

Supplementary Appendix

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This appendix has been provided by the authors to give readers additional information about the work.

Supplementary Appendix

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Materials and Methods

Ethics statement

For the use of human specimens, all protocols involving human subjects recruited at Kyoto University and Chiba University were reviewed and approved by the Institutional Review Boards of Kyoto University (approval number G0697) and Chiba University (approval number HS202103-03). All human subjects provided written informed consent.

Human sera

Peripheral blood was collected from three COVID-19 convalescents and the sera were isolated (**Table S6**). Moreover, peripheral blood was collected four weeks after the second vaccination with BNT162b2 (Pfizer-BioNTech), and the sera of fourteen vaccinated-individuals were isolated (**Table S7**). Sera were inactivated at 56°C for 30 min and stored at –80°C until use. Ten additional COVID-19 convalescent sera were purchased from RayBiotech (**Table S6**).

Epidemic data

The data of daily COVID-19 cases in Colombia (until August 31, 2021) were downloaded from Our World in Data (<https://ourworldindata.org/covid-cases>)¹ on September 2, 2021.

Viral genome sequences

SARS-CoV-2 annotation information used in this study was downloaded from the GISAID database (<https://www.gisaid.org>) as of August 30, 2021 (3,059,698 genomes). Based on the annotation by Phylogenetic Assignment of Named Global Outbreak (PANGO), we determined the daily frequency of SARS-CoV-2 Gamma (P.1), Delta (B.1.617.2, AY.4, AY.5, AY.12), Lambda (C.37), and Mu (B.1.621) variants isolated in Colombia. We also examined amino acid replacements of the spike protein of each SARS-CoV-2 variant by comparing it to the sequence of the Wuhan-Hu-1 strain (GISAID ID: EPI_ISL_1532199).

Cell culture

HEK293T cells (a human embryonic kidney cell line; ATCC CRL-3216) and HOS-ACE2/TMPRSS2 cells,^{2,3} a derivative of HOS cells (a human osteosarcoma cell line; ATCC CRL-1543) stably expressing human ACE2 and TMPRSS2, were maintained in Dulbecco's modified Eagle's medium (high glucose) (Wako, Cat# 044-29765) containing 10% fetal calf serum, 100 units penicillin and 100 ug/ml streptomycin.

Plasmid construction

Plasmids expressing the SARS-CoV-2 spike proteins of the parental D614G (B.1), Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), Epsilon (B.1.427), and Lambda (C.37) variants

were prepared in our previous studies.³⁻⁵ A plasmid expressing the spike protein of the Mu (B.1.621) variant was generated by site-directed overlap extension PCR using pC-SARS2-S D614G³ as the template and the primers listed in **Table S8**. The resulting PCR fragment was digested with KpnI and NotI and inserted into the corresponding site of the pCAGGS vector.⁶ Nucleotide sequences were determined by DNA sequencing services (Eurofins), and the sequence data were analyzed by Sequencher v5.1 software (Gene Codes Corporation).

Neutralization assay

Pseudoviruses were prepared as previously described.^{3-5,7} Briefly, lentivirus (HIV-1)-based, luciferase-expressing reporter viruses were pseudotyped with the SARS-CoV-2 spikes. HEK293T cells (1×10^6 cells) were cotransfected with 1 μ g psPAX2-IN/HiBiT,⁸ 1 μ g pWPI-Luc2,⁸ and 500 ng plasmids expressing parental S or its derivatives using PEI Max (Polysciences, Cat# 24765-1) according to the manufacturer's protocol. Two days post transfection, the culture supernatants were harvested and centrifuged. The pseudoviruses were stored at -80°C until use.

Neutralization assays were performed as previously described.^{4,7} Briefly, the SARS-CoV-2 spike pseudoviruses (counting $\sim 20,000$ relative light units) were incubated with serially diluted (40-, 120-, 360-, 1,080-, 3,240-, 9,720-, and 29,160-fold dilution at the final concentration) heat-inactivated human sera at 37°C for 1 h. Pseudoviruses without sera were included as controls. Then, an 80 μ l mixture of pseudovirus and serum was added to HOS-ACE2/TMPRSS2 cells (10,000 cells/50 μ l) in a 96-well white plate. Two days post infection, the infected cells were lysed with a One-Glo luciferase assay system (Promega, Cat# E6130), and the luminescent signal was measured using a GloMax explorer multimode microplate reader 3500 (Promega). The assay of each serum was performed in triplicate, and the 50% neutralization titer was calculated using Prism 9 (GraphPad Software).

