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Fourth mRNA vaccination increases cross-neutralizing antibody titers against SARS-CoV-2 variants, including BQ.1.1 and XBB, in a very elderly population

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ABSTRACT

Background: Omicron variants with immune evasion have emerged, and they continue to mutate rapidly, raising concerns about the weakening of vaccine efficacy, and the very elderly populations are vulnerable to Coronavirus Disease 2019 (COVID-19). Therefore, to investigate the effect of multiple doses of mRNA vaccine for the newly emerged variants on these populations, cross-neutralizing antibody titers were examined against Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) variants, including BQ.1.1 and XBB.
Methods: Blood samples were taken from residents at four long-term care facilities in Hyogo prefecture, Japan (median age, 91 years), after 3rd (n = 67) and 4th (n = 48) mRNA vaccinations, from April to October 2022. A live virus microneutralization assay was performed to determine the neutralizing antibody titers in participants' sera.
Results: After 3rd vaccination, cross-neutralizing antibody prevalence against conventional (D614G) virus, Delta, Omicron BA.2, BA.5, BA.2.75, BQ.1.1, and XBB were 100%, 97%, 81%, 51%, 67%, 4%, and 21%, respectively. After 4th vaccination, the antibody positivity rates increased to 100%, 100%, 98%, 79%, 92%, 31%, and 52%, respectively. The 4th vaccination significantly increased cross-neutralizing antibody titers against all tested variants.
Conclusion: The positivity rates for BQ.1.1 and XBB increased after 4th vaccination, although the titer value was lower than those of BA.5 and BA.2.75. Considering the rapid mutation of viruses and the efficacy of vaccines, it may be necessary to create a system that can develop vaccines suitable for each epidemic in consideration of the epidemic of the virus.

Introduction

Infection by Severe Acute Respiratory Syndrome–Coronavirus 2 (SARS-CoV-2) can cause Coronavirus Disease 2019 (COVID-19). The first case of COVID-19 pneumonia was reported in Wuhan, China, in December 2019. The virus spread so rapidly that the World Health Organization declared COVID-19 a pandemic in March, 2020 [1]. Since then, SARS-CoV-2 has undergone such mutations that the current circulating variants have striking differences from the wild type. One of its variants, Omicron BA.1, which harbors over 30 amino acid mutations in the S protein, has emerged at the end of 2021 [2], and its derivatives, which have replaced the current epidemic variants, have been appearing one after another. At the time this paper has been written, in Japan and worldwide, the dominant circulating subvariant has been Omicron BA.5. However, the other Omicron subvariants, BA.2.75, BQ.1.1 and XBB have also been circulating in smaller proportions [3]. These newly circulating variants have been reported to have a reduced susceptibility to SARS-CoV-2 neutralizing antibodies, obtained after vaccinations [4–7]. A study in 20 individuals (median age 48.5 years) in Japan reported that 4th mRNA vaccination could induce cross-neutralizing antibodies against Omicron BA.5, BQ.1.1, and XBB with 11.7-fold, 43.3-fold, and 51.6-fold reduction, respectively, compared to conventional virus [7].

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Vaccination is still considered the most reliable measure to prevent infection and to reduce the morbidity and mortality of COVID-19. In Japan, 4th vaccination of the elderly has been recommended [8]. One of the most prominent risk factors of severe and prolonged COVID-19 is advanced age [9–11]. The risk of severe COVID-19 has been reported for high age group [12]. Recent studies showed that 4th mRNA vaccination can protect elderly populations from infections, hospitalizations for mild-to-moderate illness, severe illness, and death related to COVID-19 [13–18]. Kurhade et al. reported the low cross-neutralizing antibody levels against newly appeared Omicron subvariants, BQ.1.1 and XBB.1 in a population who received 4th vaccination (median age 80 years) [19]. To assess whether 3rd and 4th vaccinations can induce neutralizing antibodies against the newly appeared Omicron subvariants for the elderly, we aimed to analyze the cross-neutralizing antibodies for several variants including Omicrons after 3rd and 4th mRNA vaccinations in a very elderly population (median age 90 years).

Material and methods

Study site and participant recruitment

Blood samples were collected from residents in 4 long-term care facilities in Hyogo prefecture, Japan (Koyukai Nishi Hospital, Subaru Uozaki-no-sato, Subaru Rokko, and Carehome Subaru). The facilities belong to Subaru Medical and Welfare Group Koyukai Medical Corporation, and widely support health of elderly including vaccination. Participants were divided into two groups based on the total number of vaccination doses they had received, namely three or four. For elderly (> 65 years old), the 3rd vaccination schedule was started from December 2021, at least 6 months after the 2nd vaccination [20]. The 4th vaccination started from May 2022, at least 5 months after 3rd vaccination [21]. The mRNA vaccines administered were Comirnaty (BNT162b2, Pfizer-BioNTech) for 1st to 3rd doses, and either Comirnaty or Spikevax (mRNA-1273, Moderna) for 4th vaccination. Blood samples in the 3rd vaccination group were taken in April 27 to May 20, 2022. Blood sampling for the 4th vaccination group was conducted from September 1 to October 6, 2022. Underlying medical conditions of participants were also documented. Some, but not all, participants of the 3rd vaccination group were included in the 4th vaccination group.

