

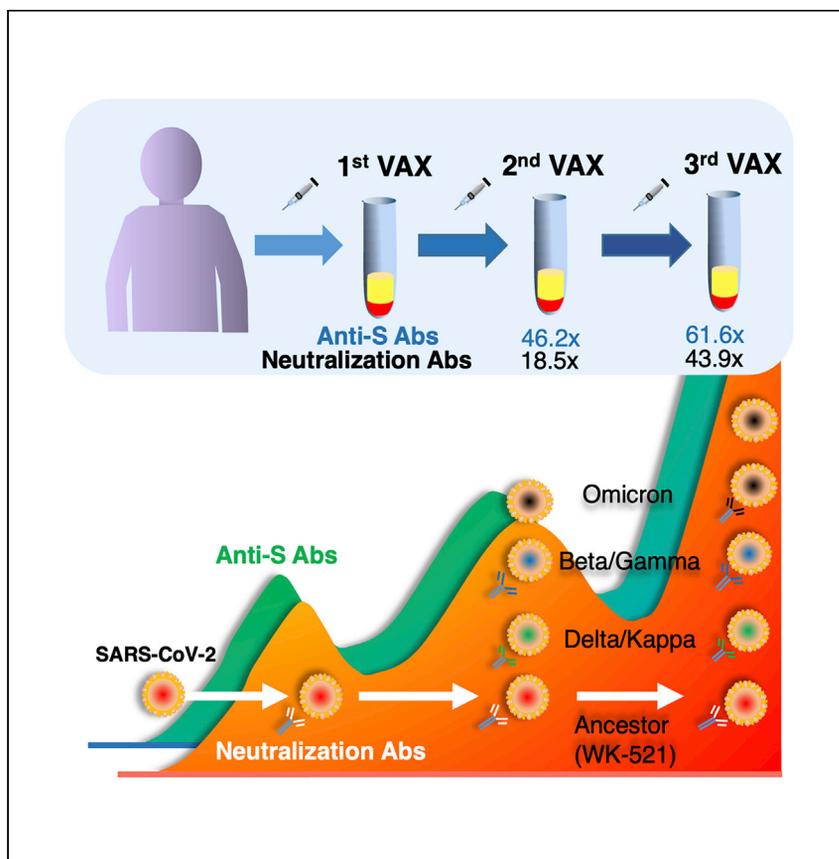


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Clinical and Translational Article

Safety and immunogenicity of the Pfizer/BioNTech SARS-CoV-2 mRNA third booster vaccine dose against the BA.1 and BA.2 Omicron variants



Seki et al. report that a three-dose Pfizer/BioNTech mRNA vaccination induced a robust increase in anti-spike antibodies and neutralization titers against the WK-521, Delta, and Omicron variants. Immunogenicity against Omicron subvariants, including BA.1, BA.1.1, and three different BA.2 subvariants did not change following the third vaccine dose.

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Highlights

Neutralization titers (NTs) against Omicron decreased after two-dose mRNA vaccination

Three-dose vaccination increased anti-spike antibody and NTs against Omicron variants

Immunogenicity against Omicron subvariants did not change after three-dose vaccination

The cytokine signature remained unchanged after three-dose vaccination



Translation to Humans

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Clinical and Translational Article

Safety and immunogenicity of the Pfizer/BioNTech SARS-CoV-2 mRNA third booster vaccine dose against the BA.1 and BA.2 Omicron variants

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SUMMARY

Background: The Omicron variant of severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) was identified in Japan in November 2021. This variant contains up to 36 mutations in the spike protein, the target of neutralizing antibodies, and can escape vaccine-induced immunity. A booster vaccination campaign began with healthcare workers and high-risk groups. The safety and immunogenicity of the three-dose vaccination against Omicron remain unknown.

Methods: A total of 272 healthcare workers were initially evaluated for long-term vaccine safety and immunogenicity. We further established a vaccinee panel to evaluate the safety and immunogenicity against variants of concern (VOCs), including the Omicron variants, using a live virus microneutralization assay.

Findings: Two-dose vaccination induced robust anti-spike antibodies and neutralization titers (NTs) against the ancestral strain WK-521, whereas NTs against VOCs were significantly lower. Within 93–247 days of the second vaccine dose, NTs against Omicron were completely abolished in up to 80% of individuals in the vaccinee panel. Booster dose induced a robust increase in anti-spike antibodies and NTs against the WK-521, Delta, and Omicron variants. There were no significant differences in the neutralization ability of sera from boosted individuals among the Omicron subvariants BA.1, BA.1.1, and BA.2. Boosting increased the breadth of humoral immunity and cross-reactivity with Omicron without changes in cytokine signatures and adverse event rate.

Conclusions: The third vaccination dose is safe and increases neutralization against Omicron variants.

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INTRODUCTION

Severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) emerged at the end of 2019, in Wuhan, China, and rapidly spread worldwide.¹ The disease was declared a pandemic by the World Health Organization (WHO) in March 2020.² In Japan, the first coronavirus disease 2019 (COVID-19) case was reported in January³ and the second in February 2020, after which a large number of COVID-19 cases were reported from the Diamond Princess cruise ship at the Port of Yokohama near Tokyo,

Context and significance

The SARS-CoV-2 Omicron variant, later named BA.1, has emerged as a highly transmissible variant due to the 36 mutations in its spike protein, which is the target of neutralizing antibodies; it can therefore escape vaccine-induced immunity. The Omicron subvariant, BA.2, was recently identified and has rapidly become a major variant of concern in many countries, including Japan. This study found that anti-spike antibody levels and neutralization ability decreased gradually 6–9 months after the second vaccination. A third dose dramatically increased the response against multiple Omicron variants. These results show that a booster shot increases neutralization antibodies against SARS-CoV-2 variants.

Japan.⁴ The number of infections increased slightly within cities, but the first major wave of infection of more than 10,000 cases per day occurred in April 2020. The Japanese government declared its first state of emergency in major cities, including Tokyo, and implemented restrictive measures. As of January 19, 2022, the Japanese government has declared a state of emergency four times following the five pandemic waves in Japan.⁵

During the second wave of the pandemic, the SARS-CoV-2 strain presenting the D614G mutation in the spike region (which affects its transmissibility⁶ and infectivity^{7,8}) emerged and rapidly increased the percentage of total SARS-CoV-2 infections. The newly emerged Alpha strain (B.1.1.7) was isolated from the United Kingdom and contained an additional E484K mutation, which gradually appeared to increase and replace the dominant endemic virus.⁹ Simultaneously, the Beta strain was isolated from South Africa with a new K417 mutation, along with three of the mutations observed in the Alpha variant (E484K, N501Y, and D614G in the spike protein), enabling the virus to escape vaccine-induced antibodies.¹⁰ The Gamma (P.1) variant, with a new K417T mutation and three previously identified mutations observed in Alpha, was also isolated in Brazil.¹¹

In late summer 2020, many countries began reviewing and authorizing new types of vaccines, such as the Pfizer/BioNTech SARS-CoV-2 mRNA vaccine,¹² the Moderna mRNA vaccine,¹³ the Janssen/Johnson & Johnson adenovirus vector DNA vaccine,¹⁴ and the AstraZeneca adenovirus vector DNA vaccine.¹⁵ In December 2020, the United States and European Union countries approved these vaccines under each entity's Emergency Use Authorization. These SARS-CoV-2 vaccines have reduced remarkably the number of COVID-19 infections, hospitalizations, and deaths in clinical trials in many countries.¹⁶ The Japanese government first approved the two mRNA vaccines in February 2021, after which the vaccination of healthcare workers and people older than age 65 years at high risk for contracting the virus was initiated. The National Hospital Organization Murayama Medical Center (NHO-MMC) is a major Japanese national organization that began two-dose vaccination as part of post-licensure safety and immunogenicity surveillance. The National Institute of Infectious Diseases (NIID) collaborated with NHO-MMC to evaluate neutralization titers (NTs) by performing live virus neutralization assays and to monitor adverse event rates. Major surveillance is ongoing; however, a vaccine panel is required to help understand the effects of vaccination against newly emerged variants of concern (VOCs).

Since August 2021, the Delta variant (B.1.617.2), which has an L452R mutation and previously identified D614G mutations, has emerged and become dominant. Because of the neutralization ability induced by mRNA vaccination,¹⁷ the number of daily Delta variant infections has gradually decreased. In November 2021, the SARS-CoV-2 Omicron variant (BA.1/B.1.1.529) was first detected by sequencing in Botswana and was found to have caused a large number of infections in South Africa. The WHO and, soon after, the Japanese government, declared the Omicron variant as a novel VOC. This strain contains more than 36 mutations in the spike protein, against which vaccines appear to be less effective at inducing immunity. A recent study showed that mutations in the receptor-binding domain (RBD) of the spike protein enable escape from vaccine-induced immunity and increase infectivity by enhancing affinity for angiotensin-converting enzymes.^{18–20} In addition, the Omicron RBD has 15 mutations, some of which overlap with previously reported variant mutations, such as K417, E484, and N501 in Beta (B.1.351) and Gamma (P.1).¹⁹ A recent study showed that the Omicron spike evasion of the virus to the Pfizer/BioNTech mRNA vaccine is 44-fold more efficient than that of the Delta

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variant.²¹ More recently, a new subvariant, BA.2, emerged and replaced more than 85% of the Omicron infections in Denmark,²² 3.6% in the United States,²³ 18.7% in the United Kingdom,²⁴ and 0.5% in Japan.²⁵ At present, 45.19% of total SARS-CoV-2 infections are BA.1, 35.48% are BA.1.1, and 15.56% are BA.2.²⁶ The pathogenicity and transmissibility of Omicron variants are still unknown.

Several studies have suggested that antibodies against SARS-CoV-2 gradually decrease after the second vaccination;²⁷ even 6 months after two-dose mRNA vaccination, durable immune memory to the SARS-CoV-2 VOC is induced.²⁸ Thus, the introduction of a booster dose may be beneficial. Some studies have suggested that booster immunization is effective against the Omicron variant according to a pseudovirus neutralization assay.²⁸ In addition, a heterologous booster can induce greater immune responses and enhance immune protection compared to homologous vaccinations,²⁹ and the immune-boosting strategy is safe for healthy adults aged 18–59 years³⁰ However, whether booster vaccination is necessary to strengthen immunity against SARS-CoV-2 infection and its safety and protection in young and old healthy people remains unclear.

