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Supplementary appendix

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Efficacy of Antiviral Agents and Bivalent mRNA Vaccines against a CH.1.1 isolate

Supplementary Appendix

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Supplementary Materials

Cells.

Vero E6-TMPRSS2-T2A-ACE2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% Fetal Calf Serum (FCS), 100 U/mL penicillin–streptomycin, and 10 μg/mL puromycin. VeroE6/TMPRSS2 (JCRB 1819) cells were propagated in the presence of 1 mg/ml geneticin (G418; Invivogen) and 5 μg/ml plasmocin prophylactic (Invivogen) in DMEM containing 10% FCS. Vero E6-TMPRSS2-T2A-ACE2 and VeroE6/TMPRSS2 cells were maintained at 37 °C with 5% CO_2. Chinese hamster ovary (CHO) cells were maintained in DMEM containing 10% FCS and antibiotics at 37 °C with 5% CO_2. Expi293F cells (Thermo Fisher Scientific) were maintained in Expi293 expression medium (Thermo Fisher Scientific) at 37 °C under 8% CO_2. The cells were regularly tested for mycoplasma contamination by using PCR, and confirmed to be mycoplasma-free.

Viruses.

The SARS-CoV-2 viruses hCoV-19/Japan/UT-OM012/2022 (Omicron CH.1.1), hCoV-19/Japan/TY41-795/2022 (Omicron XBB)[1], hCoV-19/Japan/TY41-716/2022 (Omicron BA.2.75)[2], hCoV-19/Japan/UT-NCD1288-2N/2022 (Omicron BA.2)[3], and SARS-CoV-2/UT-NC002-1T/Human/2020/Tokyo (ancestral strain) were propagated in VeroE6/TMPRSS2 cells.

All experiments with SARS-CoV-2 were performed in enhanced biosafety level 3 (BSL3) containment laboratories at the University of Tokyo and the National Institute of Infectious Diseases, Japan, which are approved for such use by the Ministry of Agriculture, Forestry, and Fisheries, Japan.

Clinical specimens.

After informed consent was obtained, plasma specimens were collected from COVID-19 convalescent individuals and vaccinees. The research protocol was approved by the Research Ethics Review Committee of the Institute of Medical Science of the University of Tokyo (approval numbers: 2019–71–0201 and 2020-740226).

Antibodies.

Amino acid sequences for the variable region of the heavy and light chains of the following human monoclonal antibodies against the S protein were used for gene synthesis: clones tixagevimab (COV2-2196/AZD8895; GenBank accession numbers QLI33947 and QLI33948), casirivimab (REGN10933; PDB accession numbers 6XDG_B and 6XDG_D), cilgavimab (COV2-2130/AZD1061; GenBank accession numbers QKY76296 and QKY75909), imdevimab (REGN10987; PDB accession numbers 6XDG_A and 6XDG_A), S309 (PDB accession numbers 6WS6_A and 6WS6_F), and bebtelovimab (LYCoV1404; PDB accession numbers 7MMO_D and 7MMO_E). An artificial signal sequence and the constant gamma heavy (IgG1, UniProtKB/Swiss-Prot accession number P01857) and kappa (UniProtKB/Swiss-Prot accession number P01834) or lambda (UniProtKB/Swiss-Prot accession number P0DOY2) light chain coding sequences were added before and after each variable region. Codon usage was optimized for expression in CHO cells. The synthesized genes were cloned into a plasmid for protein expression and transfected into CHO cells. Cell culture media were harvested after incubation for 10–14 days at 37 °C. Monoclonal antibodies were purified by using MabSelect SuRe LX (Cytiva) or a protein A column. Purity was confirmed by SDS-PAGE and/or HPLC before use. The reactivities of these antibodies against SARS-CoV-2, including the Alpha, Beta, Delta, Gamma, and Omicron variants, have been tested previously[4].

Antiviral compounds.

Active components of remdesivir and molnupiravir (i.e., GS-441524 and EIDD-1931), and nirmatrelvir (PF-07321332) were purchased from MedChemExpress. Ensitrelvir (S-217622) was kindly provided by Shionogi & Co., Ltd. All compounds were dissolved in dimethyl sulfoxide.