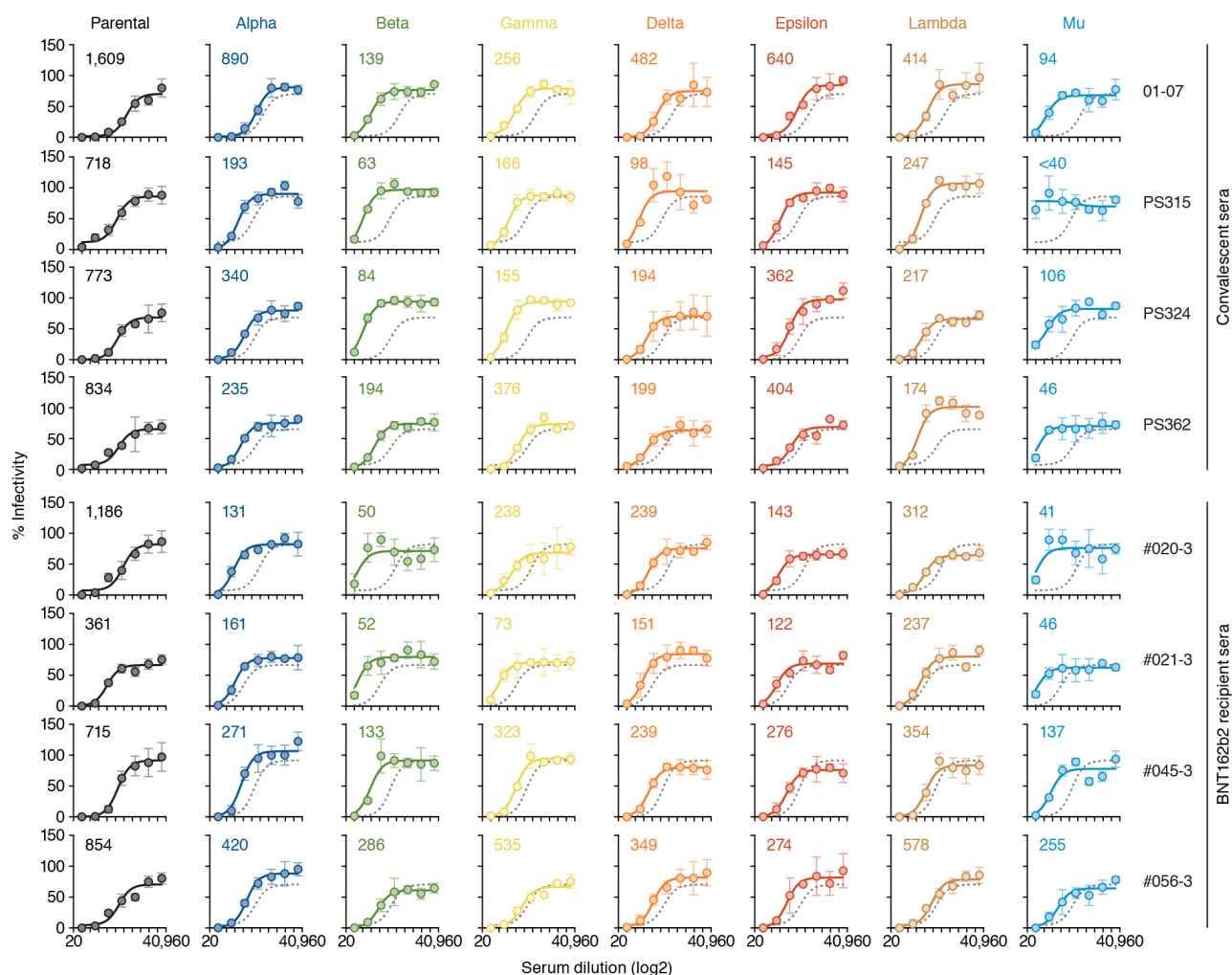


Figure S1. Representative neutralization curves. Representative results of virus neutralization assays using four convalescent sera (donors 01-07, PS315, PS324 and PS362; top panel) and four BNT162b2-vaccinated sera (donors #020-3, #021-3, #045-3 and #056-3; bottom panel) are shown. The percentages of pseudovirus infectivity compared to that without sera are shown. Assays were performed in triplicate, and data are average with standard deviation. The number in each panel indicates the 50% neutralization titers of the respective serum. The fitting curve of parental virus of respective serum is shown as broken black lines in all panels. The 50% neutralization titers are summarized in **Tables S6 and S7**.

Table S1. Number of Mu variant sequences isolated in each country.