Participants from both groups who had a history of COVID-19 infection or high serum titers of anti-nucleocapsid (N) antibody were analyzed separately from the main group. Antibodies against the N protein of SARS-CoV-2 are produced in people who have been infected by the SARS-CoV-2, but not in those receiving mRNA vaccinations. Since we aimed to evaluate neutralizing antibodies elicited by vaccination alone, participants with hybrid immunity (i.e., immunity elicited by both infection and vaccination) were analyzed separately in this study. No statistical methods were used to predetermine the sample size.

SARS-CoV-2 variants

The SARS-CoV-2 Biken-2 (B2) variant containing the S D614G mutation (whole genome sequence in DNA Data Bank of Japan accession number: LC644163) was received from BIKEN Innovative Vaccine Research Alliance Laboratories, Osaka University, Osaka, Japan, and was used as the conventional virus. The other SARS-CoV-2 variants—Delta (GISAID ID: EPI_ISL_2158617), Omicron BA.2 (GISAID ID: EPI_ISL_9595859), Omicron BA.5 (GISAID ID: EPI_ISL_13241867), Omicron BA.2.75 (GISAID ID: EPI_ISL_13969765), Omicron BQ.1.1 (GISAID ID: EPI_ISL_15579783), and Omicron XBB.1 (GISAID ID: EPI_ISL_15669344)—were received from the National Institute of Infectious Diseases, Tokyo, Japan. Each variant's mutations of the S gene were reconfirmed in our laboratory by complementary DNA sequencing.

Live virus neutralization assay

Neutralizing antibodies of human sera were detected with an in vitro neutralization assay as described in our previous studies [22–24]. Briefly, Vero E6-expressing transmembrane serine protease 2 (Vero E6/TMPRSS2) cells were seeded 4×10^4 cells/well in a 96-well microplate in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 1 g/L G418 disulfate, and were grown overnight inside an incubator (37 °C with 5% CO_2 infusion) [25]. Sera were heat-treated at 56 °C for 30 min, and then serially diluted two-fold in DMEM. In a biosafety level 3 laboratory, authentic SARS-CoV-2 solution were added at 100-median tissue culture infectious dose (100 $TCID_{50}$) per well into diluted sera and incubated at 37 °C for 1 h. The sera and virus mixture were then added to Vero E6/TMPRSS2 cells and incubated at 37 °C with 5% CO_2 infusion for 6 days. In this system, failure to neutralize results in infection, which can be visualized as a cytopathic effect. The neutralizing antibody titer was defined as the lowest concentration (i.e., highest dilution) of sera that could completely inhibit cytopathic effects. The cutoff titer was set at 2 as the detection limit as in our previous study [24]. Neutralizing antibody was considered positive if the titer was 2 or more.

Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) method was used to detect anti-SARS-CoV-2-S or -N immunoglobulin (IgG) antibodies in human sera as described in our previous study [26]. Briefly, the wells of a 96-well ELISA microplate (Corning) were coated with S- or N protein (100 ng/well) in a coating buffer (0.252% w/v sodium carbonate, 0.84% w/v sodium bicarbonate, pH 9) and incubated at 4 °C overnight. The plate was washed twice with phosphate-buffered saline containing 0.1% Tween 20 (PBST). The plate was blocked with blocking buffer (phosphate-buffered saline supplemented with 1% w/v bovine serum albumin) at 4 °C for 2 h and then washed twice with PBST. Sera were serially diluted 1:40 to 1:5120 in 100 μl dilution buffer (PBST supplemented with 1% w/v bovine serum albumin) and then added to the ELISA plate. The plate was incubated at 37 °C for 1 h and then washed twice with PBST. Goat anti-human IgG-HRP (abcam) was diluted 1:10000 in dilution buffer, then added 100 μl per well into the ELISA plate. The plate was incubated in 37 °C for 1 h and then washed twice with PBST. ABTS solution (Roche) was used for color development at room temperature for 40 min. The reaction was stopped using stop buffer (1.5% w/v oxalic acid dehydrate).

Optical density was measured at 405 nm using the plate reader Multiskan FC (Thermofisher Scientific). A cut-off OD value of 0.3 for 1:40 serum dilution was used to define reactivity to S- or N protein considering the average OD values of naïve samples and standard deviation [26]. Antibody titers are shown as area-under-the-curve (AUC) values.

S proteins expression

S proteins used for ELISA were prepared in-house using a recombinant expression system similar to that previously reported by another research group [27]. This protein was also used for our previous project [26]. Briefly, the gene sequence of the SARS-CoV-2 S ectodomain (amino acids 1–1213) was subcloned into a pCAGGS vector [28] containing a puromycin-resistance gene. Other than specific mutations of each variant, the S sequence used here includes mutations R682del, R683del, R685del, F817P, A892P, A899P, A942P, K986P, and V987P to remove a furin cleavage site and to stabilize the

prefusion state, as well as additional sequences: namely, an HRV-3 C recognition site, a T4 foldon, and a His-tag at the C-terminal side. The sequence was confirmed by the capillary electrophoresis sequencer DS3000 (Hitachi High-Tech). The S protein was expressed using the Expi293 expression system (Thermofisher Scientific) according to the manufacturer's instructions. The culture supernatant was collected at five days post transfection. The His-tagged S protein was purified by Ni-NTA agarose (Qiagen). Purity of the eluted S protein was confirmed by SDS-PAGE.

N protein expression

A pET21b vector containing the SARS-CoV-2 N gene (amino acids 1–419) was kindly provided by Prof. Yoshiharu Matsuura from Research Institute for Microbial Diseases, Osaka University. An additional HRV-3 C restriction site was added into the N gene fragment, and the fragment was then subcloned into a pMAL-c2 vector [29,30]. The recombinant plasmid was propagated and subsequently transformed into competent *Escherichia coli* strain BL21 for protein expression.