Supplementary Methods

Whole genome sequencing

Viral RNA was extracted by using a QIAamp Viral RNA Mini Kit (QIAGEN). The whole genome of SARS-CoV-2 was amplified by using a modified ARTIC network protocol in which some primers were replaced or added. Briefly, viral cDNA was synthesized from the extracted RNA by using a LunarScript RT SuperMix Kit (New England BioLabs). The DNA was amplified by performing a multiplexed PCR in two pools using the ARTIC-N5 primers and the Q5 Hot Start DNA polymerase (New England BioLabs). The DNA libraries for Illumina NGS were prepared from pooled amplicons by using a QIAseq FX DNA Library Kit (QIAGEN) and were then analyzed by using the iSeq 100 System (Illumina). To determine the virus sequences, the reads were assembled by CLC Genomics Workbench (version 22, Qiagen) with the Wuhan/Hu-1/2019 sequence (GenBank accession no. MN908947) as a reference. The sequence of CH.1.1 (hCoV-19/Japan/UT-OM012/2022) was deposited in the Global Initiative on Sharing All Influenza Data (GISAID) database with accession ID: EPI_ISL_16893733 and in GenBank with accession ID: OQ421449.

Focus reduction neutralisation test (FRNT).

Neutralisation activities of monoclonal antibodies and human plasma were determined by using a focus reduction neutralisation test as previously described[5]. Serially diluted antibodies (starting concentration, 50,000 ng/ml) were mixed with 100–400 focus-forming units (FFU) of virus/well and incubated for 1 h at 37 ℃. The antibody-virus mixture (50 μl) was then inoculated onto Vero E6-TMPRSS2-T2A-ACE2 cells or VeroE6/TMPRSS2 cells in 96-well plates in triplicate. After a 1-h incubation at 37 ℃, 100 μl of 1.5% Methyl Cellulose 400 (FUJIFILM Wako Pure Chemical Corporation, Japan) in culture medium was added to each well. The cells were incubated for 14–18 h at 37 ℃ and then fixed with formalin. For human plasma, the samples were first incubated at 56 ℃ for 1 h. Then, the treated plasma samples were serially diluted five-fold with DMEM containing 2% FCS in 96-well plates and mixed with 100–400 FFU of virus/well, followed by incubation at 37 ℃ for 1 h. The plasma-virus mixture was inoculated onto Vero E6-TMPRSS2-T2A-ACE2 cells in 96-well plates in duplicate. After a 1-h incubation at 37 ℃, 100 μl of of 1.5% Methyl Cellulose 400 (FUJIFILM Wako Pure Chemical Corporation) in culture medium was then added to each well. The cells were incubated for 14–18 h at 37 ℃ and then fixed with formalin.

After the formalin was removed, the cells were immunostained with a mouse monoclonal antibody against SARS-CoV-2 nucleoprotein [N45 (TAUNS Laboratories, Inc., Japan)], followed by a horseradish peroxidase-labeled goat anti-mouse immunoglobulin (Jackson ImmunoResearch Laboratories Inc.). The infected cells were stained with TrueBlue Substrate (SeraCare Life Sciences) and then washed with distilled water. After cell drying, the focus numbers were quantified by using an ImmunoSpot S6 Analyzer, ImmunoCapture software, and BioSpot software (Cellular Technology). The results are expressed as the 50% focus reduction neutralisation titre ($FRNT_{50}$). The $FRNT_{50}$ values were calculated by using GraphPad Prism (GraphPad Software). Samples under the detection limit (<10-fold dilution) were assigned an $FRNT_{50}$ of 10.

Inhibitory effect of compounds against SARS-CoV-2 *in vitro.*

Antiviral susceptibilities of SARS-CoV-2 were determined by applying a focus reduction assay as previously described[1,6]. Vero E6-TMPRSS2-T2A-ACE2 cells in 96-well plates were infected with 100–400 FFU of virus/well. Virus adsorption was carried out for 1 h at 37 ℃ and then the inoculum was removed and 1% Methyl Cellulose 400 (FUJIFILM Wako Pure Chemical Corporation) in culture medium containing serial dilutions of antiviral compounds was added to each well in triplicate. The cells were incubated for 18 h at 37 ℃ and then fixed with formalin. After the formalin was removed, immunostaining was performed as described for the FRNT. The results are expressed as the 50% inhibitory concentration (IC_{50}). The IC_{50} values were calculated by using GraphPad Prism (GraphPad Software).