Country	B.1.621 (Mu)	B.1.621.1
USA	1,492	505
Colombia	844	8
Spain	344	122
Mexico	338	6
Ecuador	168	0
Aruba	74	5
Chile	70	2
Netherlands	68	2
Italy	63	14
United Kingdom	48	35
Costa Rica	46	0
Canada	43	4
Switzerland	23	25
Belgium	22	12
Portugal	21	3
Curacao	14	5
France	14	1
Brazil	11	0
Denmark	7	0
Germany	7	7
Peru	6	0
Poland	6	0
Bonaire	5	1
Venezuela	5	0
Finland	3	0
Sweden	3	0
Dominican Republic	2	41
Hong Kong	2	0
Ireland	2	2
Japan	2	0
Slovakia	2	1
Turkey	2	0
Austria	1	48
Romania	1	0
Sint Maarten	1	1
Liechtenstein	1	0
Turks and Caicos Islands	1	0
Luxembourg	0	1
Malta	0	1

Table S2. Weekly new cases and numbers of different VOCs/VOIs in Colombia.

Week number	Date from (MM/DD)	Weekly new cases	Number					Percentage				
			Gamma	Delta	Lambda	Mu	Others	Gamma	Delta	Lambda	Mu	Others
1	01/04	111,080	4	0	0	0	41	8.9	0	0	0	91.1
2	01/11	121,513	1	0	0	1	44	2.2	0	0	2.2	95.7
3	01/18	107,072	8	0	0	0	29	21.6	0	0	0	78.4
4	01/25	79,399	2	0	0	0	16	11.1	0	0	0	88.9
5	02/01	62,332	5	0	0	0	6	45.5	0	0	0	54.5
6	02/08	37,823	2	0	0	0	8	20.0	0	0	0	80.0
7	02/15	31,223	5	0	0	1	6	41.7	0	0	8.3	50.0
8	02/22	25,428	0	0	0	5	16	0	0	0	23.8	76.2
9	03/01	24,966	4	0	0	8	36	8.3	0	0	16.7	75.0
10	03/08	26,488	8	0	0	13	22	18.6	0	0	30.2	51.2
11	03/15	34,086	8	0	0	7	19	23.5	0	0	20.6	55.9
12	03/22	45,500	5	0	0	11	30	10.9	0	0	23.9	65.2
13	03/29	63,489	16	0	1	23	31	22.5	0	1.4	32.4	43.7
14	04/05	89,979	111	0	8	85	152	31.2	0	2.2	23.9	42.7
15	04/12	116,749	95	0	6	62	85	38.3	0	2.4	25.0	34.3
16	04/19	121,517	36	0	3	54	35	28.1	0	2.3	42.2	27.3
17	04/26	119,191	50	0	5	47	50	32.9	0	3.3	30.9	32.9
18	05/03	109,103	40	0	5	78	42	24.2	0	3.0	47.3	25.5
19	05/10	115,668	37	0	5	56	29	29.1	0	3.9	44.1	22.8
20	05/17	114,030	32	0	0	51	12	33.7	0	0	53.7	12.6
21	05/24	150,823	10	0	1	18	3	31.3	0	3.1	56.3	9.4
22	05/31	187,788	3	0	0	15	6	12.5	0	0	62.5	25.0
23	06/07	182,157	10	0	3	33	7	18.9	0	5.7	62.3	13.2
24	06/14	191,942	6	0	0	22	2	20.0	0	0	73.3	6.7
25	06/21	213,550	7	0	1	20	1	24.1	0	3.4	69.0	3.4
26	06/28	191,779	4	1	4	49	5	6.3	1.6	6.3	77.8	7.9
27	07/05	161,465	2	2	1	30	5	5.0	5.0	2.5	75.0	12.5
28	07/12	127,506	2	5	1	39	5	3.8	9.6	1.9	75.0	9.6
29	07/19	88,380	0	3	1	26	0	0	10.0	3.3	86.7	0
30	07/26	66,568	0	3	0	3	0	0	50.0	0	50.0	0
31	08/02	44,570	0	0	0	1	0	0	0	0	100.0	0

Table S3. Haplotypes of the spike protein of Mu variant.