E. coli BL21 containing the recombinant plasmid was shake-flask cultured (200 rotation per minute at 37 °C) in Luria Bertani broth supplemented with 50 μg/ml carboxy-benzylpenicillin as a selective agent. Isopropyl β-d-1-thiogalactopyranoside was added when the culture had reached an absorbance of 0.6–0.7 at 600 nm. The suspension was further cultured at 32 °C for 3 h, or 19 °C overnight. The cell pellet was concentrated and lysed to release the expressed protein into the supernatant. The supernatant was then separated from the pellet. The maltose-binding protein (MBP)-bound N protein was purified from the supernatant using amylose resin beads (NEB); the eluted N protein was then cleaved from MBP using HRV-3 C protease. The N protein was purified by cation exchange using SP Sepharose Fast Flow (GE Healthcare), and the purified N protein was confirmed by SDS-PAGE.

Statistical analysis

Data were analyzed using Prism 8 (GraphPad) software. The Mann-Whitney or Kruskal-Wallis test was used to compare the neutralizing antibody titers against each variant in each group. If a significant difference was found, the Dunn multiple comparison test was then performed. Fold-change was calculated as the ratio of geometric mean of each data with respect to that of reference data. The reference data were defined in each analysis. Spearman's rank correlation coefficient was used to determine the strength of the correlation between the ELISA AUC value and the neutralizing antibody titer for each variant. P values < 0.05 (two-tailed) were considered statistically significant.

Results

The 3rd vaccination group consisted of 67 participants with an age range of 80–103 years (median age 92 years). The 4th vaccination group consisted of 48 participants with an age range of 69–103 years (median age 91 years old). There were 43 individuals from the 3rd vaccination group who were included in the 4th vaccination group (Fig. 1). Participants' characteristics are described in Table 1.

Neutralizing antibody titers

We tested sera neutralizing antibody titers against seven variants of SARS-CoV-2, i.e., conventional virus, Delta, Omicron BA.2, BA.5, BA.2.75, BQ.1.1, and XBB, at two time points: 103 days after the 3rd vaccination and 48 days after the 4th vaccination. After the 3rd vaccination, positivity rates of neutralizing antibodies against conventional virus, Delta, Omicron BA.2, BA.5, BA.2.75, BQ.1.1, and XBB were 100%, 97%, 81%, 51%, 67%, 4%, and 21%, respectively (Fig. 2A). Neutralizing antibodies against BQ.1.1 seemed to be barely positive, but still to be remained positive against XBB. After the 4th vaccination, the positivity rates against conventional virus, Delta, Omicron BA.2, BA.5, BA.2.75, BQ.1.1, and XBB increased to 100%, 100%, 98%, 79%, 92%, 31%, and 52%, respectively (Fig. 2C), while the neutralizing antibody titers increased significantly after the 4th vaccination: 2.6-fold, 2.0-fold, 4.2-fold, 3.5-fold, 2.7-fold, 1.9-fold, and 2.0-fold, respectively (Fig. 2E). The neutralizing antibody titer's potency decreased with each emerging variant (Fig. 2B, D). Among them, neutralizing antibody titers of BA.5 was maintained at a certain level, compared to that of BA.2. But especially for BQ.1.1, although the neutralizing antibody positivity rate increased with 4 doses of vaccine, the titers was significantly lower than that of BA.2 (Fig. 2D).

To investigate the influence of age in the humoral immune response to the 3rd and 4th vaccinations, we separately analyzed neutralizing antibody titers for participants aged under 90 years old and 90 years or more. Positivity rates of neutralizing antibody against conventional virus, Omicron BA.2, BA.5, BA.2.75, BQ.1.1, and XBB after 3rd vaccination were similar between participants under 90 years old and older (Fig. 3A, C). Cross-neutralizing antibody titers against conventional virus, Omicron BA.2, BA.5, BA.2.75, BQ.1.1, and XBB were significantly increased after the 4th vaccination: 2.6-fold, 4.4-fold, 2.6-fold, 3.1-fold, 1.8-fold, and 2.0-fold, respectively, for participants under 90 years; and 2.6-fold, 4.1-fold, 4.1-fold, 2.5-fold, 1.9-fold, and 2.0-fold, respectively, for participants aged 90 years or more. In participants aged 90 years or more, the neutralizing antibody titers after 3rd vaccination were lower than those of participants aged under 90 years. However, after the 4th vaccination, the neutralizing antibody titers between these two age groups were

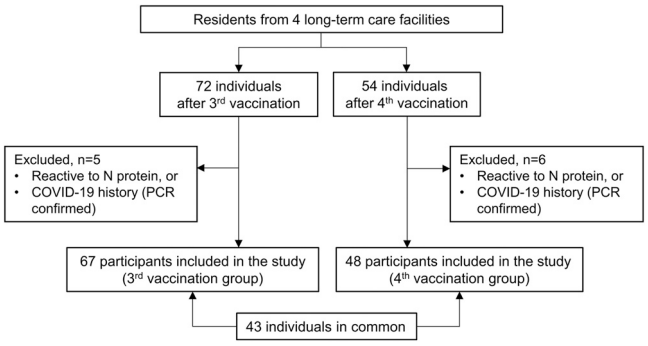

Fig. 1. Participant enrollments for the 3rd and 4th vaccination groups. PCR, polymerase chain reaction.