In this study, we performed a longitudinal analysis on a vaccinee panel to monitor the safety and immunogenicity of three-dose vaccination with the Pfizer/BioNTech SARS-CoV-2 mRNA vaccine. We show that booster vaccination in Japanese healthcare workers increased the breadth of humoral immunity and cross-reactivity against the SARS-CoV-2 ancestral strain WK-521, as well as the newly emerged Delta and Omicron VOCs, including BA.1, BA.1.1, and BA.2. The cytokine signatures following the three booster vaccinations were unchanged, and adverse event reports suggest that the three-dose vaccinations are effective and safe.

RESULTS

Establishment of a vaccinee panel from long-term three-dose vaccination study in Japanese healthcare workers

We collected 259 samples after the first and second doses of the Pfizer/BioNTech SARS-CoV-2 mRNA vaccine (Comirnaty) from Japanese healthcare workers (long-term safety and immunogenicity [LT-SI] study, [Figure 1](#)). Anti-spike antibody (SAb) titers were measured using the Roche Elecsys Anti-SARS-CoV-2 (RUO), and NTs were measured using the live virus for the original ancestral vaccine strain, WK-521, of SARS-CoV-2, as described previously.^{31–33} Although anti-SAb and NTs showed a weak correlation at the second vaccination in our preliminary analysis ([Figure 2A](#)), each anti-SAb titer was normally distributed in the NT, 20x, 40x, 80x, 160x, 320x, and 640x groups. We selected 10–14 representative samples from approximately the mean anti-SAb value for each NT ([Figure 2B](#)). Details on the participants in the LT-SI study are listed in [Table 1](#), and the characteristics of the selected vaccinee panel study (VP-SI study) are described in [Table S1](#); briefly, we selected 34–38 samples from a population that reflected the original LT-SI study. We also included individuals who developed adverse events, including fever, headache, fatigue, and injection site pain, matching the frequency of these events initially detected in the LT-SI study 12 days after the second (2nd VAX) and third vaccinations (3rd VAX). There was no significant difference in the anti-SAb and NT distributions between the LT-SI and VP-SI groups ([Figures 2C and 2D](#)).

Three-dose vaccinations induce higher anti-SARS-CoV-2 spike IgG and neutralization antibodies

In our VP-SI study, anti-SAb levels were significantly increased (46x) after the second and third Pfizer/BioNTech SARS-CoV-2 mRNA doses ([Figure 3A](#)) compared the titers

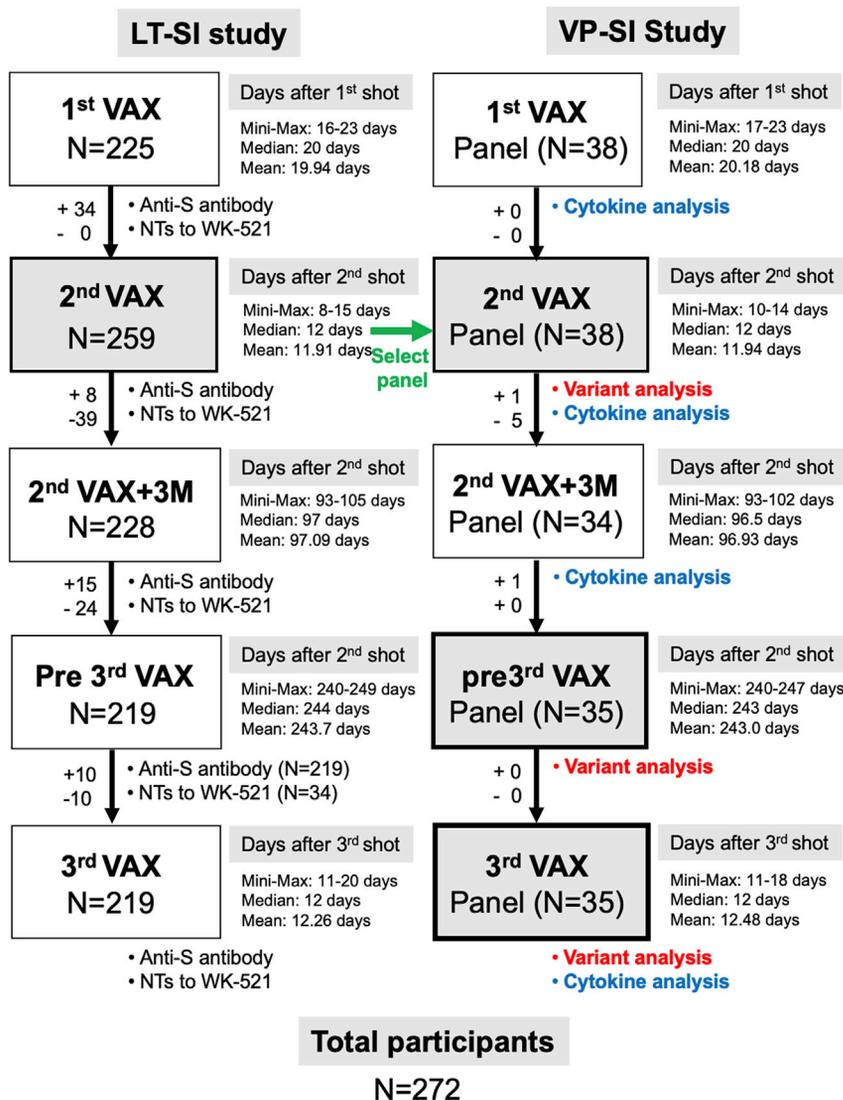


Figure 1. Flowchart of participant enrollment and establishment of vaccine panel

The long-term safety and immunogenicity (LT-SI) study was conducted by National Hospital Organization Murayama Medical Center in collaboration with the National Institute of Infectious Diseases. A total of 272 participants were enrolled in the LT-SI study. The vaccine panel (VP-SI) study was composed of 34–38 selected samples from participants in the LT-SI study matching the original population at the time of second vaccination.

in response to the first dose. Although anti-SAb levels slightly decreased at 97 (2nd VAX + 3M) and 243 days (“pre-3rd” VAX) after the second vaccination, they remained significantly higher than those after the 1st VAX. A similar trend was observed for NT (Figure 3B), which significantly increased (18.5×) after the 2nd VAX and decreased in the months following. The third dose of the mRNA vaccine dramatically and significantly increased both anti-SAb levels (61.6×) and NTs (43.9×) compared to that of the pre-3rd VAX.

Second dose of vaccination was effective for some variants but not for Omicron

The Pfizer mRNA vaccine shows 95% effectiveness against the ancestral strain, WK-521.¹² However, some groups reported that NTs against the Beta and Kappa strains

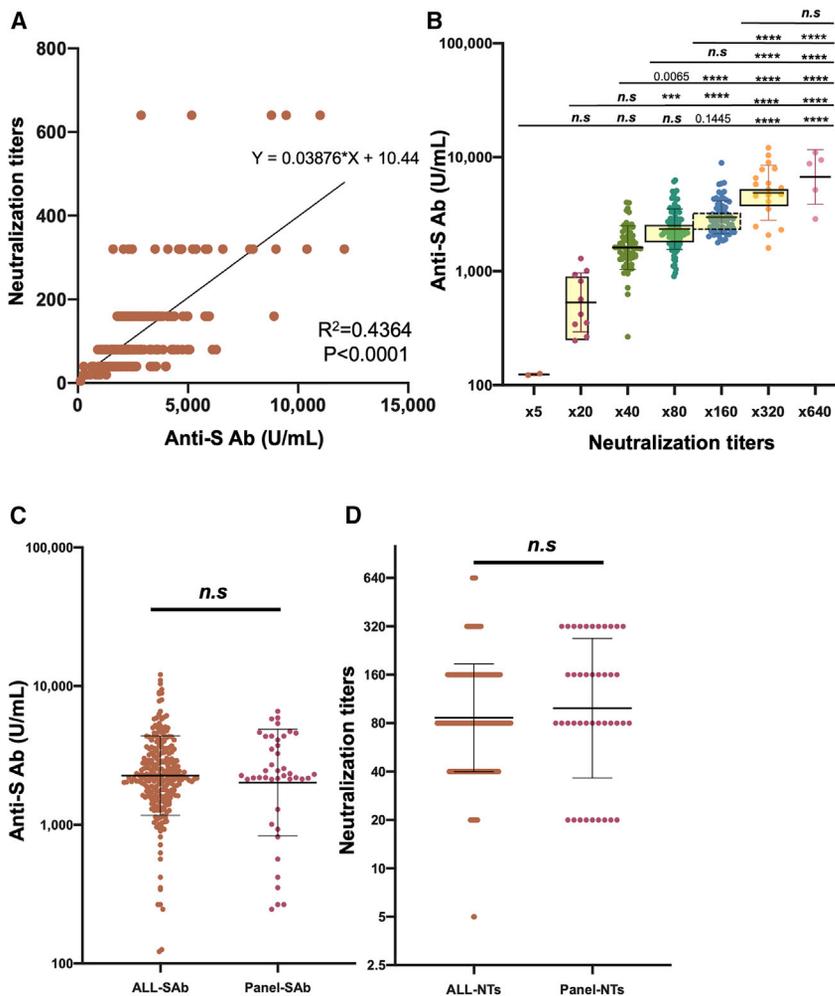


Figure 2. Establishment of vaccine panel

(A and B) Weak correlation of anti-spike antibodies (anti-SAb) and microneutralization titer (NTs) were observed at the second vaccination ($n = 259$) (A). (B) Anti-SAb titers were plotted in each microneutralization titer group, $5 \times$ ($n = 2$), $20 \times$ ($n = 10$), $40 \times$ ($n = 63$), $80 \times$ ($n = 93$), $160 \times$ ($n = 67$), $320 \times$ ($n = 19$), and $640 \times$ ($n = 5$), respectively. At least 10 samples showing values near mean NTs in each titer group were selected.

(C) Comparison of anti-SAb in LT-SI ($n = 259$) and VP-SI ($n = 42$) studies.

(D) Comparison of NTs ($n = 259$) in LT-SI ($n = 42$) and VP-SI studies. *** $p < 0.001$, **** $p < 0.0001$, n.s., not significant.