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Author Contributions
R.U.: conceptualization, formal analysis, validation, visualization, and writing the first draft. M. Ito, M. Kiso: data curation, formal analysis, and methodology. S. Yamayoshi: conceptualization and methodology. K.I-H.: resources and validation. Y.S-T., Y.F., M. Imai, M. Koga, S. Yamamoto, Y. Kashima, E.A., M.S., T.T, A.O., T.K., H.Y.,Y.S.: resources. Y.Kawaoka: conceptualization, supervision, writing (review and editing), and funding acquisition. R.U., M. Ito, and M. Kiso contributed equally.

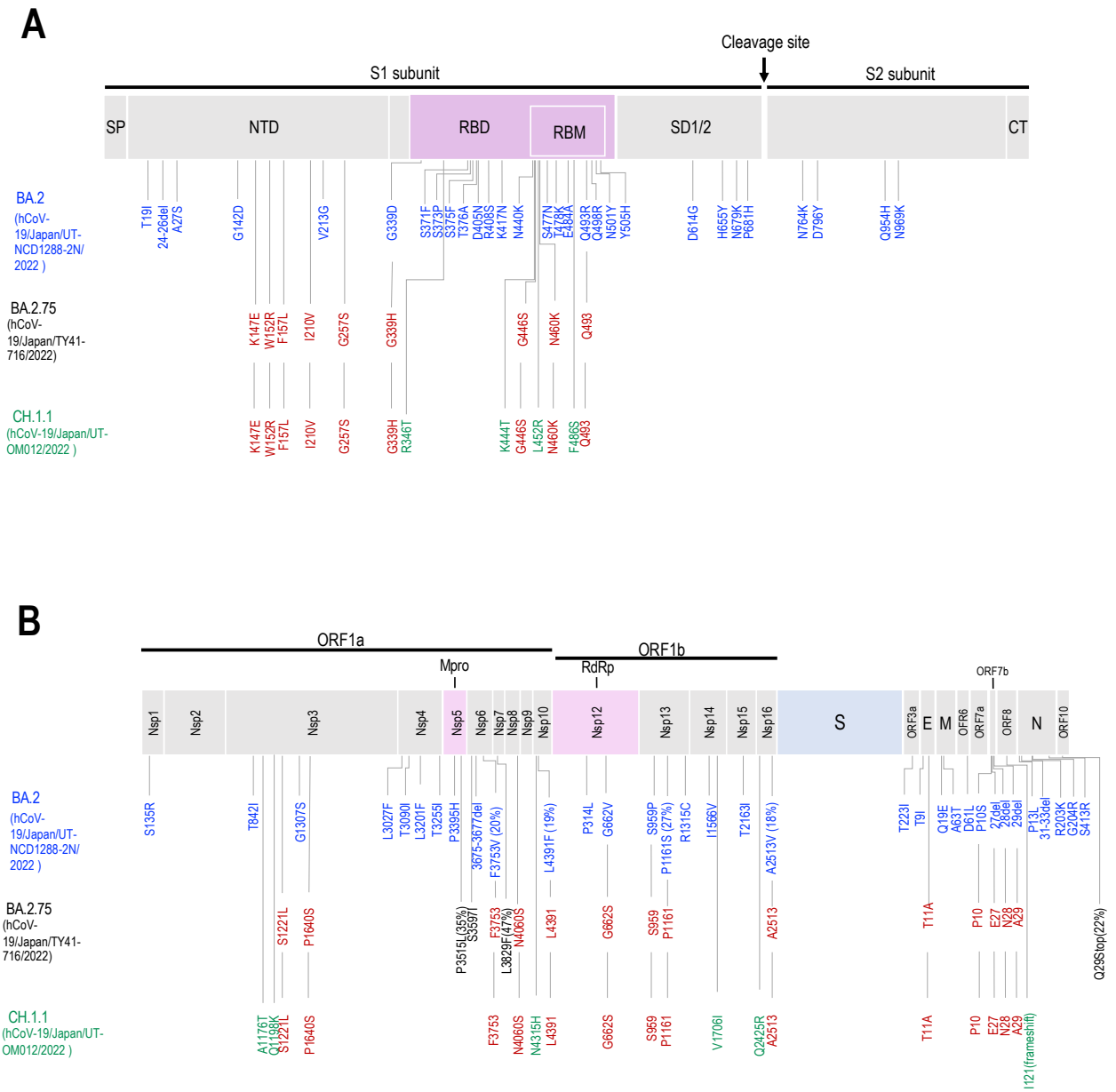
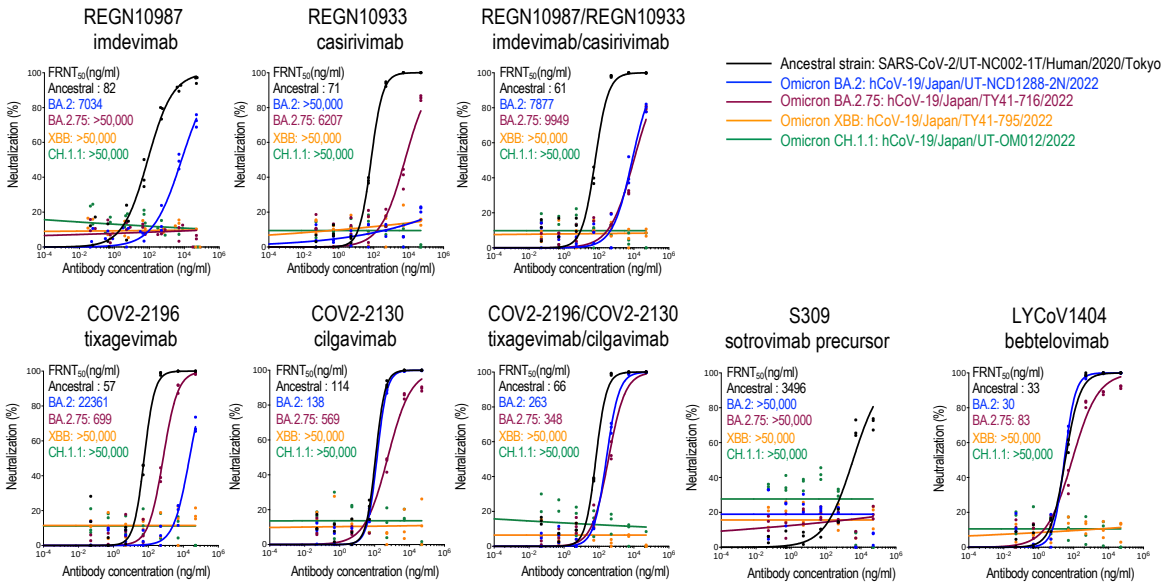