Table 1
Participant characteristics in the 3rd vaccination and 4th vaccination groups.

Participants Characteristics	3rd vaccination (n = 67)	4th vaccination (n = 48)
Age, median (IQR), years	92 (88–96)	91 (87–96)
Male, n (%)	8 (12)	8 (17)
Days from 3rd vaccination, median (IQR)	103 (98–111)	
Days from 4th vaccination, median (IQR)		48.5 (42–92.5)
Medical history:		
Hypertension, n (%)	47 (70)	33 (69)
Hyperlipidemia, n (%)	10 (15)	8 (17)
Diabetes mellitus, n (%)	17 (25)	13 (27)
Chronic heart disease, n (%)	26 (39)	17 (35)
Respiratory disease, n (%)	6 (9)	5 (10)
Cerebrovascular disease, n (%)	21 (31)	16 (33)
Cancer, n (%)	8 (12)	4 (8)

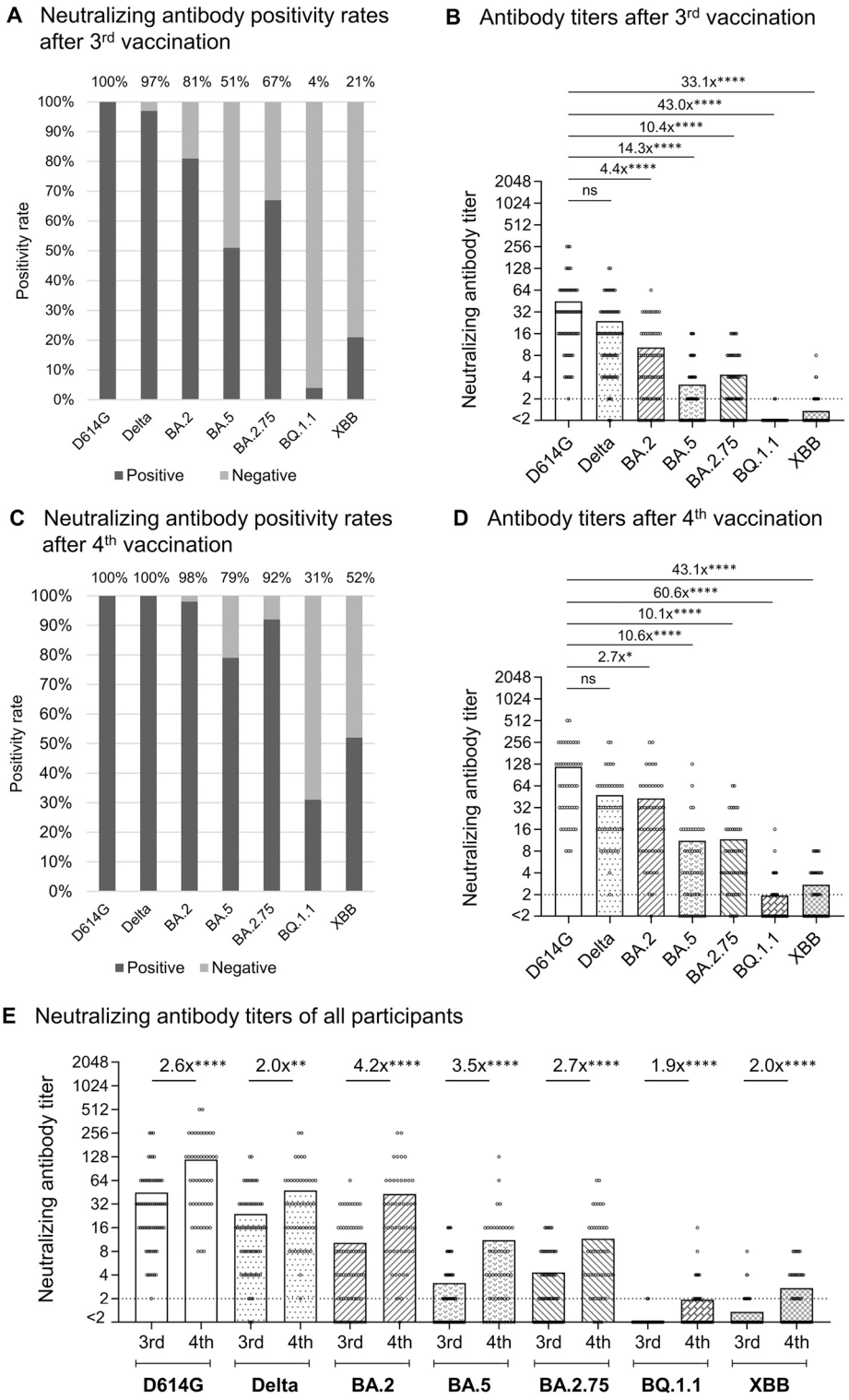

Fig. 2. Neutralizing antibody positivity rates and titers against SARS-CoV-2 conventional virus (D614G), Delta, Omicron BA.2, BA.5, BA.2.75, BQ.1.1 and XBB after the 3rd and 4th mRNA vaccinations: (A) Neutralizing antibody positivity rates after 3rd vaccination (n = 67), (B) Neutralizing antibody titers after 3rd vaccination, (C) Neutralizing antibody positivity rates after 4th vaccination (n = 48), (D) Neutralizing antibody titers after 4th vaccination; Kruskal-Wallis test was used to compare the neutralizing antibody titers against each variant in each group. If a significant difference was found, the Dunn multiple comparison test was then performed. (E) All participants at both time points, Mann-Whitney test was used to compare the neutralizing antibody titers between 3rd and 4th vaccination groups. In (B) and (D), relative fold-changes were calculated as the ratios of geometric means of D614G to those of indicated data. In (E), relative fold-changes were calculated as the ratios of geometric means of 4th vaccination to those of 3rd vaccination. Statistical significance was also shown. ns, not significant; * p < 0.05; ** p < 0.01; **** p < 0.0001.

similar (Fig. 3B, D), indicating that 4th vaccination is important to increase the neutralizing antibody of people aged 90 years or more.