Haplotype	Sequence number	Percentage
T95I YY144-145TSN R346K E484K N501Y D614G P681H D950N	1488	39.6
T95I YY144-145TSN R346K K417N E484K N501Y D614G P681H D950N	189	5.0
T95I YY144-145TSN R346K E484K N501Y D614G P681H	129	3.4
T95I YY144-145TSN R346K E484K N501Y D614G P681H D950N M1229I	128	3.4
T95I YY144-145SN R346K E484K N501Y D614G P681H D950N	111	3.0
T95I V143VT Y145N R346K E484K N501Y D614G P681H D950N	108	2.9
T95I R346K E484K N501Y D614G P681H D950N	76	2.0
T95I YY144-145TSN R346K E484K N501Y T572I D614G P681H D950N	58	1.5
L5F T95I YY144-145TSN R346K E484K N501Y D614G P681H D950N	45	1.2
T95I YY144-145TSN R346K D614G P681H D950N	43	1.1

Table S4. Mutations in the spike protein of Mu variant.

Mutation	Sequence number	Percentage
D614G	3,755	99.8
P681H	3,743	99.5
R346K	3,684	97.9
N501Y	3,607	95.9
T95I	3,604	95.8
E484K	3,591	95.5
D950N	3,385	90.0
Y145N	3,264	86.8
Y144S	3,053	81.2
V143VT	2,952	78.5
K417N	302	8.0
M1229I	192	5.1
Y144T	97	2.6
T572I	96	2.6
Y145SN	71	1.9
M1237I	64	1.7
L5F	64	1.7
E583D	41	1.1
E1258D	39	1.0

Table S5. Mutations in the spike proteins of SARS-CoV-2 variants used in this study.

Classification	Parental	Alpha	Beta	Gamma	Delta	Epsilon	Lambda	Mu
PANGO lineage	B.1	B.1.1.7	B.1.351	P.1	B.1.617.2	B.1.427	C.37	B.1.621
Mutations	D614G	HV69-70del	L18F	L18F	T19R	S13I	G75V	T95I
		Y144del	D80A	T20N	G142D	W152C	T76I	YY144-145TSN
		N501Y	D215G	P26S	EFR156-158G	L452R	RSYLTPGD246-253N	R346K
		A570D	R246I	D138Y	L452R	D614G	L452Q	E484K
		D614G	K417N	R190S	T478K		F490S	N501Y
		P681H	E484K	K417T	D614G		D614G	D614G
		T716I	N501Y	E484K	P681R		T859N	P681H
		S982A	D614G	N501Y	D950N			D950N
		D1118H	A701V	D614G				
				H655Y				
				T1027I				

Table S6. Summary of COVID-19 convalescent sera used in this study.

Donor ID	Sex	Age	Severity	Date of test (MM/DD/YY)	Date of sampling (MM/DD/YY)	50% neutralization titer							
						Parental	Alpha	Beta	Gamma	Delta	Epsilon	Lambda	Mu
01-07	Male	57	Severe	09/01/20	11/04/20	1,609	890	139	256	482	640	414	94
03-03	Male	58	Severe	09/30/20	12/04/20	500	158	<40	131	92	200	163	<40
12-01	Male	86	Moderate	08/19/20	09/18/20	293	370	117	95	222	260	159	44
PS315 ^a	Female	61	NA ^b	04/01/20	05/03/20	718	193	63	166	98	145	247	<40
PS324 ^a	Female	76	NA ^b	04/01/20	05/03/20	773	340	84	155	194	362	217	106
PS329 ^a	Female	92	NA ^b	04/02/20	05/03/20	7,834	2,801	1,365	2,697	5,039	3,613	4,442	844
PS362 ^a	Female	52	NA ^b	04/17/20	05/17/20	834	235	194	376	199	404	174	46
PS364 ^a	Female	74	NA ^b	04/17/20	05/17/20	2,579	893	320	537	503	624	489	260
PS313 ^a	Female	69	NA ^b	04/01/20	05/03/20	327	147	48	225	88	278	76	47
PS314 ^a	Female	64	NA ^b	04/02/20	05/03/20	318	132	172	377	129	877	129	<40
PS330 ^a	Female	60	NA ^b	04/02/20	05/03/20	430	194	42	420	106	260	84	<40
PS340 ^a	Female	73	NA ^b	04/02/20	05/03/20	349	210	68	542	117	371	214	48
PS354 ^a	Male	71	NA ^b	04/17/20	05/17/20	1,762	2,054	973	1,777	501	1,502	569	265
Geometric mean						806	378	153	357	235	471	257	111

^aPurchased from RayBiotech.^bNot applicable.

Table S7. Summary of BNT162b2-vaccinated sera used in this study.