Data are represented as the geometric mean with geometric SD. (A) Simple linear regression, (B) one-way ANOVA, (C and D) unpaired t test.

were lower^{34–36} after two-dose Pfizer mRNA vaccination, as well as after other approved vaccines such as the Moderna mRNA³⁷ and AstraZeneca adenovirus vector DNA vaccines.³⁸ Thus, we tested whether two-dose vaccinations exhibited neutralization activity against SARS-CoV-2 VOCs, including the recently emerged Omicron strain (Figure 4A). Two doses of the Pfizer mRNA vaccine resulted in significantly lower NTs against the Beta and Kappa strains, as well as the Delta variant, which was endemic in Japan as of December 2021. Two doses of Pfizer mRNA vaccination resulted in dramatically lower NTs against Omicron strains BA.1(TY38-873) and BA.1.1 (TY38-871).

Table 1. Characteristics of study subjects vaccinated with Pfizer/BioNTech BNT162b2 SARS-CoV-2 mRNA

	Male (%)	Female (%)	Total (%)
Total number	103 (38)	169 (62)	272 (100)
Age, y, mean	42.1	40.0	40.8
Age group, y			
20–29	15 (25)	45 (75)	60 (22)
30–39	33 (48)	36 (52)	69 (25)
40–49	26 (34)	51 (66)	77 (28)
50–59	21 (43)	28 (57)	49 (18)
60–64	8 (47)	9 (53)	17 (6)

To characterize the neutralization patterns observed in individuals who were vaccinated with the second dose of the Pfizer mRNA vaccine, we directly compared NTs against wild-type and VOCs using a heatmap of the Spearman's rank correlation coefficient (Figure 4B). We found that NTs against the ancestral strain from individuals administered their second vaccination dose correlated with cross-neutralizations against Alpha, Beta, Gamma, R.1, Kappa, and Delta variants, and weakly correlated with cross-neutralizations against two Omicron variants. These data suggest that the two-dose vaccination induced high NTs against the vaccine strain WK-521 but not against the Omicron variant.

Cytokine signature was stable during vaccinations 1–3

The Pfizer/BioNtech mRNA vaccine is a new modality used in Japan and other countries. In this LT-SI study, we collected information on adverse events and summarized vaccine safety data at each time point after vaccination (Table S1). Briefly, there were no serious adverse events associated with this vaccine compared with those in previous phase II/III clinical studies in Japan and other countries. In addition, we collected 2nd VAX, 2nd VAX + 3M, and 3rd VAX samples to measure 48 cytokine profiles. During vaccination, enhanced cytokine production, including that of some inflammatory cytokines, was not observed at any time point (Figure S3). The cytokine levels of eotaxin in the 3rd VAX group were significantly higher than those in the 2nd VAX and 2nd VAX + 3M groups. The cytokine levels of CTACK, a cutaneous T cell-attracting chemokine, in the 2nd VAX + 3M group were significantly lower than those in the 1st and 2nd VAX groups. The cytokine levels of macrophage inhibitory factor and macrophage inhibitory protein-1 β in the 3rd VAX group were significantly lower than those in the 1st VAX group.

We divided the subjects into adverse event-positive and adverse event-negative groups, namely high-fever ($\geq 39^{\circ}\text{C}$), low-fever ($\geq 37^{\circ}\text{C}$), and no-fever groups, and observed no differences in the cytokine signatures among these groups (Figure S4). There was no correlation between body temperature and cytokine levels in the 2nd and 3rd VAX groups. In addition, we analyzed the correlation between anti-SAb and each cytokine level and found that only the eotaxin level was weakly correlated with the anti-SAb titer in the 3rd VAX group.

Three doses of vaccination increase both anti-SAb and NTs that sufficiently cross-react with some variants

In Japan, the third booster vaccination was initiated in November 2021 for health-care workers only. We collected samples before (pre-3rd VAX") and two weeks after ("3rd VAX") a third vaccine dose from the same participants in our panel. We first analyzed NTs against the original vaccine strain, WK-521. NTs against WK-521

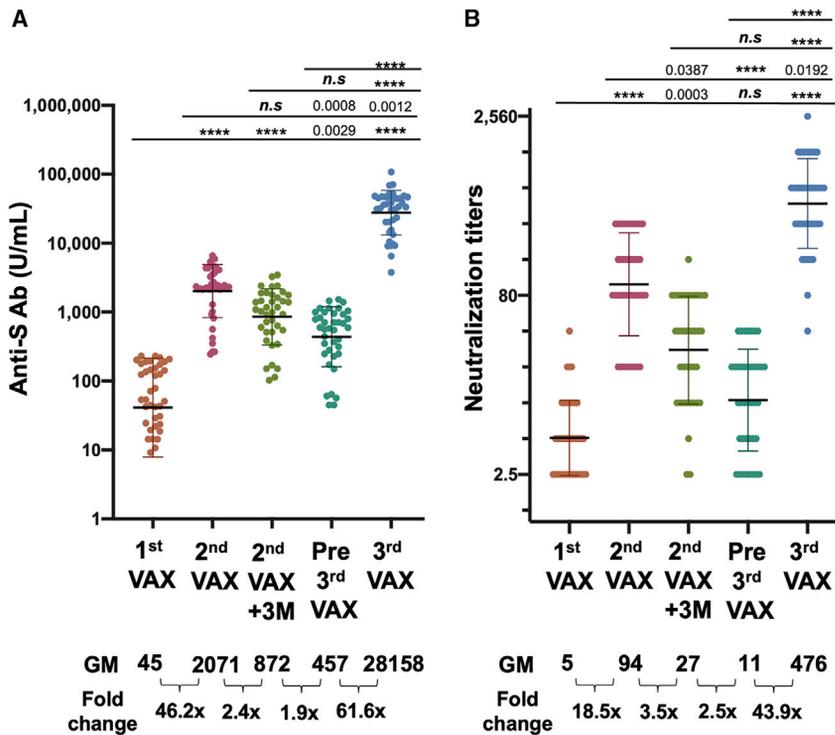


Figure 3. Anti-SAb and micro-NTs after three-dose vaccination with Pfizer/BioNTech mRNA vaccine

Antibody responses at 12 days after the first vaccination (1st VAX) (n = 38), 20 days after the second vaccination (2nd VAX) (n = 38), 20 days after the second vaccination (2nd VAX + 3M) (n = 34), 244 days after the second vaccination (pre-3rd VAX) (n = 35), and 12 days after the third vaccination (3rd VAX) (n = 35) were plotted.

(A) Anti-SAb (U/mL) was measured using a Roche Elecsys anti-SARS-CoV-2 S assay.

(B) Micro-NTs were measured with live virus, WK-521, and the dot plot shows the data of each participant. Geometric mean and SD are indicated. Fold change is the value compared to the previous time point. ***p < 0.001, ****p < 0.0001.

(A and B) Kruskal-Wallis test. The values of each geometric mean of NT were rounded to the nearest whole number; fold change, rounded to two decimal places.

were significantly higher than after second dose (Figure 5A). Some participants showed NTs that exceeded 2,560, and the geometric mean of the cohort was 476 after the third dose. Samples collected after the third dose showed a 43.9-fold NT increase compared to the pre-third dose time point. A similar result was obtained for the Delta variant (Figure 5B). The geometric mean of NTs was 238 after the third dose, showing a 36.0-fold increase compared to the pre-third dose samples. In the most recently identified VOC, Omicron, there were six NTs after two doses; this number decreased to three NTs 3 months later. These values are both lower than those of the WK-521 and Delta variants (Figure 5C). Most NTs in the pre-3rd VAX were below the detection limit of 2.5; however, they increased 33.3-fold after third dose administration, leading to higher NTs than those detected against WK-521 after the second dose. A similar result was obtained using a commercially available multiplex SARS-CoV-2 neutralization antibody detection assay kit. Compared to the response after the second dose, neutralizing antibodies against wild-type S1, Alpha S1, Beta S1, and D614G S1 were significantly increased by 1.96- to 2.98-fold and neutralizing antibodies against the wild-type RBD, Gamma RBD, Kappa RBD, Epsilon RBD, N501Y RBD, K417RBD, and E484K RBD significantly increased by 2.35- to 3.15-fold (Figure 5D).