Participants' medical conditions were disclosed during the recruitment process; these included hypertension, hyperlipidemia, diabetes mellitus, chronic heart disease, chronic respiratory disease, cerebrovascular disease, and malignancy. We analyzed the influence of participants' medical conditions to neutralizing antibody titers after the 3rd and 4th vaccinations using the Mann-Whitney test.

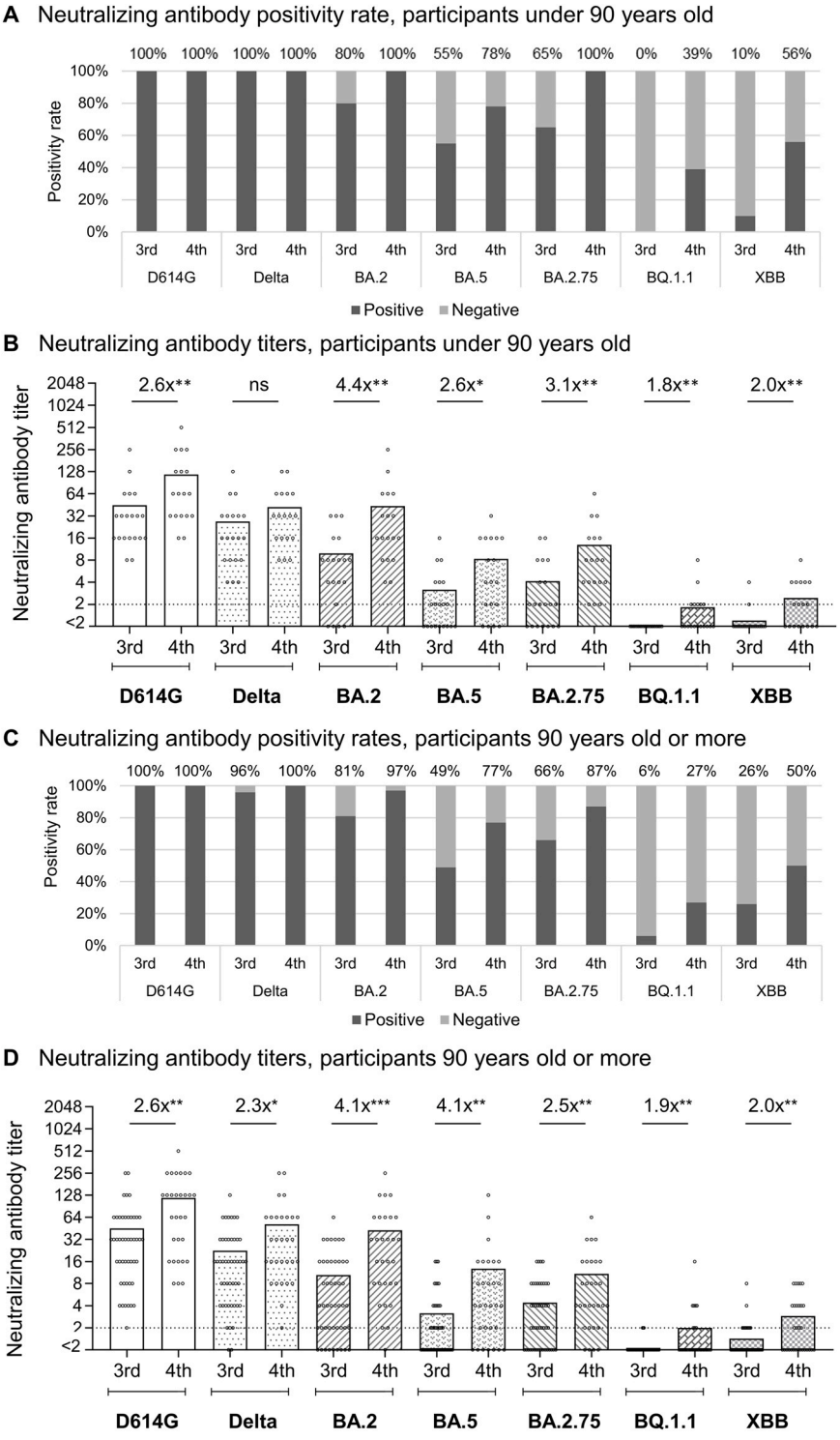

Fig. 3. Neutralizing antibody positivity rates and titers against SARS-CoV-2 conventional virus (D614G), Delta, Omicron BA.2, BA.5, BA.2.75, BQ.1.1 and XBB after the 3rd and 4th mRNA vaccinations, based on age: Participants under 90 years old (3rd vaccination n = 20, 4th vaccination n = 18), and Participants aged 90 years old or more (3rd vaccination n = 47, 4th vaccination n = 30). (A) Neutralizing antibody positivity rates, participants under 90 years old, (B) Neutralizing antibody titers, participants under 90 years old, (C) Neutralizing antibody positivity rates, participants aged 90 years old or more, (D) Neutralizing antibody titers, participants aged 90 years old or more. Mann-Whitney test was used to compare the neutralizing antibody titers between 3rd and 4th vaccination groups. In (B) and (D), relative fold-changes were calculated as the ratios of geometric means of 4th vaccination to those of 3rd vaccination and expressed together with statistical significance. ns, not significant; * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

There was no significant difference found in neutralizing antibody titers after the 3rd or 4th vaccination among the various medical conditions (Fig. 4A-B).