Figure S1. Mutations of Omicron subvariants.

(A) Spike (S) protein substitutions in the CH.1.1 clinical isolate used in this study. The BA.2 (hCoV-19/Japan/UT-NCD1288-2N/2022) isolate possesses 31 amino acid changes in its S protein relative to the reference strain Wuhan/Hu-1/2019. Compared with BA.2 (hCoV-19/Japan/UT-NCD1288-2N/2022), substitutions are shown in black for BA.2.75 (hCoV-19/Japan/TY41-716/2022) and green for CH.1.1(hCoV-19/Japan/UT-OM012/2022). The conserved substitutions between BA.2.75 and CH.1.1 are shown in red. The S protein comprises two subunits, S1 and S2. The arrow indicates the S1/S2 proteolytic cleavage site. SP, signal peptide; NTD, N-terminal domain; RBD, receptor-binding domain; RBM, receptor-binding motif; SD1/2, subdomain 1 and 2; and CT, cytoplasmic tail. (B) Non-spike protein substitutions in the CH.1.1 clinical isolate used in this study. Compared with the reference strain Wuhan/Hu-1/2019, the BA.2 (hCoV-19/Japan/UT-NCD1288-2N/2022) isolate possesses 37 amino acid changes in regions other than the S protein. Compared with BA.2 (hCoV-19/Japan/UT-NCD1288-2N/2022), substitutions are shown in black for BA.2.75 (hCoV-19/Japan/TY41-716/2022) and green for CH.1.1(hCoV-19/Japan/UT-OM012/2022). The conserved substitutions between BA.2.75 and CH.1.1 are shown in red. ORF, open reading frame; Mpro, main protease; RdRp, RNA-dependent RNA polymerase; S, Spike; E, Envelope; M, Membrane; and N, Nucleocapsid.