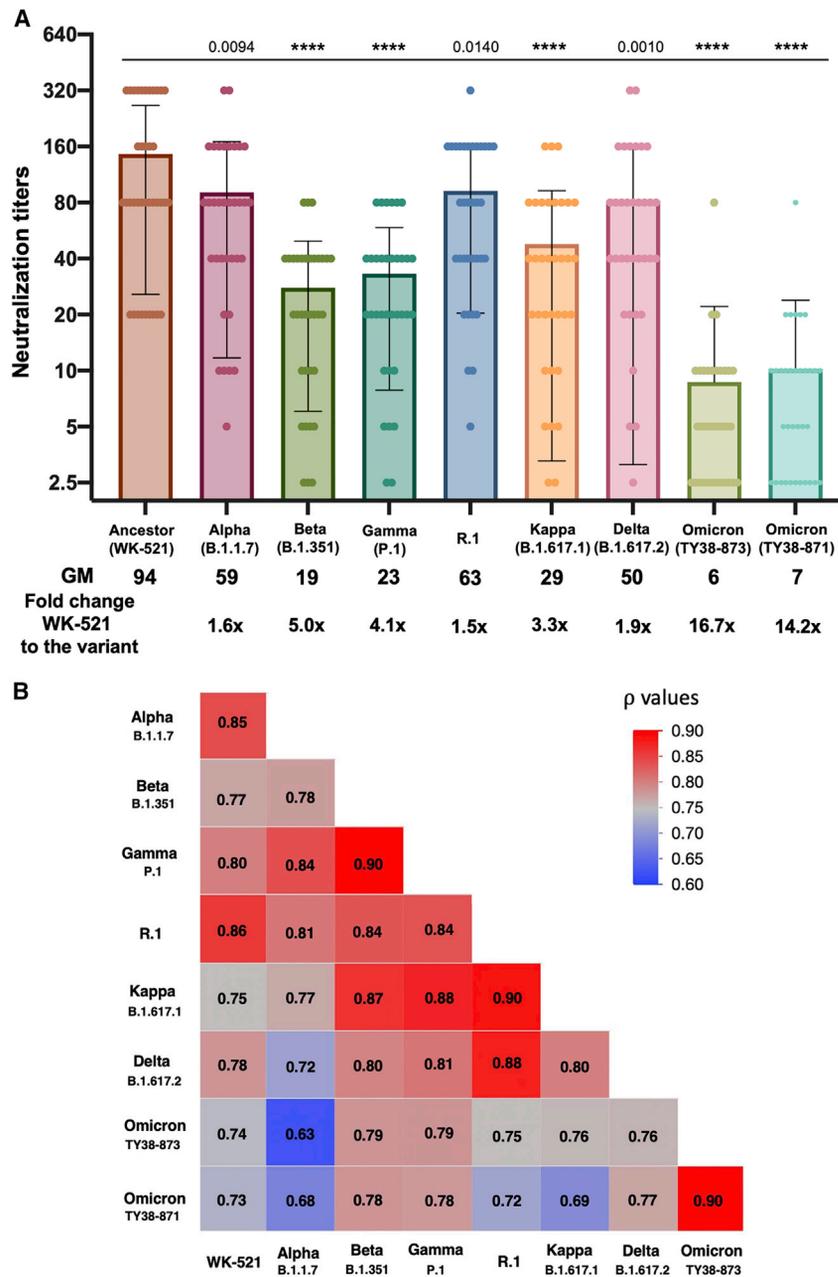
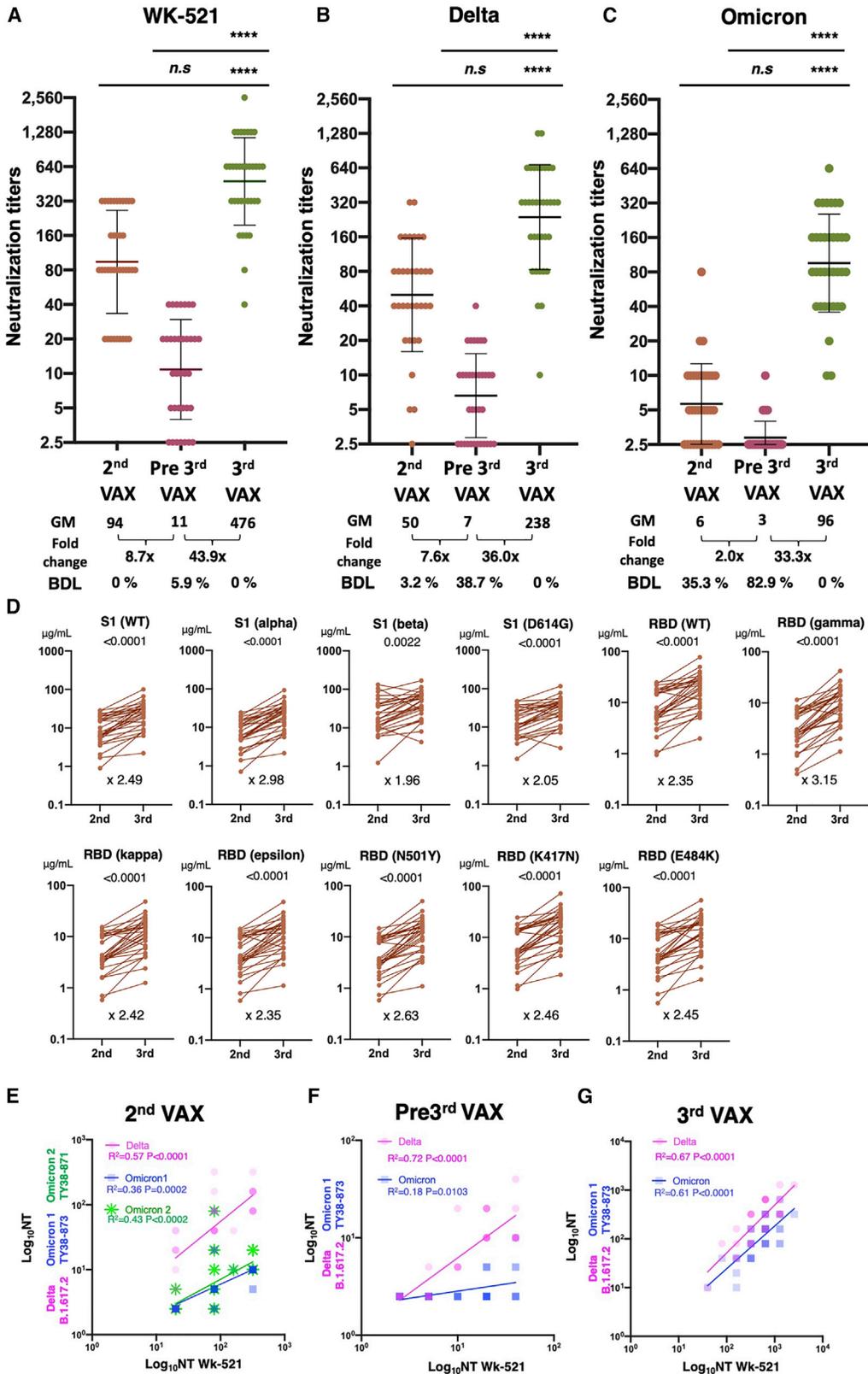


Figure 4. Comparison of neutralization ability in vaccine sera from two-dose vaccination

(A) The microneutralization titer of 2nd VAX against WK-521 (n = 38), Alpha (n = 34), Beta (n = 34), Gamma (n = 34), R.1 (n = 34), Kappa (n = 34), Delta (n = 34), and two Omicron variants (n = 34) is shown. ***p < 0.001, ****p < 0.0001. The values of each geometric mean of NTs were rounded to the nearest whole number; fold change, rounded to two decimal places. Data are represented as the geometric mean with geometric SD.

(B) Cross-reactivity of NTs (n = 34) in WK-521 and SARS-CoV-2 variants. Heatmap of Spearman's rank correlation coefficients among the SARS-CoV-2 variants of concern (VOCs) after 2nd VAX. Colors represent the ρ (rho) values of the Spearman correlations (i.e., the strength of the correlations among the VOCs). Red (value of 1) represents a stronger correlation coefficient and a more significant p value. Dark blue represents weaker correlation coefficients and p values. NTs against wild-type WK-521 correlated with Alpha, Gamma, and R.1. Weak correlation to Beta, Kappa, Delta, and Omicron variant.

(A) One-way ANOVA, (B) Spearman's rank correlation.



Three-dose vaccination increased breadth of humoral immunity and cross-reactivity against SARS-CoV-2 variant

To characterize the neutralization patterns in individuals who were booster vaccinated with the Pfizer mRNA vaccine, we directly compared wild-type NTs with those developed against the Delta and Omicron strains (Figures 5E–5G). We found that NTs against the ancestral strain from individuals administered their second dose vaccination were weakly correlated with Delta variant cross-neutralization and not correlated with TY38-871 or TY38-873 Omicron variants cross-neutralization in the 2nd VAX and pre-3rd VAX groups. In contrast, wild-type neutralization of boosted individuals correlated with Delta and Omicron variant cross-neutralization. These data suggest that booster vaccination induces not only higher NTs against the vaccine strain WK-521 but also increases the breadth of humoral immunity and cross-reactivity against the highly mutated SARS-CoV-2 Omicron variant.

Differences in cross-neutralization ability against SARS-CoV-2 Omicron subvariants using serum samples from three vaccination doses

Since the genetic sequence of the SARS-CoV-2 Omicron variant was first isolated and determined, subvariant strains have been isolated in many countries. At present, Omicron is composed of several sublineages, such as BA.1, BA.1.1 (or Nextstrain clade 21 K), and BA.2 (or Nextstrain clade 21 L). Studies have shown that BA.2 has a growth advantage over BA.1, and some data suggest that BA.2 is inherently more transmissible than BA.1. Some studies have shown that reinfection with BA.2 following infection with BA.1 can occur.³⁹ Thus, comparing the cross-neutralization ability among the Omicron subvariants such as BA.1, BA.1.1, and BA.2 is crucially important to understanding the merit of three-dose vaccination. Thus, we compared NTs against five SARS-CoV-2 Omicron subvariants in samples collected from individuals who had received three vaccine doses. Although the NTs against BA1.1 were much lower than against the other variants, there were no significant differences among these Omicron subvariants (Figure 6A). We compared NTs against ancestral and Omicron subvariants using a heatmap of the Spearman's rank correlation coefficient and found that booster vaccination increased the correlation coefficient between the ancestral strain (WK-521) and Omicron subvariants; in addition, there were no differences in cross-reactivity among Omicron subvariants (Figure 6B).

DISCUSSION

We first determined the changes in anti-SAb and NTs against Omicron variants in Japanese healthcare workers receiving a three-dose vaccination with the Pfizer/BioNTech SARS-CoV-2 mRNA vaccine. The second vaccine dose dramatically increased both anti-SAb and anti-NT against the vaccine strain WK-521. The effectiveness of this vaccine has already been established,¹² preventing more than 95% of COVID-19 cases in phase II/III clinical studies. In this study, we compared NTs against VOCs after the second vaccination dose. Our results agree with those of a previous study showing that NTs were severely reduced for the Beta and Gamma strains, which exhibit three-vaccine escape mutations in the spikeRBD domain.¹⁹

Figure 5. Neutralization antibody against WK-521, the Delta variant, and the Omicron variant after 3-dose vaccination

(A) NTs against WK-521 at 2nd VAX (n = 38), pre-3rd VAX (n = 35), and 3rd VAX (n = 35).

(B) NTs against the Delta variant at 2nd VAX (n = 34), pre-3rd VAX (n = 35), and 3rd VAX (n = 35).

(C) NTs against the Omicron variant at 2nd VAX (n = 34), pre-3rd VAX (n = 35), and 3rd VAX (n = 35). Values of each geometric mean of NTs were rounded to the nearest whole number; fold change, rounded to 2 decimal places.

(D–G) Comparison of neutralization antibody measured with the Bio-Plex SARS-CoV-2 Neutralization Antibody Assay panel at 2nd VAX (n = 18) and 3rd VAX (n = 18). Data are represented as geometric means with geometric SDs. Linear regression analysis of wild-type versus Omicron at 2nd VAX (E) (n = 34), pre-3rd VAX (F) (n = 34), and 3rd VAX (G) (n = 35). NTs against wild-type WK-521 correlated with those against Omicron at booster vaccination. (A–C) One-way ANOVA, (D) paired t test, and (E–G) simple linear regression.