Anti-SARS-CoV-2-S antibody titers

IgG against the S protein of the SARS-CoV-2 variants was measured by ELISA, and the titers are shown as areas under the curve (AUCs). Anti-S IgG was detected in all participants' samples. The binding affinity of anti-S IgG was significantly increased for the

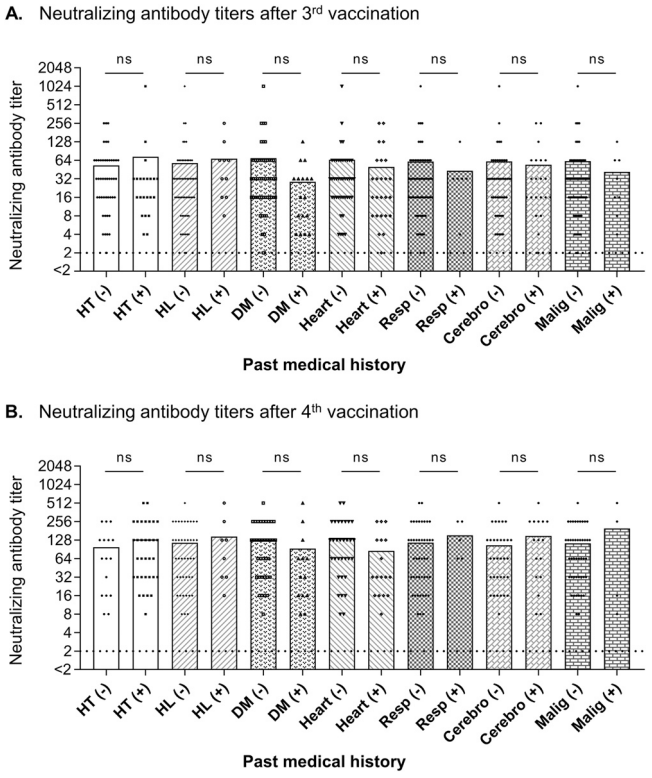

Fig. 4. Comparation of neutralizing antibody titers against conventional (D614G) virus after 3rd and 4th mRNA vaccination for each of participants' medical condition. (A) Neutralizing antibody titer after 3rd vaccination (n = 67), (B) Neutralizing antibody titer after 4th vaccination (n = 48). Mann-Whitney test was used to compare the neutralizing antibody titers in the absence and presence of disease. Abbreviations: ns, not significant; HT, hypertension; HL, hyperlipidemia; DM, diabetes mellitus; Heart, chronic heart disease; Resp, chronic respiratory disease; Cerebro, cerebrovascular disease; Malig, malignancy; (-) disease not present, (+) disease present.

SARS-CoV-2 S protein of the conventional virus, Omicron BA.2, BA.5, and BA.2.75 variants, respectively, after the 4th vaccination (S Fig. 1). The correlation between ELISA AUCs and neutralizing antibody titers was analyzed by Spearman's rank correlation coefficient method for all samples. We found a moderate-to-strong positive correlation of anti-S titers and neutralizing antibody titers, with correlation coefficients (r) of 0.55, 0.77, 0.73, and 0.74 for conventional virus, Omicron BA.2, BA.5, and BA.2.75, respectively (S Fig. 2A-D).

Reactivity to N protein as exclusion criteria

During participant recruitment, we excluded some participants from the 3rd vaccination (n = 5) and 4th vaccination (n = 6) groups based on their COVID-19 history and sera reactivity towards the SARS-CoV-2 N protein (S Fig. 3). Antibodies against the N protein may be elicited after infection with SARS-CoV-2, but not after COVID-19 mRNA vaccination. An excluded participant who was infected in April 2021 (before the emergence of Delta variant) had a high titer of anti-N IgG at both time points, i.e., after the 3rd and 4th vaccinations. But two other excluded participants who were infected after the emergence of Delta and Omicron variants, i.e., in 2022, displayed low titer of anti-N.

Neutralizing antibody titers of excluded participants

We also performed neutralization assay for excluded participants (3rd vaccination group, n = 5; 4th vaccination group, n = 6), i.e., who have either history of COVID-19 or high reactivity to N protein (Fig. 5A-D). After 3rd vaccination, the positivity rates of cross-neutralizing antibodies against conventional virus, Delta, Omicron BA.2, BA.5, BA.2.75, BQ.1.1, and XBB were 100%, 100%, 100%, 60%, 80%, 40%, and 40%, respectively. After 4th vaccination, the rates increased to 100%, 100%, 100%, 100%, 100%, 50%, and 83%, respectively. Even after 4th vaccination, cross-neutralizing antibody titers against BQ.1.1 and XBB variants were low, compared to that against conventional virus, however their positivity rates increased when compared to those of the 3rd vaccination group.