A

Vero E6-TMPRSS2-T2A-ACE2 cells



B

Vero E6-TMPRSS2 cells

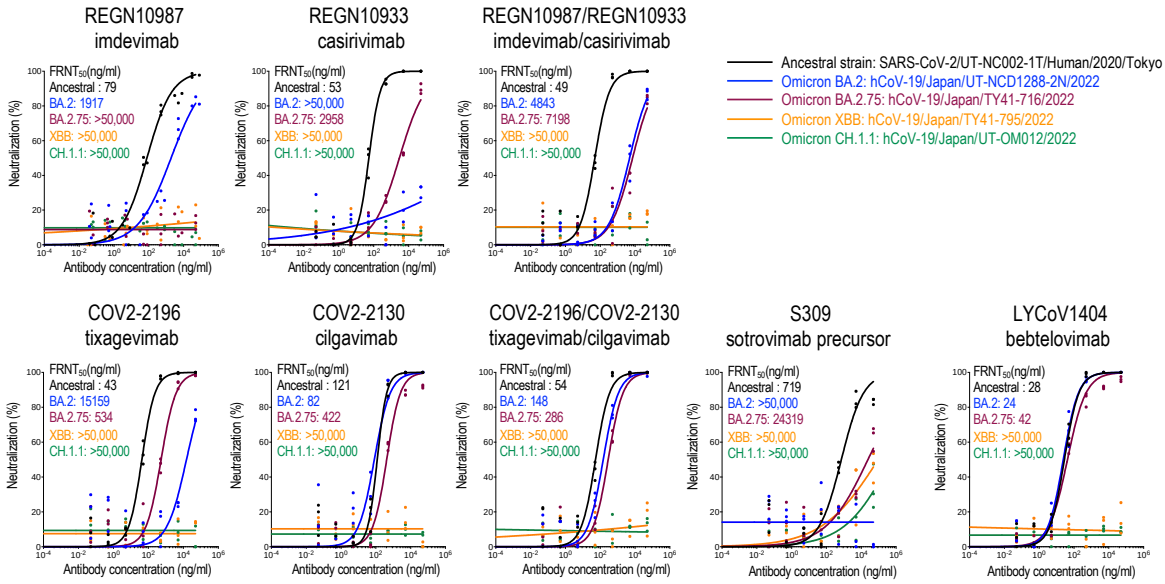


Figure S2. *In vitro* neutralising activity of therapeutic monoclonal antibodies against Omicron subvariants.

The 50% focus reduction neutralisation test titres ($FRNT_{50}$) of therapeutic monoclonal antibodies were determined in Vero E6-TMPRSS2-T2A-ACE2 cells (A) or VeroE6/TMPRSS2 cells (B). The antibodies used in this assay were produced in the authors' laboratories and are not identical to the commercially available products. Data are the mean values for triplicate experiments. Statistical analysis of the data was not performed.

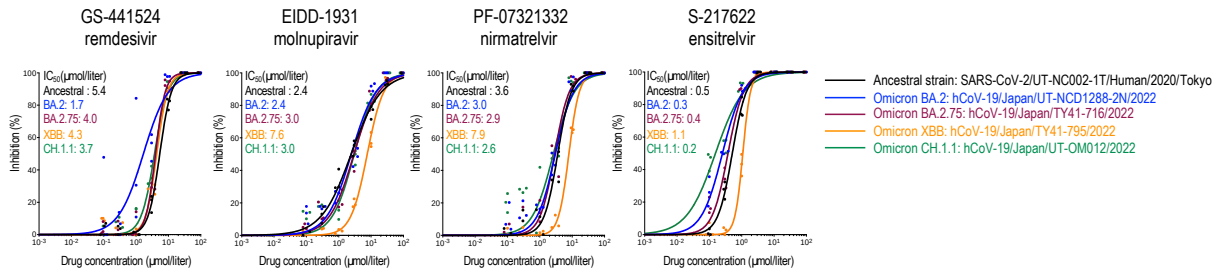


Figure S3. *In vitro* inhibitory activity of antiviral drugs against Omicron subvariants.

The *in vitro* 50% inhibitory concentration (IC_{50}) values were determined in Vero E6-TMPRSS2-T2A-ACE2 cells. GS-441524 (the main metabolite of remdesivir) and EIDD-1931 (the active form of molnupiravir) are RNA-dependent RNA polymerase inhibitors. PF-07321332 (nirmatrelvir) and S-217622 (ensitrelvir) are inhibitors of Mpro (also called 3CLpro). Data are the mean values for triplicate experiments. Statistical analysis of the data was not performed.