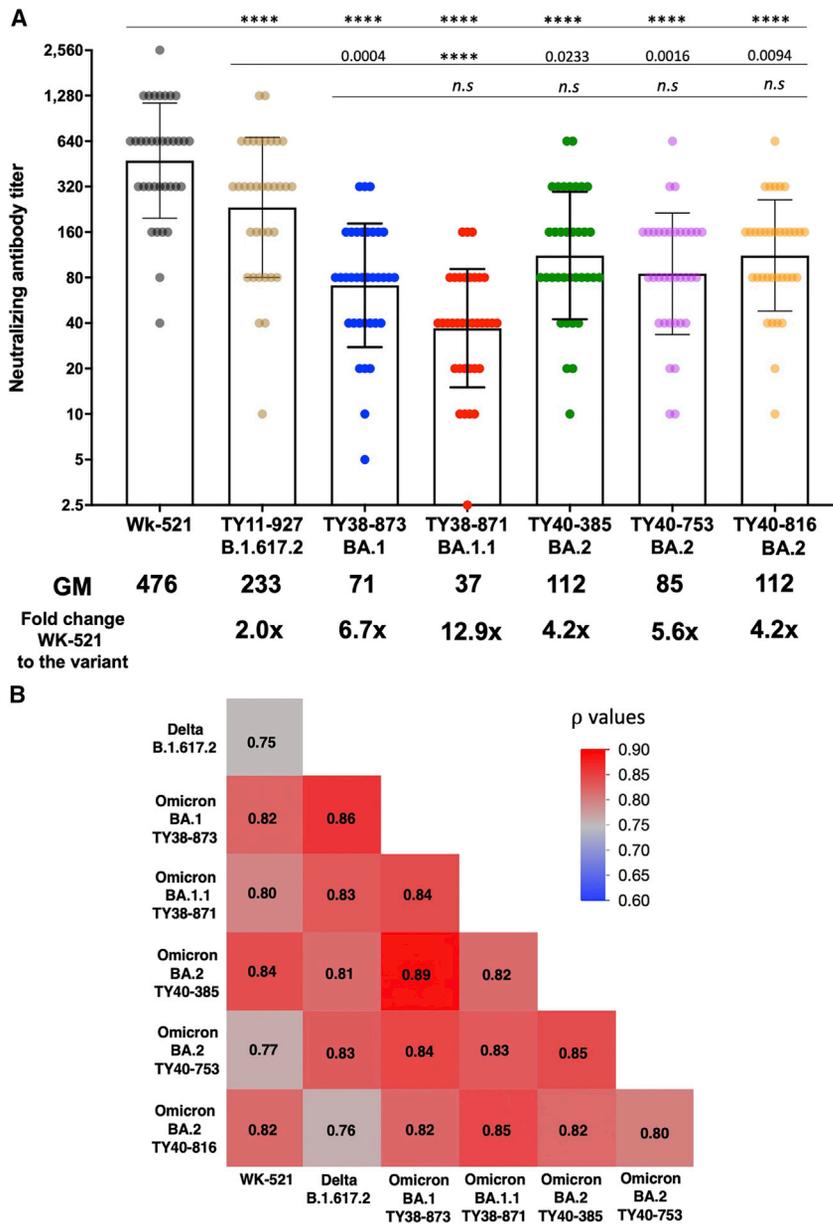


Figure 6. Neutralization antibody against the SARS-CoV-2 Omicron variants in the vaccine sera from 3-dose booster vaccination

(A) The microneutralization titer of 3rd VAX (n = 35) in WK-521, Delta (B.1.617.2), Omicron BA.1 (TY38-873), Omicron BA.1.1 (TY38-871), Omicron BA.2 (TY40-385), Omicron BA.2 (TY40-753), and Omicron BA.2 (TY40-816) are shown. Data are represented as geometric means with geometric SDs. ****p < 0.0001. Values of each geometric mean NT were rounded to the nearest whole number; fold change, rounded to 2 decimal places.

(B) Cross-reactivity of NTs against WK-521 (n = 35) and SARS-CoV-2 Omicron subvariants (n = 35). Heatmap of Spearman's rank correlation coefficients among the SARS-CoV-2 VOCs at 2nd VAX. Colors represent the p values of the Spearman correlations (i.e., the strength of the correlations among the VOCs). Red (value of 1) represents a stronger correlation coefficient and a more significant p value. Dark blue represents weaker correlation coefficients and p values.

(A) One-way ANOVA, (B) Spearman's rank correlation.

Similarly, NTs against Delta and Gamma were slightly decreased compared to those against the ancestral strain WK-521. In November 2021, the SARS-CoV-2 Omicron variant (BA.1/B.1.1.529), which contains more than 15 mutations in the spike RBD and overlaps the three sites mutated in Gamma and Beta (K417, E484, and N501), including vaccine escape mutations, was detected using sequencing in Botswana. This variant was reported to the WHO in November 2021 as a novel VOC, harboring up to 36 mutations with immune-evasive potential in the spike protein, the target of neutralizing antibodies, and spread rapidly worldwide. These mutations in Omicron indicate the potential for antibody escape from mRNA vaccine-induced immunity and are more resistant to neutralization by the mRNA vaccine sera.³¹ In Japan, the first Omicron case was reported in December 2021. We isolated and sequenced two Omicron strains with R346K mutations in the spike RBD domain, which have been observed at relatively low frequency (<10%) within the Omicron lineage but that are known as key targets for escaping neutralizing monoclonal antibodies such as AZD1061/cilgavimab.^{40–43} Using the two newly isolated Omicron strains, we observed a severe reduction in the NTs against the Omicron strain. Neutralizing titers in sera from 35% of participants were below the detection limit (NTs = 2.5), indicating low or no cross-reactivity against Omicron, even at 10–12 days after the second vaccination. Both NTs against the WK-521, Delta, and Omicron strains drastically decreased 244 days after the second vaccination, as indicated in [Figures 3](#) and [5](#). However, the percentage of NTs below the detection limit was 6% in the WK-521 strain, 39% in the Delta strain, and 83% in the Omicron strain. These NT monitoring data clearly show that the effectiveness of the second vaccine dose was not maintained at 243 days or later.

Cellular and natural immunity are involved in protecting against SARS-CoV-2 infection after mRNA vaccination and disease progression,^{44,45} thus, the NT data may not fully reflect actual immunity against SARS-CoV-2. Regardless, many countries recommend booster vaccination 6 months after the second vaccination. In our study, three-dose vaccination significantly and dramatically increased anti-SAb and NTs against WK-521, Delta, and Omicron. NTs against Delta upon booster vaccination were higher than those against WK-521 after the second dose. NTs against Omicron in the samples collected after the booster dose were comparable to those against WK-521 after the second dose. These data would suggest that similar effectiveness can be expected with three-dose vaccinations against both the ancestral strain and the current variants. Several studies have suggested that mRNA vaccination induces a humoral immune response and immune memory.^{44,45} Booster vaccination may affect the ongoing antibody maturation that occurs during vaccination and the late convalescent phase beyond 3 months after COVID-19 symptom onset.^{31,46–48} Thus, booster vaccination may reduce the risk of infection with the newly emerged SARS-CoV-2 Omicron variants.

After the first isolation of the SARS-CoV-2 Omicron variant, a subvariant strain has also been isolated in many countries. BA.2 is almost dominant in Denmark²² and has increased its prevalence in Omicron infections detected in the United States²³ and the United Kingdom.²⁴ In our study, we found that a three-dose vaccination increases neutralizing antibodies against Omicron subvariants and observed no significant differences in neutralization ability among the Omicron subvariants.

In addition to immunogenicity, safety is a critical issue, as some people consider that emergency use authorization means that complete safety research has not been performed, resulting in vaccine hesitancy. A Phase II/III study suggested that the booster shot does not change the adverse event rate or specific adverse events in

short-term analysis.⁴⁹ One to 2 weeks after the third vaccination dose, abnormal or prolonged cytokine production was not observed in healthy participants (Figure S3) or in those who developed fever (Figure S4), headache, fatigue, or injection site pain. Only eotaxin levels increased after the third dose compared to the post-second dose time point. Eotaxin, a chemokine ligand for CCR3, is a chemoattractant for eosinophils, basophils, and T helper 2 (Th2) lymphocytes, and is released from endothelial and epithelial cells. Eotaxin recruits eosinophils to the site of inflammation and releases reactive oxygen species, causing tissue damage during chronic inflammatory responses. Several studies have shown that eotaxin levels increased after the smallpox vaccination, correlating to adverse event presentation.⁵⁰ Thus, eotaxin levels may be related to adverse events such as injection site pain and redness after the third vaccine dose. However, damage-associated inflammatory cytokine levels induced by eosinophils were unchanged in our samples, as were key pathways involving eotaxin, such as interferon- γ and interleukin-4. These data suggest that eotaxin alone is not an indicator of serious adverse events. Multiple pathway analyses are required to determine the relationship between cytokine levels and adverse events. Overall, the cytokine data suggest no signs of adverse events.

Taken together, booster vaccination induced an increase not only in the anti-spike IgG titer but also in the breadth of humoral immunity and cross-reactivity against newly emerged Omicron variants without causing specific adverse events or abnormal or prolonged cytokine production.

Limitations of the study

This study has several limitations. First, the number of samples evaluated in this study for the analysis of cross-reactivity of neutralizing antibodies against several SARS-CoV-2 variants was small compared to the original survey. Thirty samples were selected as representative of the original survey (N = 272) based on the correlations in anti-S antibodies and neutralizing antibodies to the ancestral strain WK-521, and the distribution of sex, age, and frequency of adverse events after the second vaccination. Second, we could not take gender, age, ethnicity, and socioeconomic status into consideration when enrolling the participants because our study was initiated upon the government's directive that COVID-19 vaccinations first be administered only to healthcare workers, the aged, and people at high risk. The sample reflects variations in the gender, ethnicity, and socioeconomic status of hospital healthcare workers. We did not set typical exclusion criteria for enrollment. Vaccination-responsible doctors were able to decide who to exclude, but no one was excluded in this study, except for participants delivering a positive PCR test after the second vaccination. Third, the vaccine sera used in this study were collected at only one time point, 1–2 weeks after the first, second, and third vaccinations. Thus, we could not analyze the differences in anti-SAb and neutralizing antibody titers in the short term, especially in the early phase after vaccination. In the same way, we could not analyze the early phases of inflammation status after vaccination. We focused only on the late-phase effects after vaccination. In addition, we could not collect mononuclear cells from each participant; thus, we could not analyze the cellular immunity against SARS-CoV-2 variants. T cell immunity against SARS-CoV-2 variants, including Omicron variants, is important for protection against SARS-CoV-2.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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Supplemental information can be found online at <https://doi.org/10.1016/j.medj.2022.04.013>.