Discussion

Our result showed that after 3rd vaccination, the proportion and titers of neutralizing antibody against conventional virus (D614G) and the Delta variant were higher than those against Omicron variants (Fig. 2A-B). As shown by us and the others [7,31], adequate neutralizing antibodies for Omicron BA.2 were induced after 3rd vaccination. In addition, those for BA.5 and BA.2.75 were also induced, albeit at lower levels than BA.2. However, they were strongly boosted by 4th vaccination, indicating that the 4th vaccination is important to increase neutralizing antibodies against Omicrons BA.5 and BA.2.75 which have more mutations than BA.2 [32]. On the other hands, our results show that although the neutralizing antibodies for Omicrons BQ.1.1 and XBB, which have further mutations compared to BA.5 and BA.2, respectively, were induced in some individuals by the 3rd vaccination, the proportion was very small, and even though induced, the titers were considerably lower than those of BA.5 and BA.2.75. The titers for BQ.1.1 and XBB increased and their positivity rates also increased by the 4th vaccination, although the titer value and positivity rates were significantly lower than BA.5 and BA.2.75 (Fig. 2C-D). Two other studies which tested neutralizing antibodies in sera or plasma of younger individuals who received 4th mRNA vaccination also reported similar results [7,33].

The distance mutations of S Omicron BA.2, BA.5, BA.2.75, BQ.1.1, and XBB from S Wuhan-Hu-1 were 31, 34, 33, 37, and 41 mutations, respectively [32]. Since the mRNA vaccines, Comirnaty and Spikevax, were made based on S Wuhan-Hu-1 with the addition of 2 proline mutations [34,35], the rapid mutations in Omicron subvariants result in immune escape from vaccination and infection. Omicron BQ.1.1 is a descendant of BA.5, while XBB is a mixture product of BA.2.10.1 and BA.2.75. Specifically, the Spike proteins of BQ.1.1 and XBB have the same R346T, N460K, and F486X mutations, with additional K444T mutation in BQ.1.1 and V445P, G446S, and F490S mutations in XBB, which conferred resistance to many monoclonal antibodies [31].

Some of the excluded participants in our study had relatively low reactivity to N protein, specifically those who had COVID-19 onset after the emergence of Delta and Omicron variants, when compared to another participant who had COVID-19 before the emergence of Delta variant. SARS-CoV-2 N protein is mostly conserved among coronaviruses. However, a few mutations of this structural protein have been reported. Delta has D63G, R203M, G215C, and D377Y mutations in its N protein. Most Omicron subvariants have P13L, del31/33, R203K, G204R, and S413R in their N protein [36–39]. From our perspective, the discrepancy of N protein titers between previously and recently infected individuals may need further investigation.

Neutralization assay for excluded participants' sera showed that after 3rd and 4th vaccination, the positivity rates of cross-neutralizing antibody against all tested variants were higher (Fig. 5A, C) than naïve vaccinated individuals (Fig. 2A, C), indicating the immune booster effects for the different variants by the infection, although even after 4th vaccination in previously infected group, cross-neutralizing antibody titers against BQ.1.1 and XBB were still low (Fig. 5B, D). Other studies reported that people who have received three doses of mRNA vaccine and experienced breakthrough infection by BA.2 variant also have low titers of neutralizing antibody