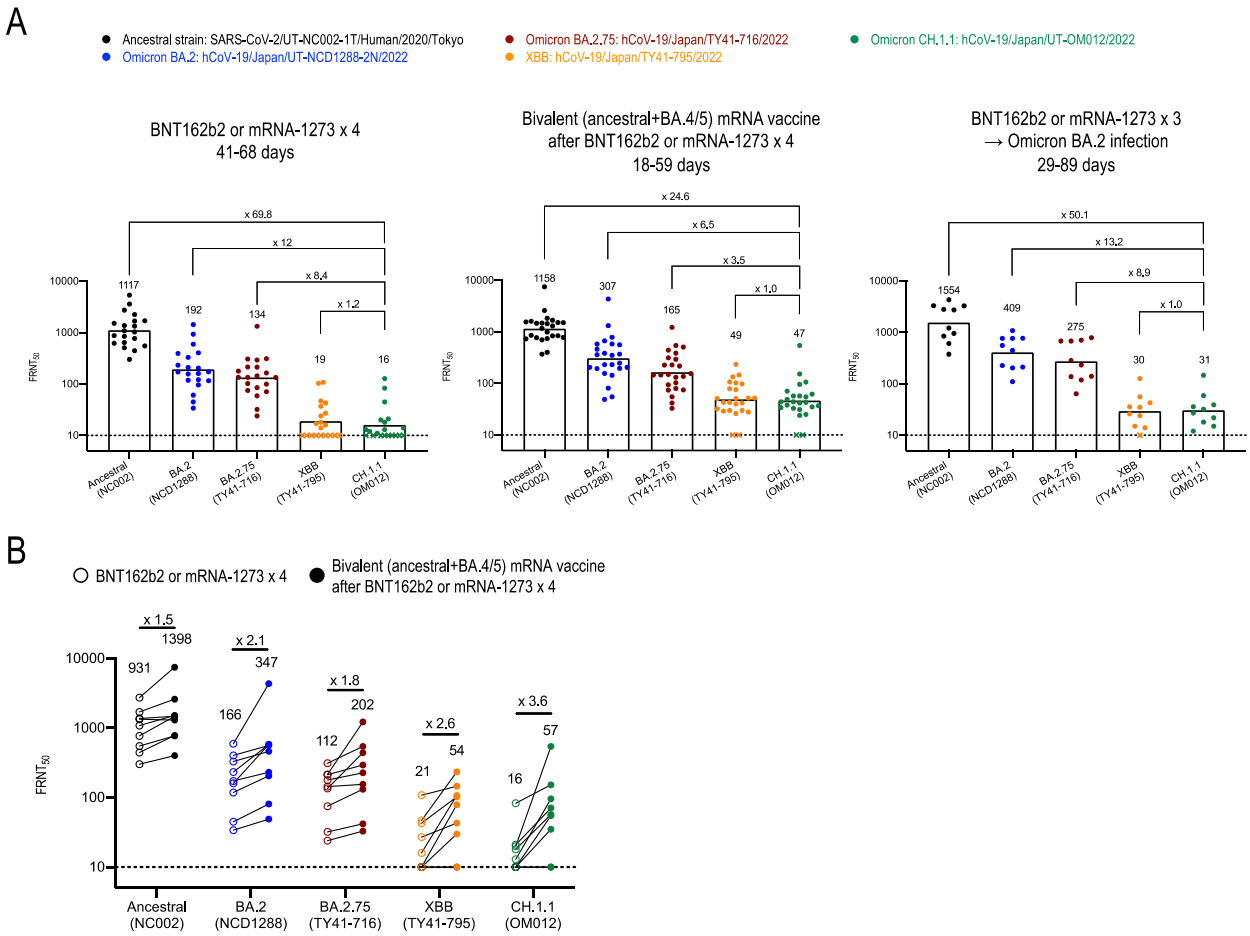


Figure S4. *In vitro* neutralising activity of plasma against SARS-CoV-2 omicron variants
(A) The neutralising titres of plasma samples obtained from individuals who had received four doses of BNT162b2 or mRNA-1273 vaccine (n=20), individuals immunized with the bivalent (ancestral and BA.4/5) vaccine as a fifth dose (n=24), and patients who were infected with the omicron BA.2 subvariant after receiving either the BNT162b2 or mRNA-1273 vaccine (n=10). The range in days is range for the number of days since the last vaccination was received. Detailed information about the participants is provided in Tables S1, S2, and S3. $FRNT_{50}$ values were determined in Vero E6-TMPRSS2-T2A-ACE2 cells. Each dot represents data from one individual. The lower limit of detection (value=10) is indicated by the horizontal dashed line. Samples under the detection limit (<10-fold dilution) were assigned an $FRNT_{50}$ of 10 and are represented by X. Geometric mean titres are shown. (B) The neutralising titres of plasma samples from the same individuals (n=9) after receiving four doses of BNT162b2 or mRNA-1273 vaccine compared with those after receiving the bivalent (ancestral and BA.4/5) vaccine as a fifth dose. Geometric mean titres are shown. Each line represents data from one individual.

Table S1. Neutralising antibody titres of human plasma from individuals who received four doses of COVID-19 vaccine

Sample ID	Age	Gender	Plasma collection day post-final vaccination	Vaccine	FRNT50: 50% focus reduction neutralisation titre				
