39	M	BNT162b2	JUL-2021	NOV-2021	28	BA.1	EPI_ISL_8604916	No	Yes
14	20-29	M			NOV-2021	15	N/A	N/A	No	Yes
15	60-69	F	BNT162b2	JUL-2021	DEC-2021	25	BA.1	EPI_ISL_8604920	No	Yes
16	60-69	M	BNT162b2	DEC-2021	DEC-2021	10	BA.1	EPI_ISL_8578311	No	No
17	30-39	M			DEC-2021	19	BA.1	EPI_ISL_8604923	No	No
18	60-69	F			DEC-2021	23	BA.1	EPI_ISL_8578312	Yes	Yes
19	30-39	M			DEC-2021	27	BA.1	EPI_ISL_8604924	No	Yes
20	20-29	F			DEC-2021	24	BA.1	EPI_ISL_8604911	No	Yes
21	20-29	M			DEC-2021	21	BA.1	EPI_ISL_8604922	No	No
22	30-39	F	AD26.COV2	AUG-2021	DEC-2021	23	BA.1	EPI_ISL_8693907	No	Yes
23	20-29	F			DEC-2021	10	BA.1	EPI_ISL_8604902	No	No
24	50-59	M	BNT162b2	AUG-2021	DEC-2021	18	N/A	N/A	No	Yes
25	30-39	F	AD26.COV2	APR-2021	DEC-2021	24	BA.1	EPI_ISL_8604914	No	No
26	50-59	F	AD26.COV2	JUL-2021	DEC-2021	23	N/A	N/A	No	No
27	50-59	F			DEC-2021	28	N/A	N/A	Yes	Yes
28	80-89	F	BNT162b2	JUL-2021	JAN-2022	22	N/A	N/A	Yes	Yes
29	60-69	M	BNT162b2	JUL-2021	DEC-2021	32	N/A	N/A	Yes	Yes
30	40-49	M			DEC-2021	13	BA.1	EPI_ISL_8604909	No	No
31	30-39	F			DEC-2021	22	N/A	N/A	Yes	Yes
32	50-59	F			DEC-2021	36	BA.1	EPI_ISL_8578347	No	Yes
33	20-29	F			DEC-2021	18	N/A	N/A	No	Yes
34	30-39	F			DEC-2021	30	N/A	N/A	No	Yes
35	50-59	F			DEC-2021	27	BA.1	EPI_ISL_8578342	Yes	Yes
36	20-29	F			DEC-2021	23	N/A	N/A	No	Yes
37	50-59	F			DEC-2021	30	N/A	N/A	No	Yes
38	30-39	M			DEC-2021	31	N/A	N/A	No	Yes
39	50-59	F			DEC-2021	30	N/A	N/A	Yes	Yes

Symp.: Symptoms date. Supp O<sub>2</sub>: participant required supplemental oxygen during the study. Hosp.: participant hospitalized during the study. N/A: Not available; sequencing failed, usually due to insufficient virus substrate. \*Boosted with Ad26.CoV2.S in Nov-2021.