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AUTHOR CONTRIBUTIONS

Y.S., K.N., T.M., Y.Y., and I.H. conceived of the study. Y.S., K.N., and T.M. designed the study. Y.S., K.N., H.M., and T.M. performed the experiments and analyzed the data. S.F. and K.M. provided isolated SARS-CoV-2 variants and consulted on the analysis and results. T.M. performed the statistical analyses and wrote the first draft. The first draft was reviewed by all of the authors and then revised to the final manuscript by T.M. A.W., N.N., K.S., T.K., and Y.Y. curated the clinical data and reviewed the results. S.M., Y.T., and T.S. provided the monoclonal antibody and the rabbit polyclonal antibody against SARS-CoV-2 RBD and consulted on the experiment and analysis. T.M., Y.Y., and I.H. had unrestricted access to all of the data. All of the authors have reviewed the manuscript and approved the final article and take responsibility for its content.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

- Johns Hopkins University Coronavirus Resource Center (2020). COVID-19 Dashboard by the Center for Systems Science and Engineering (CSSE) at (Johns Hopkins University). <https://coronavirus.jhu.edu/map.html>.
- World Health Organization (2020). WHO Director-General's opening remarks at the media briefing on COVID-19 — 11 March 2020. <https://www.who.int/director-general/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19—11-march-2020>.
- Ministry of Health, Labor, and Welfare (2020). Report of Pneumonia Associated with the Novel Coronavirus. (accessed December 1, 2021). (in Japanese). https://www.mhlw.go.jp/stf/newpage_08906.html.
- Nakazawa, E., Ino, H., and Akabayashi, A. (2020). Chronology of COVID-19 cases on the Diamond Princess cruise ship and ethical consideration: a report from Japan. *Disaster Med. Public Health Prep.* 14, 506–513.
- Song, P., and Karako, T. (2021). The strategy behind Japan's response to COVID-19 from 2020-2021 and future challenges posed by the uncertainty of the Omicron variant in 2022. *Biosci. Trends* 15, 350–352.
- Volz, E., Hill, V., McCrone, J.T., Price, A., Jorgensen, D., Southgate, J., Johnson, R., Jackson, B., Nascimento, F.F., Rey, S.M., et al. (2021). Evaluating the effects of SARS-CoV-2 spike mutation D614G on transmissibility and pathogenicity. *Cell* 184, 64–75.
- Hou, Y.J., Chiba, S., Halfmann, P., Ehre, C., Suzuki, T., Dinno 3rd, K.H., Leist, S.R., Nakajima, N., Takahashi, K., Lee, R.E., et al. (2020). SARS-CoV-2 D614G variant exhibits efficient replication *ex vivo* and transmission *in vivo*. *Science* 370, 1464.
- Yurkovetskiy, L., Wang, X., Pascal, K.E., Tomkins-Tinch, C., Nyalile, T.P., Wang, Y., Baum, A., Diehl, W.E., Dauphin, A., Carbone, C., et al. (2020). Structural and functional analysis of the D614G SARS-CoV-2 spike protein variant. *Cell* 183, 739–751.e8.
- Vöhringer, H.S., Sanderson, T., Sinnott, M., De Maio, N., Nguyen, T., Goater, R., Schwach, F., Harrison, I., Hellewell, J., Ariani, C.V., et al. (2021). Genomic reconstruction of the SARS-CoV-2 epidemic in England. *Nature* 600, 506–511.
- Tegally, H., Wilkinson, E., Lessells, R.J., et al. (2021). Sixteen novel lineages of SARS-CoV-2 in South Africa. *Nat. Med.* 27, 440–446.
- Faria, N.R., Mellan, T.A., Whittaker, C., Claro, I.M., Candido, D.D.S., Mishra, S., Crispim, M.A.E., Sales, F.C., Hawrylyuk, I., McCrone, J.T., et al. (2021). Genomics and epidemiology of the P.1 SARS-CoV-2 lineage in Manaus, Brazil. *Science* 372, 815–821.
- Polack, F.P., Thomas, S.J., Kitchin, N., Absalon, J., Gurtman, A., Lockhart, S., Perez, J.L., Pérez Marc, G., Moreira, E.D., Zerbini, C., et al. (2020). Safety and efficacy of the BNT162b2 mRNA Covid-19 vaccine. *N. Engl. J. Med.* 383, 2603–2615.
- Baden, L.R., El Sahly, H.M., Essink, B., Kotloff, K., Frey, S., Novak, R., Diemert, D., Spector, S.A., Roupael, N., Creech, C.B., et al. (2021). Efficacy and safety of the mRNA-1273 SARS-CoV-2 vaccine. *N. Engl. J. Med.* 384, 403–416.
- Sadoff, J., Gray, G., Vandebosch, A., Cárdenas, V., Shukarev, G., Grinsztejn, B., Goepfert, P.A., Truyers, C., Fennema, H., Spiessens, B., et al. (2021). Safety and efficacy of single-dose Ad26.COV2.S vaccine against Covid-19. *N. Engl. J. Med.* 384, 2187–2201.
- Voysey, M., Costa Clemens, S.A., Madhi, S.A., Weckx, L.Y., Folegatti, P.M., Aley, P.K., Angus, B., Baillie, V.L., Barnabas, S.L., Bhorat, Q.E., et al. (2021). Single-dose administration and the influence of the timing of the booster dose on immunogenicity and efficacy of ChAdOx1 nCoV-19 (AZD1222) vaccine: a pooled analysis of four randomised trials. *Lancet* 397, 881–891.
- Tregoning, J.S., Flight, K.E., Higham, S.L., Wang, Z., and Pierce, B.F. (2021). Progress of the COVID-19 vaccine effort: viruses, vaccines and variants versus efficacy, effectiveness and escape. *Nat. Rev. Immunol.* 21, 626–636.
- Lopez Bernal, J., Andrews, N., Gower, C., Gallagher, E., Simmons, R., Thelwall, S., Stowe, J., Tessier, E., Groves, N., Dabrera, G., et al. (2021). Effectiveness of Covid-19 vaccines against the B.1.617.2 (Delta) variant. *N. Engl. J. Med.* 385, 585–594.
- Cele, S., Gazy, I., Jackson, L., Hwa, S.-H., Tegally, H., Lustig, G., Giandhari, J., Pillay, S., Wilkinson, E., Naidoo, Y., et al. (2021). Escape of SARS-CoV-2 501Y.V2 from neutralization by convalescent plasma. *Nature* 593, 142–146.
- García-Beltrán, W.F., Lam, E.C., St. Denis, K., Nitido, A.D., García, Z.H., Hauser, B.M., Feldman, J., Pavlovic, M.N., Gregory, D.J., Poznansky, M.C., et al. (2021). Multiple SARS-CoV-2 variants escape neutralization by vaccine-induced humoral immunity. *Cell* 184, 2372–2383.e9.
- Zhou, D., Dejnirattisai, W., Supasa, P., Liu, C., Mentzer, A.J., Ginn, H.M., Zhao, Y., Duyvesteyn, H.M.E., Tuekprakhon, A., Nitalai, R., et al. (2021). Evidence of escape of SARS-CoV-2 variant B.1.351 from natural and vaccine-induced sera. *Cell* 184, 2348–2361.e6.
- Hoffmann, M., Krüger, N., Schulz, S., Cossmann, A., Rocha, C., Kempf, A., Nehlmeier, I., Graichen, L., Moldenhauer, A.S., Winkler, M.S., et al. (2021). The Omicron variant is highly resistant against antibody-mediated neutralization: implications for control of the COVID-19 pandemic. *Cell* 185, 447–456.
- Statens Serum Institut (2022). Ugentlige Tendenser: Covid-19 Og Andre Luftvejsinfektioner Uge 6. <https://www.ssi.dk/-/media/cdn/files/covid19/tendensrapport/rapport/ugentlige-tendenser-covid19-andre-luftvejs-uge6-2022-8d2k.pdf?la=da>.
- CDC variant proportions; 2022. Accessed 25 February 2022 (<https://covid.cdc.gov/covid-data-tracker/#variant-proportions>).
- UKHSA (2022). SARS-CoV-2 Variants of Concern and Variants under Investigation in England Technical Briefing 36. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/1056487/Technical-Briefing-36-22.02.22.pdf.
- The National Institute of Infectious Diseases. SARS-CoV-2 variant B.1.1.529 (Omicron). 8th edition. Article in Japanese. https://www.niid.go.jp/niid/images/cepr/covid-19/b11529_8.pdf.
- WHO (2022). Weekly Epidemiological Update on COVID-19 - 8 February 2022. <https://www.who.int/publications/m/item/weekly-epidemiological-update-on-covid-19—8-february-2022>.
- Israel, A., Shenhar, Y., Green, I., Merzon, E., Golan-Cohen, A., Schäfer, A.A., Ruppin, E., Vinker, S., and Magen, E. (2022). Large-scale study of antibody titer decay following BNT162b2 mRNA vaccine or SARS-CoV-2 infection. *Vaccines* 10, 64.
- García-Beltrán, W.F., St Denis, K.J., Hoelzemer, A., Lam, E.C., Nitido, A.D., Sheehan, M.L., Berrios, C., Ofoman, O., Chang, C.C., Hauser, B.M., et al. (2022). mRNA-based COVID-19 vaccine boosters induce neutralizing immunity against SARS-CoV-2 Omicron variant. *Cell* 185, 457–466.
- Pozzetto, B., Legros, V., Djebali, S., et al. (2021). Immunogenicity and efficacy of heterologous ChAdOx1-BNT162b2 vaccination. *Nature* 600, 701–706.
- Ai, J., Zhang, H., Zhang, Q., et al. (2022). Recombinant protein subunit vaccine booster following two-dose inactivated vaccines dramatically enhanced anti-RBD responses and neutralizing titers against SARS-CoV-2 and Variants of Concern. *Cell Res.* 32, 103–106.
- Moriyama, S., Adachi, Y., Sato, T., Tonouchi, K., Sun, L., Fukushi, S., Yamada, S., Kinoshita, H., Nojima, K., Kanno, T., et al. (2021). Temporal maturation of neutralizing antibodies in COVID-19 convalescent individuals improves potency and breadth to circulating SARS-CoV-2 variants. *Immunity* 54, 1841–1852.e4.
- Onodera, T., Kita, S., Adachi, Y., Moriyama, S., Sato, A., Nomura, T., Sakakibara, S., Inoue, T., Tadokoro, T., Anraku, Y., et al. (2021). A SARS-CoV-2 antibody broadly neutralizes SARS-related coronaviruses and variants by coordinated recognition of a virus-vulnerable site. *Immunity* 54, 2385–2398.e10. Epub 2021 Aug 24. <https://doi.org/10.1016/j.immuni.2021.08.025>.
- Miyamoto, S., Arashiro, T., Adachi, Y., Moriyama, S., Kinoshita, H., Kanno, T., Saito, S., Katano, H., Iida, S., Ainai, A., et al. (2022). Vaccination-infection interval determines cross-neutralization potency to SARS-CoV-2 Omicron after breakthrough infection by other variants. Preprint at MedRxiv. <https://doi.org/10.1101/2021.12.28.21268481>.
- Collier, D.A., De Marco, A., Ferreira, I.A.T.M., Meng, B., Dattir, R.P., Walls, A.C., Kemp, S.A., Bassi, J., Pinto, D., Silacci-Fregni, C., et al. (2021). Sensitivity of SARS-CoV-2 B.1.1.7 to mRNA vaccine-elicited antibodies. *Nature* 593, 136–141. Epub 2021 Mar 11. PMID: 33706364. <https://doi.org/10.1038/s41586-021-03412-7>.