					SARS-CoV-2/UT-NC002-1T/Human/2020/Tokyo (A)	hCoV-19/Japan/UT-NCD1288-2N/2022 (Omicron BA.2)	hCoV-19/Japan/TY41-716/2022 (Omicron BA.2.75)	hCoV-19/Japan/TY41-795/2022 (Omicron XBB)	hCoV-19/Japan/UT-OM012/2022 (Omicron CH.1.1)
HP(H)-032	39	M	53	BNT162b2 x 3, mRNA-1273 x 1	849	116	135	18	13
HP(H)-058	58	F	48	BNT162b2 x 3, mRNA-1273 x 1	5346	1433	1327	103	128
HP(H)-088	46	F	43	BNT162b2 x 3, mRNA-1273 x 1	1371	233	213	43	21
HP(H)-113	62	M	41	BNT162b2 x 3, mRNA-1273 x 1	1516	172	134	17	<10
HP(H)-158	29	F	41	BNT162b2 x 3, mRNA-1273 x 1	552	45	24	<10	<10
HP(H)-182	52	F	54	BNT162b2 x 4	2250	395	293	24	14
HP(H)-183	47	F	44	BNT162b2 x 3, mRNA-1273 x 1	1685	204	85	<10	<10
HP(H)-185	48	F	50	BNT162b2 x 3, mRNA-1273 x 1	2730	596	215	47	20
HP(H)-189	57	F	68	BNT162b2 x 4	1085	173	144	27	18
HP(H)-198	33	F	45	BNT162b2 x 3, mRNA-1273 x 1	875	121	102	<10	<10
HP(H)-220	34	M	44	BNT162b2 x 3, mRNA-1273 x 1	640	96	71	<10	11
HP(H)-228	49	F	51	BNT162b2 x 3, mRNA-1273 x 1	1335	331	179	<10	<10
HP(H)-241	62	F	57	BNT162b2 x 4	771	160	135	16	13
HP(H)-250	35	F	49	BNT162b2 x 3, mRNA-1273 x 1	3601	930	305	37	45
HP(H)-255	51	F	42	BNT162b2 x 3, mRNA-1273 x 1	1034	211	164	14	12
HP(H)-264	43	M	41	BNT162b2 x 3, mRNA-1273 x 1	623	61	59	<10	<10
HP(H)-282	43	M	48	BNT162b2 x 3, mRNA-1273 x 1	444	118	75	<10	<10
HP(H)-297	56	M	45	BNT162b2 x 3, mRNA-1273 x 1	1700	405	312	108	83
HP(H)-299	53	F	53	BNT162b2 x 3, mRNA-1273 x 1	303	34	32	<10	<10
HP(H)-303	51	M	49	BNT162b2 x 4	507	157	100	<10	<10

Table S2. Neutralising antibody titres of human plasma from individuals who received the bivalent (ancestral and BA.4/5) mRNA vaccine after four doses of COVID-19 vaccine