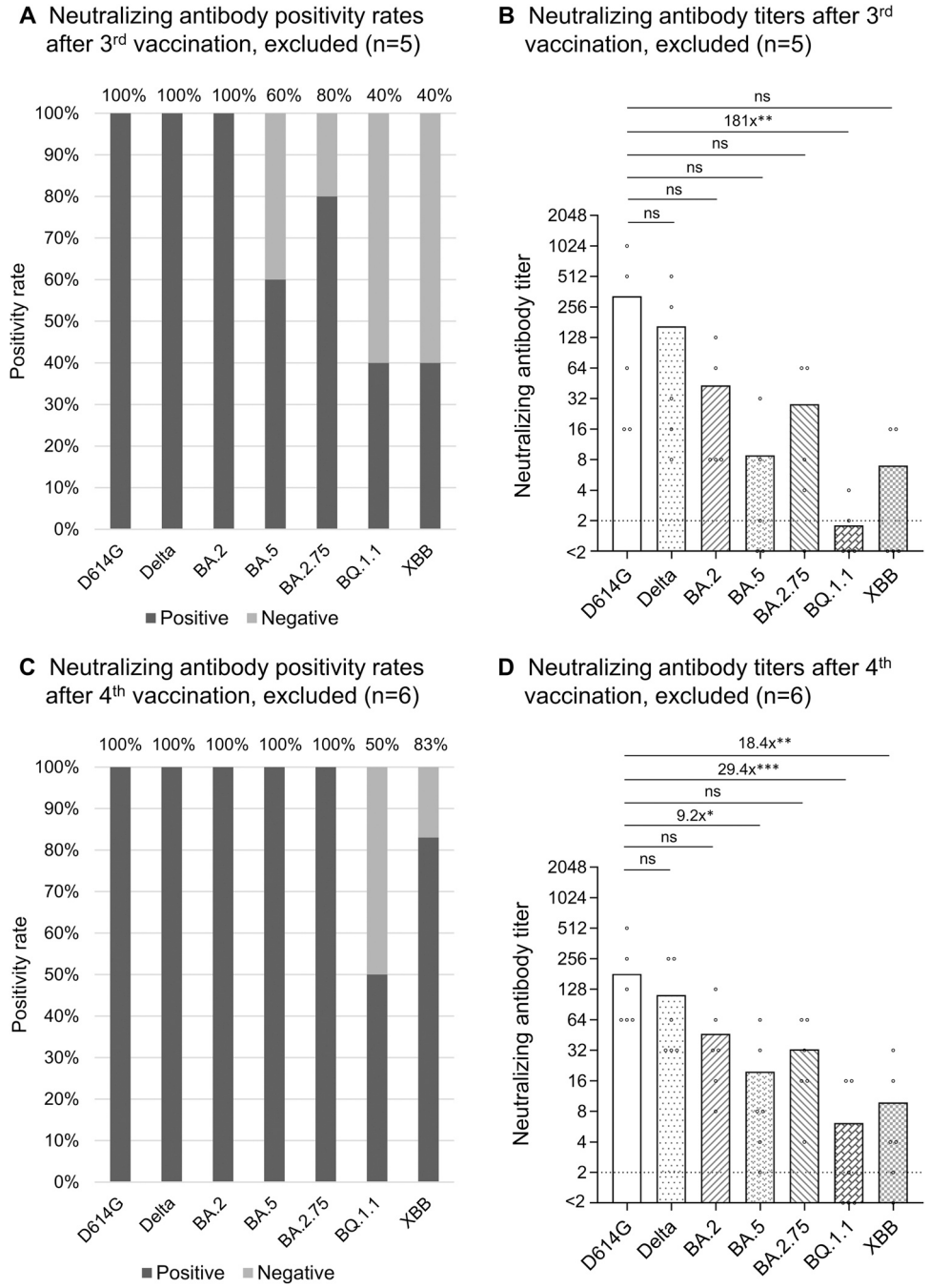

Fig. 5. Excluded participants' neutralizing antibody positivity rates and titers against SARS-CoV-2 conventional virus (D614G), Delta, Omicron BA.2, BA.5, and BA.2.75 after the 3rd and 4th vaccinations. (A) Neutralizing antibody positivity rates after 3rd vaccination, (B) Neutralizing antibody titers after 3rd vaccination, (C) Neutralizing antibody positivity rates after 4th vaccination, (D) Neutralizing antibody titers after 3rd vaccination; Kruskal-Wallis test was used to compare the neutralizing antibody titers against each variant in each group. If a significant difference was found, the Dunn multiple comparison test was then performed. In (B) and (D), relative fold-changes were calculated as the ratios of geometric means of D614G to those of indicated data and expressed together with statistical significance. ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

against BQ.1.1 and XBB compared to conventional virus [7]. Because in our study, the sample number was too few to draw any conclusion, further study would be required to conclude it.

In this study, we reported that 4th mRNA vaccination can readily induce cross-neutralizing antibodies against many SARS-CoV-2 variants in the very elderly population. However, it may not be enough to protect them from newly emerged variants, e.g., BQ.1.1 and XBB. Importantly, we reported that the use of N protein-based serology tests to determine infection history may not be accurate in elderly populations who have received multiple vaccinations.

One of the limitations of this study is the lack of a control population (i.e., those not receiving vaccinations) at the same time point. Due to the imbalance of gender proportion in our participants, we also did not analyze the influence of sex in our study. Comorbidities of participants possibly cause a limitation, because they may have influence on immune responses for vaccination, that is, the neutralizing antibody titers reported in this study. Finally, there was a discrepancy in the timings of blood sampling after the 3rd and 4th vaccinations, with more than twice as many days having elapsed after the 3rd vaccination. However, there is no doubt that the very elderly population benefits from a 4th vaccination.

Conclusion

Our participants developed higher titers and positivity rates of neutralizing antibodies against SARS-CoV-2 all variants after the 4th vaccination, but the induction of those for BQ.1.1 and XBB which are highly variable was weak. Our analysis on cross-neutralizing antibody proportions and titers induced by 4th vaccination in the elderly population aged under 90 years old were similar to those aged 90 years old or more. Although protection against infection is orchestrated by both cellular and humoral immune responses, our results may provide essential information about the role of 4th mRNA vaccination to optimize humoral immune responses in the very elderly population. Considering the rapid mutation of viruses and the efficacy of vaccines [7,19], it may be necessary to create a system that can develop vaccines suitable for each epidemic in consideration of the epidemic of the virus.

Ethics statement

This study was approved by the Ethical Committee of Kobe University Graduate School of Medicine (approval no. B200200) on 15 April, 2022. All participants of this study have given their written informed consent.

Author contributions

All authors contributed to the concept of this article. SS drafted the manuscript, and YM revised the manuscript. KF collected samples. SS, KF, YK, MIM, GBE, NH, and HI performed experiments. SS, KF, HI, MN, JA, and YM analyzed the data. YM supervised experiments. YM oversaw the project. All authors approved the final version of the manuscript.

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Declaration of Competing Interest

This statement is to certify that all Authors have seen and approved the manuscript being submitted. We warrant that the article is the Authors' original work. We warrant that the article has not received prior publication and is not under consideration for publication elsewhere. On behalf of all Co-Authors, the corresponding Author shall bear full responsibility for the submission.

This research has not been submitted for publication nor has it been published in *whole* or in part elsewhere. We attest to the fact that all Authors listed on the title page have contributed significantly to the work, have read the manuscript, attest to the validity and legitimacy of the data and its interpretation, and agree to its submission to the *Journal of Infection and Public Health.*

All authors agree that author list is correct in its content and order and that no modification to the author list can be made without the formal approval of the Editor-in-Chief, and all authors accept that the Editor-in-Chiefs decisions over acceptance or rejection or *in* the event of any breach of the Principles of Ethical Publishing in the *Journal of lnfection and Public Health* being discovered of retraction are final.

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Disclosure statement

All authors have no reported conflicts of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jiph.2023.05.004.

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