35. Wang, P., Nair, M.S., Liu, L., Iketani, S., Luo, Y., Guo, Y., Wang, M., Yu, J., Zhang, B., Kwong, P.D., et al. (2021). Antibody resistance of SARS-CoV-2 variants B.1.351 and B.1.1.7. *Nature* 593, 130–135. Epub 2021 Mar 8. PMID: 33684923. <https://doi.org/10.1038/s41586-021-03398-2>.
36. Abu-Raddad, L.J., Chemaitelly, H., and Butt, A.A.; National Study Group for COVID-19 Vaccination (2021). Effectiveness of the BNT162b2 Covid-19 vaccine against the B.1.1.7 and B.1.351 variants. *N. Engl. J. Med.* 385, 187–189. Epub 2021 May 5. PMID: 33951357; PMCID: PMC8117967. <https://doi.org/10.1056/NEJMc2104974>.
37. Wu, K., Werner, A.P., Koch, M., Choi, A., Narayanan, E., Stewart-Jones, G.B.E., Colpitts, T., Bennett, H., Boyoglu-Barnum, S., Shi, W., et al. (2021). Serum neutralizing activity elicited by mRNA-1273 vaccine. *N. Engl. J. Med.* 384, 1468–1470. Epub 2021 Mar 17. PMID: 33730471; PMCID: PMC8008744. <https://doi.org/10.1056/NEJMc2102179>.
38. Emary, K.R.W., Golubchik, T., Aley, P.K., Ariani, C.V., Angus, B., Bibi, S., Blane, B., Bonsall, D., Cicconi, P., Charlton, S., et al. (2021). COVID-19 Genomics UK consortium; AMPHEUS Project; Oxford COVID-19 Vaccine Trial Group. Efficacy of ChAdOx1 nCoV-19 (AZD1222) vaccine against SARS-CoV-2 variant of concern 202012/01 (B.1.1.7): an exploratory analysis of a randomised controlled trial. *Lancet* 10, 1351–1362. [https://doi.org/10.1016/S0140-6736\(21\)00628-0](https://doi.org/10.1016/S0140-6736(21)00628-0).
39. WHO (2022). Statement on Omicron Sublineage BA.2. <https://www.who.int/news/item/22-02-2022-statement-on-omicron-sublineage-ba-2>.
40. Schmidt, F., Weisblum, Y., Rutkowska, M., Poston, D., DaSilva, J., Zhang, F., Bednarski, E., Cho, A., Schaefer-Babajew, D.J., Gaebler, C., et al. (2021). High genetic barrier to SARS-CoV-2 polyclonal neutralizing antibody escape. *Nature* 600, 512–516.
41. Barnes, C.O., Jette, C.A., Abernathy, M.E., Dam, K.-M.A., Esswein, S.R., Gristick, H.B., Malyutin, A.G., Sharaf, N.G., Huey-Tubman, K.E., Lee, Y.E., et al. (2020). SARS-CoV-2 neutralizing antibody structures inform therapeutic strategies. *Nature* 588, 682–687.
42. Dong, J., Zost, S.J., Greaney, A.J., Starr, T.N., Dingsen, A.S., Chen, E.C., Chen, R.E., Case, J.B., Sutton, R.E., Gilchuk, P., et al. (2021). Genetic and structural basis for SARS-CoV-2 variant neutralization by a two-antibody cocktail. *Nat. Microbiol.* 6, 1233–1244.
43. Greaney, A.J., Loes, A.N., Crawford, K.H.D., Starr, T.N., Malone, K.D., Chu, H.Y., and Bloom, J.D. (2021a). Comprehensive mapping of mutations in the SARS-CoV-2 receptor-binding domain that affect recognition by polyclonal human plasma antibodies. *Cell Host Microbe* 29, 463–476.e6.
44. Goel, R.R., Painter, M.M., Apostolidis, S.A., Mathew, D., Meng, W., Rosenfeld, A.M., Lundgreen, K.A., Reynaldi, A., Khoury, D.S., Pattekar, A., et al. (2021). mRNA vaccines induce durable immune memory to SARS-CoV-2 and variants of concern. *Science* 374, abm0829. Epub 2021 Dec 3. PMID: 34648302. <https://doi.org/10.1126/science.abm0829>.
45. Turner, J.S., O'Halloran, J.A., Kalaidina, E., Kim, W., Schmitz, A.J., Zhou, J.Q., Lei, T., Thapa, M., Chen, R.E., Case, J.B., et al. (2021). SARS-CoV-2 mRNA vaccines induce persistent human germinal centre responses. *Nature* 596, 109–113. Epub 2021 Jun 28. PMID: 34182569. <https://doi.org/10.1038/s41586-021-03738-2>.
46. Turner, J.S., Halloran, J.A., Kalaidina, E., Kim, W., Schmitz, A.J., Zhou, J.Q., Lei, T., Thapa, M., Chen, R.E., Case, J.B., et al. (2021). SARS-CoV-2 mRNA vaccines induce persistent human germinal centre responses. *Nature* 596, 109–113.
47. Muecksch, F., Weisblum, Y., Barnes, C.O., Schmidt, F., Schaefer-Babajew, D., Wang, Z., C Lorenzi, J.C., Flyak, A.I., DeLaitch, A.T., Huey-Tubman, K.E., et al. (2021). Affinity maturation of SARS-CoV-2 neutralizing antibodies confers potency, breadth, and resilience to viral escape mutations. *Immunity* 54, 1853–1868.
48. Gaebler, C., Wang, Z., Lorenzi C C, J., Muecksch, F., Finkin, S., Tokuyama, M., Cho, A., Jankovic, M., Schaefer-Babajew, D., Oliveira, T.Y., et al. (2021). Evolution of antibody immunity to SARS-CoV-2. *Nature* 591, 639–644.
49. Intapiboon, P., Seepathomnarong, P., Ongarj, J., Surasombatpattana, S., Uppanisakorn, S., Mahasirimongkol, S., Sawaengdee, W., Phumiamorn, S., Sapsutthipas, S., Sangsupawanich, P., et al. (2021). Immunogenicity and safety of an intradermal BNT162b2 mRNA vaccine booster after two doses of inactivated SARS-CoV-2 vaccine in healthy population. *Vaccines (Basel)* 9, 1375. PMID: 34960122; PMCID: PMC8703694. <https://doi.org/10.3390/vaccines9121375>.
50. McKinney, B.A., Reif, D.M., Rock, M.T., Edwards, K.M., Kingsmore, S.F., Moore, J.H., and Crowe, J.E., Jr. (2006). Cytokine expression patterns associated with systemic adverse events following smallpox immunization. *J. Infect. Dis.* 194, 444–453. Epub 2006 Jul 13. PMID: 16845627; PMCID: PMC1620015. <https://doi.org/10.1086/505503>.
51. Fenwick, C., Turelli, P., Pellaton, C., Farina, A., Campos, J., Raclot, C., Pojer, F., Cagno, V., Pantaleo, G., and Trono, D. (2021). A multiplexed high-throughput neutralization assay reveals a lack of activity against multiple variants after SARS-CoV-2 infection. Preprint at MedRxiv. <https://doi.org/10.1101/2021.04.08.21255150>.
52. Goel, R.R., Painter, M.M., Apostolidis, S.A., Mathew, D., Meng, W., Rosenfeld, A.M., Lundgreen, K.A., Reynaldi, A., Khoury, D.S., Pattekar, A., et al. (2021). mRNA vaccines induce durable immune memory to SARS-CoV-2 and variants of concern. *Science* 374, abm0829.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	RESOURCE	IDENTIFIER
Bacterial and virus strains		
hCoV-19/Japan/TY-WK-521/2020	National Institute of Infectious Diseases	EPI_ISL_408667
hCoV-19/Japan/QHN002/2020	National Institute of Infectious Diseases	EPI_ISL_804008
hCoV-19/Japan/TY7-503/2021	National Institute of Infectious Diseases	EPI_ISL_877769
hCoV-19/Japan/TY8-612-P1/2021	National Institute of Infectious Diseases	EPI_ISL_1123289
hCoV-19/Japan/TY8-524-P0/2021	National Institute of Infectious Diseases	EPI_ISL_1358213
hCoV-19/Japan/TY11-330-P1/2021	National Institute of Infectious Diseases	EPI_ISL_2158613
hCoV-19/Japan/TY11-927-P1/2021	National Institute of Infectious Diseases	EPI_ISL_2158617
hCoV-19/Japan/TY38-873.2P0/2021	National Institute of Infectious Diseases	EPI_ISL_7571617
hCoV-19/Japan/TY38-871P0/2021	National Institute of Infectious Diseases	EPI_ISL_7571618
hCoV-19/Japan/TY40-385-P1/2022	National Institute of Infectious Diseases	EPI_ISL_9595859
hCoV-19/Japan/TY40-753-P1/2022	National Institute of Infectious Diseases	EPI_ISL_9595860
hCoV-19/Japan/TY40-816-P1/2022	National Institute of Infectious Diseases	EPI_ISL_9595861
Biological Samples		
Human serum samples obtained from vaccinated healthcare workers	This Paper	N/A
Chemical, peptides, and recombinant proteins		
SARS-CoV-2 RBD	Moriyama et al., 2021 ³¹	N/A
Critical commercial assay system		
Bio-Plex Pro Human SARS-CoV-2 Variant Neutralization Antibody, 11-plex	Bio-Rad	#12016897
Bio-Plex Pro Human Cytokine Screening Panel 48-plex	Bio-Rad	#12007283
Elecsys® Anti-SARS-CoV-2 S RUO	Roche	REF 9203095190
Experimental models: Cell lines		
VeroE6/TMPRSS2 cells	JCRB Cell Bank	JCRB1819
Software and algorithms		
Bio-Plex Manager Software version 6.0	Bio-Rad	N/A
CellSence 3.1.1	Olympus	N/A
JMP 14	SAS Institute Inc	N/A
Graphpad Prism 9	Graphpad	N/A
Other		
Bio-Plex MAGPIX system	Bio-Rad	#171015033J2
Olympus CKX41	Olympus	N/A
Olympus DP80	Olympus	N/A

RESOURCE AVAILABILITY

Lead contact

Additional information and requests for resources and reagents should be directed to the lead contact, Takuo Mizukami (tmiz@nih.gov).