Sample ID	Age	Gender	Plasma collection day post-final vaccination	Vaccine	FRNT50: 50% focus reduction neutralisation titre				
					SARS-CoV-2/UT-NC002-1T/Human/2020/Tokyo (A)	hCoV-19/Japan/UT-NCD1288-2N/2022 (Omicron BA.2)	hCoV-19/Japan/TY41-716/2022 (Omicron BA.2.75)	hCoV-19/Japan/TY41-795/2022 (Omicron XBB)	hCoV-19/Japan/UT-OM012/2022 (Omicron CH.1.1)
HP(H)-019	57	F	57	BNT162b2 x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	731	197	137	52	44
HP(H)-088	46	F	18	BNT162b2 x 3, mRNA-1273 x 1, bivalent (Moderna)	1485	581	295	102	71
HP(H)-101	49	M	18	BNT162b2 x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	994	194	146	42	43
HP(H)-148	45	F	21	BNT162b2 x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	1172	205	74	29	49
HP(H)-152	54	F	21	BNT162b2 x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	1513	673	218	98	57
HP(H)-158	29	F	21	BNT162b2 x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	769	81	33	<10	<10
HP(H)-172	53	F	19	BNT162b2 x 3, mRNA-1273 x 1, bivalent (Moderna)	701	144	75	32	31
HP(H)-173	40	M	59	BNT162b2 x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	1833	788	313	51	33
HP(H)-179	52	F	21	BNT162b2 x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	743	370	150	30	44
HP(H)-185	49	F	21	BNT162b2 x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	7480	4351	1226	234	543
HP(H)-189	58	F	22	BNT162b2 x 4, bivalent (Pfizer/BioNTech)	1518	231	155	43	57
HP(H)-215	49	M	21	BNT162b2 x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	1677	1319	508	134	106
HP(H)-228	49	F	18	BNT162b2 x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	1309	463	227	79	55
HP(H)-230	35	M	18	BNT162b2 x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	1555	248	80	40	25
HP(H)-235	43	F	42	BNT162b2 x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	370	55	55	26	38
HP(H)-241	62	F	18	BNT162b2 x 4, bivalent (Pfizer/BioNTech)	1476	585	441	107	96
HP(H)-247	49	F	28	BNT162b2 x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	841	231	146	43	24
HP(H)-248	60	F	18	BNT162b2 x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	838	360	103	28	37
HP(H)-252	49	M	21	BNT162b2 x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	859	156	94	51	42
HP(H)-282	44	M	21	BNT162b2 x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	779	205	132	30	35
HP(H)-297	57	M	18	BNT162b2 x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	2601	562	544	146	152
HP(H)-299	54	F	30	BNT162b2 x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	401	49	42	<10	<10
HP(H)-300	72	F	21	BNT162b2 x 4, bivalent (Pfizer/BioNTech)	1440	385	387	76	62
HP(H)-305	71	M	34	BNT162b2 x 4, bivalent (Pfizer/BioNTech)	1613	351	228	52	34

Table S3. Neutralising antibody titres of human plasma from individuals who were infected with the Omicron BA.2 variant after three doses of COVID-19 vaccine

Sample ID	Age	Gender	Plasma collection day post-onset	Vaccine	FRNT50: 50% focus reduction neutralisation titre				
					SARS-CoV-2/UT-NC002-1T/Human/2020/Tokyo (A)	hCoV-19/Japan/UT-NCD1288-2N/2022 (Omicron BA.2)	hCoV-19/Japan/TY41-716/2022 (Omicron BA.2.75)	hCoV-19/Japan/TY41-795/2022 (Omicron XBB)	hCoV-19/Japan/UT-OM012/2022 (Omicron CH.1.1)
HPCo-383	56	F	29	mRNA-1273 x 2, BNT162b2 x 1	376	110	64	<10	18
HP-S(H)0377	29	M	44	BNT162b2 x 3	609	227	119	36	27
HP-S(H)0380	22	M	42	BNT162b2 x 3	2804	448	233	26	22
HP-S(H)0381	22	M	44	BNT162b2 x 3	959	204	138	15	12
HP-S(H)0382	21	M	44	BNT162b2 x 3	4311	769	680	43	59
HP-S(H)0383	18	M	42	BNT162b2 x 3	1219	557	284	24	36
HP-S(H)0882	23	M	89	BNT162b2 x 3	849	212	140	14	15
HP-S(H)0883	24	M	84	BNT162b2 x 3	2735	765	679	35	32
HP-S(H)0888	21	M	89	BNT162b2 x 3	3317	767	705	56	39
HP-S(H)1056	22	M	42	BNT162b2 x 3	3287	1087	793	127	147

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