Donor ID	Sex	Age	Date of 2nd vaccination (MM/DD/YY)	Date of sampling (MM/DD/YY)	50% neutralization titer							
					Parental	Alpha	Beta	Gamma	Delta	Epsilon	Lambda	Mu
#005-3	Female	34	04/30/21	05/27/21	555	440	64	168	84	225	228	<40
#006-3	Female	41	04/30/21	05/27/21	2,417	880	248	1,171	623	641	1,062	259
#007-3	Female	35	04/26/21	05/24/21	613	415	128	202	260	138	219	<40
#020-3	Female	43	04/30/21	05/28/21	1,186	131	50	238	239	143	312	41
#021-3	Female	47	04/27/21	05/25/21	361	161	52	73	151	122	237	46
#026-3	Female	28	04/26/21	05/25/21	206	179	58	289	149	581	127	69
#027-3	Male	41	04/27/21	05/25/21	368	227	205	300	96	89	195	52
#028-3	Female	42	04/28/21	05/26/21	595	597	<40	112	520	463	434	112
#030-3	Female	29	04/27/21	05/25/21	256	81	97	112	224	319	272	59
#036-3	Male	34	04/27/21	05/27/21	448	192	54	334	218	179	358	70
#045-3	Female	34	04/30/21	05/24/21	715	271	133	323	239	276	354	137
#056-3	Male	38	04/30/21	05/26/21	854	420	286	535	349	274	578	255
#078-3	Female	32	04/22/21	05/06/21	1,260	691	141	291	265	347	579	121
#103-3	Male	35	04/07/21	05/07/21	313	1,407	66	112	511	203	406	44
Geometric mean					575	324	101	233	239	243	332	85

Table S8. Primers used for the construction of Mu spike expression plasmid.

Primer name	Sequence (5'-to-3')
T95I Fwd	TCTACTTTGCCAGCAttGAGAAGAGCAACATC
T95I Rev	GATGTTGCTCTTCTCaaTGCTGGCAAAGTAGA
YY144-145TSN Fwd	CCATTCCTGGGAGTCacctccaacCACAAGAACAACAAG
YY144-145TSN Rev	CTTGTTGTTCTTGTGggttgaggGACTCCCAGGAATGG
R346K Fwd	GTTCAATGCCACCAaGTTTGCCTCTGTCT
R346K Rev	AGACAGAGGCCAAACtGGTGGCATTGAAC
E484K Fwd	CCATGTAATGGAGTGaAGGGCTTCAACTGTT
E484K Rev	AACAGTTGAAGCCCTtCACTCCATTACATGG
N501Y Fwd	TGGCTTCCAACCAACCTATGGAGTGGGCTA
N501Y Rev	TAGCCCACTCCATaGGTTGGTTGGAAGCCA
P681H Fwd	CCCAGACCAACAGCCatAGGAGGGCAAGGTCT
P681H Rev	AGACCTTGCCCTCCTatGGCTGTTGGTCTGGG
D950N Fwd	CTGGGCAAACCTCCAAaATGTGGTGAACCAG
D950N Rev	CTGGTTCACCACATtTTGGAGTTTGCCCAG

Consortia

The Genotype to Phenotype Japan (G2P-Japan) Consortium

Institute of Medical Science, University of Tokyo, Japan

Jumpei Ito, Daichi Yamasoba, Yusuke Kosugi, Mai Suganami, Akiko Oide,
Miyabishara Yokoyama, Mika Chiba

Tokai University, Japan

Jiaqi Wu, Miyoko Takahashi

Kyoto University, Japan

Yasuhiro Kazuma, Ryosuke Nomura, Yoshihito Horisawa

Chiba University, Japan

Motoaki Seki, Ryoji Fujiki, Tadanaga Shimada, Kiyoshi Hirahara, Koutaro Yokote,
Toshinori Nakayama

Hiroshima University, Japan

Takashi Irie, Ryoko Kawabata, Nanami Morizako

Hokkaido University, Japan

Takasuke Fukuhara, Kenta Shimizu, Kana Tsushima, Haruko Kubo

Kumamoto University, Japan

Terumasa Ikeda, Chihiro Motozono, Hesham Nasser, Ryo Shimizu, Yue Yuan,
Kazuko Kitazato, Haruyo Hasebe, Takamasa Ueno

University of Miyazaki, Japan

Akatsuki Saito, Erika P Butlertanaka, Yuri L Tanaka

National Institute of Infectious Diseases, Japan

Kenzo Tokunaga, Seiya Ozono

Tokyo Metropolitan Institute of Public Health, Japan

Kenji Sadamasu, Hiroyuki Asakura, Isao Yoshida, Mami Nagashima, Kazuhisa Yoshimura

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