Materials availability

The SARS-CoV-2 viruses used in this study are available from NIID under a material transfer agreement with NIID, Tokyo, Japan.

Data and code availability

- All data reported in this paper will be shared by the [lead contact](#) upon request.
- This study did not generate any new codes.
- Any additional information required to reanalyze the data reported in this work is available from the [lead contact](#) upon request.

EXPERIMENTAL MODELS AND SUBJECT DATA

Study design, human subjects and collection

This study was part of a long-term safety and immunogenicity study (LT-SI study) aimed at evaluating anti-spike antibody titers (anti-SAb) and NTs after vaccination for comparison with the adverse event rate during three-dose vaccination with the Pfizer/BioNTech SARS-CoV-2 mRNA vaccine (Comirnaty) in healthcare workers. The vaccine was approved for emergency use with special authorization from the Japanese government in February 2021. Participants who had not been previously infected with SARS-CoV-2 were administered the SARS-CoV-2 mRNA vaccine. We collected 259 samples at each vaccination step (VAX) from the National Hospital Organization Murayama Medical Center (NHO-MMC). The study design is shown in [Figure 1](#). We first evaluated the anti-SAb IgG and neutralization antibody titers (NTs) at an average of 20 days after the first and 12 days after the second vaccination (1st VAX and 2nd VAX). We prepared a post-vaccination serum panel by selecting representative samples to determine the median anti-SAb titers in each NT against WK-521 after the second vaccination ([Figure 2](#)). We selected 10–14 samples from the x320, x80, and x20 NTs groups and confirmed that they showed a normal distribution within each NT. The same vaccines were used after the second vaccination, including after 3 months (2nd VAX+3M), pre-third vaccination (pre-3rd VAX) 243 days after the second vaccination, and after the third vaccination (3rd VAX). Because of changes in the personnel within the hospital groups, we could not obtain serial data from four participants after the 2nd VAX; therefore, we added another four representatives from the x160 NT population. To form a similar composition of sex and age in our panel and to avoid overlap of anti-SAb titers in different NTs, we did not select a sample from x40 NT. A total of 272 participants were enrolled in this study. The adverse events (AEs) are collected 2 weeks after the second and third booster vaccination using participant questionnaires. After the second vaccination, we found that one healthcare worker tested positive for COVID-19 via PCR testing. This breakthrough-infected participant was not included in our third-dose vaccination study.

Ethics statement

All samples, protocols, and procedures were approved by the Medical Research Ethics Committee of NHO-MMC (#21-01, #21-04, #21-08) and NIID Institutional Review Boards (Approval number #1270 and #1339) for the use of human subjects in accordance with the Helsinki Declaration.

SARS-CoV-2 virus

We used the SARS-CoV-2 ancestral strain WK-521 (lineage A, GISAID ID: EPI_ISL_408667), Alpha variant QHN002 (lineage B.1.1.7, GISAID ID: EPI_ISL_804008), Gamma variant TY7-503 (lineage P.1, GISAID ID: EPI_ISL_877769), Beta variant TY8-612 (B.1.351, GISAID ID: EPI_ISL_1123289), R.1 variant TY8-524 (R.1, GISAID ID: EPI_ISL_1358213), Delta variant TY11-330 (B.1.617.1, GISAID ID: EPI_ISL_2158613), Delta variant TY11-927 (B.1.617.2, GISAID ID: EPI_ISL_2158617), Omicron variant TY38-873 (BA.1, GISAID ID: EPI_ISL_7571617), Omicron variant TY38-871 (BA.1.1, GISAID ID: EPI_ISL_7571618), Omicron variant TY40-385 (BA.2, GISAID ID: EPI_ISL_9595859), Omicron variant TY40-753 (BA.2, GISAID ID: EPI_ISL_9595860), and Omicron variant TY40-816 (BA.2, GISAID ID: EPI_ISL_9595861) as live viruses. These viruses were isolated from VeroE6/TMPRSS2 cells using respiratory specimens collected from individuals screened at an airport quarantine facility in Japan at NIID with ethical approval from the Medical Research Ethics Committee of NIID for the use of human subjects (#1178). All isolated viruses were sequenced at NIID. TY38-871 was found to contain an additional R346K mutation.

METHOD DETAILS

Anti-SAb evaluation

The anti-spike antibody titer was measured using the Elecsys anti-SARS-CoV-2 S assay (Roche Diagnostics International Ltd., Basel, Switzerland), which is an electrochemiluminescence immunoassay with a double-antigen sandwich design used to detect immunoglobulins in the RBD of the spike protein. This kit primarily detects IgG, IgA, and IgM. Serum samples were prepared according to the manufacturer's instructions and analyzed using the Roche Cobas e411 platform. According to the manufacturer's guidelines, sample values of ≥ 0.8 AU/mL were classified as positive for anti-SARS-CoV-2 antibodies.

Live virus microneutralization assay

Live virus neutralization assays were performed as previously described.^{31–33} Briefly, serum samples were serially diluted (two-fold dilution starting from 1:5) in high-glucose Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum and 100 U/mL penicillin/streptomycin and then mixed with 100 \times ancestral strain, QHN002 (Alpha variant), TY7-503 (Gamma variant), TY8-612 (Beta variant), TY8-524 (R.1 variant), TY11-330 (Delta variant), TY11-927 (Delta variant), TY38-873 (Omicron variant BA.1), TY38-871 (Omicron variant BA.1.1), TY40-385 (Omicron variant BA.2), TY40-753 (Omicron variant BA.2), and TY40-816 (Omicron variant BA.2), followed by incubation at 37°C for 1 h. The virus-serum mixture was placed on VeroE6/TMPRSS2 cells (JCRB1819) seeded in 96-well plates and cultured at 37°C with 5% CO₂ for 5 days. The timing of the appearance of cytopathic effects (CPEs) was unchanged among all the SARS-CoV-2 variants over days 5 post infection (Figures S1 and S2). After culture, the cells were fixed with 20% formalin (Fujifilm Wako Pure Chemicals, Osaka, Japan) and stained with crystal violet (Sigma-Aldrich, St. Louis, MO, USA). The mean cutoff dilution index with the >50% cytopathic effect from two multiplicate series is presented as the NT. The NT titer of the sample below the detection limit (1:5 dilution) was set as 2.5. To calculate the geometric mean of the microneutralization (MN) titers, an MN titer of 0 was considered as 2.5. This is described in the figures as below the detection limit. All experiments using live viruses were performed in a biosafety level 3 facility at the NIID. In each assay, back titrations of SARS-CoV-2 infectivity were performed to demonstrate infection with ~ 100 TCID₅₀ in each well.

Multiplex SARS-CoV-2 neutralization antibodies detection assay

SARS-CoV-2 neutralizing antibodies in serum samples were analyzed using the Bio-Plex Pro Human SARS-CoV-2 Variant Neutralization Antibody, 11-plex (Bio-Rad) according to the manufacturer's instructions and a previous report.⁵¹ Briefly, after determination of the optimal concentration of some typical serum samples, 25- μ L serum samples were mixed with SARS-CoV-2 antigen-coupled beads in a 96-well plate. After incubation on a shaker at 850 rpm for 30 min at room temperature, 25 μ L of biotinylated detection angiotensin-converting enzyme 2 receptor was added and the mixture was incubated at 850 rpm for 30 min. After washing, 50 μ L of streptavidin-phycoerythrin was added, and the mixture was incubated on a shaker at 850 rpm for 10 min at room temperature. The beads were washed and resuspended in 125 μ L of assay buffer. Fluorescence intensities were measured using a Bio-Plex MAGPIX multiplex reader. Data were analyzed using Bio-Plex Manager Software version 6.0 (Bio-Rad).

Cytokine assay

To monitor abnormal and prolonged cytokine production, the cytokine levels in serum samples 1–2 weeks after the second and third vaccination doses were

analyzed using the Bio-Plex Pro Human Cytokine Screening Panel 48-plex (#12007283) (Bio-Rad) according to the manufacturer's instructions (Figures S3 and S4). Briefly, 50 μ L of the serum sample was mixed with capture antibody-coupled beads in a 96-well plate and incubated on a shaker at 850 rpm for 1 h at room temperature. The beads were washed, mixed with 50 μ L of biotinylated detection antibodies, and incubated on a shaker at 850 rpm for 30 min at room temperature. After washing, 50 μ L of streptavidin-phycoerythrin reporter dye was added and incubated on a shaker at 850 rpm for 10 min at room temperature. The beads were washed and resuspended in 125 μ L assay buffer. Fluorescence intensities were measured using a Bio-Plex MAGPIX multiplex reader (Bio-Rad) and the data were analyzed using Bio-Plex Manager Software version 6.0.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistics

All statistical experimental designs and data analyses were performed using JMP software version 14.2.0 (SAS Institute, Cary, NC, USA) and GraphPad Prism 9.0.2 software (GraphPad, Inc., San Diego, CA, USA). To assess the correlations, simple linear regression was carried out with GraphPad Prism. To assess statistical significance, one-way ANOVA and Kruskal-wallis test (comparison of $3 \geq$ groups), unpaired t test and paired T test (comparison between 2 groups) were carried out with GraphPad Prism. A heatmap of Spearman's rank correlation coefficients was evaluated with JMP software and used to evaluate cross reactivity among the SARS-CoV-2 variants using the sera after the second and third vaccination doses. For all figures, p values are represented as follows: ***p < 0.001, ****p < 0.0001. Statistical details of our experiments and analyses can be found in each figure legends.