Journal of Infection 89 (2024) 106310

Contents lists available at ScienceDirect

Journal of Infection

journal homepage: www.elsevier.com/locate/jinf

Viruses and Viral Diseases

Synergistic evolution: The dynamic adaptation of SARS-CoV-2 and human protective immunity in the real world

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ARTICLE INFO

Article history:
Accepted 30 September 2024
Available online 10 October 2024

Keywords:
SARS-CoV-2
Evolution
Proteomics
Neutralizing antibody
Hybrid immunity

SUMMARY

Objectives: SARS-CoV-2 is continually evolving with new variants to evade protective immunity and cause new infections. This study aimed to assess infection-acquired immunity and hybrid immunity against re-infection or severe COVID-19.

Methods: During 2020–2023, we collected 890 serum samples from individuals infected with SARS-CoV-2 variants including wild type, D614G, Alpha, Delta, BA.1, BA.2, BA.2.76, BA.5.2, BF.7, XBB, and EG.5. The levels of serum neutralizing antibodies (NAbs) against 18 diverse SARS-CoV-2 variants were determined using a bead-based high-throughput broad neutralizing-antibody assay.

Results: In the initial wave of the COVID-19 pandemic, > 75% of the patients demonstrated robust NAb responses against the ancestral SARS-CoV-2, during a period when vaccines were not yet available. After the emergence of the Omicron variant, the seroprevalence of anti-Omicron NAbs among the patients increased rapidly. By April 2023, when XBB variant was predominant, approximately 80% of the patients demonstrated > 50% neutralization against the highly immune-evasive XBB lineages. Three serotypes of SARS-CoV-2, namely non-Omicron, Omicron, and XBB serotypes, were identified, with the strong likelihood of further changes occurring as the virus mutating. Generally, NAbs elicited by a previous serotype could not typically effectively protect against another serotype that emerges later in the evolutionary stages.

Conclusion: Our results firstly demonstrated the synergistic evolution between host immunity and SARS-CoV-2 variants in the real world, which would be helpful to develop future vaccines and public health strategies.

Introduction

Since the emergence of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) in December 2019, the virus has rapidly spread globally, causing the COVID-19 pandemic. As of September 1, 2024, the contagion has caused > 770 million confirmed cases and > 7 million deaths worldwide (https://covid19.who.int/). Throughout the pandemic, the SARS-CoV-2 genome has continuously mutated, resulting in a succession of variant replacements and a resurgence of SARS-CoV-2 infections.

In early 2020, an important mutation occurred in the SARS-CoV-2 genome, giving rise to the D614G variant, characterized by increased viral stability, transmissibility, and infectivity.[1] During the early stages of the adaptive evolution, variants of concern (VOCs), such as Alpha, Beta, and Delta, emerged in sequence, quickly spreading locally and shortly thereafter globally.[2–4] Although these VOCs originated from distinct phylogenetic lineages within the SARS-CoV-2 clade and demonstrated varying degrees of resistance to antibody-mediated neutralization,[5] vaccine-induced immune responses have

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https://doi.org/10.1016/j.jinf.2024.106310

proven effective in mitigating disease severity and preventing hospitalization.[6] However, the arrival of the Omicron (B.1.1529) variant in late 2021 led to the emergence of numerous sub-lineages with altered biological properties. These variants challenged the population humoral immunity established via vaccinations or infections, triggering an unprecedented surge in global SARS-CoV-2 infections. Multiple studies have confirmed that Omicron variants substantially evade most known antibodies. The initial Omicron BA.1 variant was able to evade the neutralizing effects of two doses of the mRNA vaccines, but this significant immune evasion was largely overcome by booster doses of these vaccines.[7] With the emergence of Omicron BA.2, various sub-variants with virally advantageous characteristics circulated simultaneously, engaging in competition within the so-called "variant soup",[8] disrupting the pattern of sequential lineage replacement seen previously. The BA.2-derived sub-lineages BA.4 and BA.5 accelerated SARS-CoV-2 evolution, as evidenced by the significant prevalence of their descendants BF.7 and BQ.1. In addition, another BA.2-derived sub-lineage, called XBB, which resulted from a recombination event between BA.2.10.1 and BA.2.75, has been suggested to be associated with an increased risk of re-infection.[9] The latest SARS-CoV-2 variant, JN.1, was first identified in September 2023 and has since become the dominant variant in the US. Notably, the World Health Organization has added it to the list of "variants of interests" (VOI).[10]

The evolution of SARS-CoV-2 is primarily driven by two forces—ACE2 binding affinity and immune evasion. Early mutations that enhanced ACE2 binding provided a higher transmission advantage than immune-evasive mutations and thereby promoted viral fitness at the onset of the contagion. As the mutation pattern shifted, the proportion of non-synonymous mutations in the receptor-binding domain (RBD) increased over time. Positive selection pressure on this region may have conferred the virus with the ability to escape immune defenses and spread to other cells.[11] Research indicated that immune evasion exhibited a stronger correlation with mutation occurrence, although its correlation with viral fitness was less significant compared to ACE2 binding affinity.[11] Recent Omicron sub-lineages displayed a pattern of convergent evolution, highlighting the effect of population humoral immune pressure in SARS-CoV-2 evolution,[12] likely driven by increasing rates of vaccination and infection. Neutralizing antibodies (NAbs) serve as key effector molecules produced by B cells in response to viral infections or vaccinations, and are considered a hallmark of the immune protection against SARS-CoV-2 infection.[13] Successive infections and vaccinations have made humoral immune responses increasingly complex and heterogeneous. Previous studies focused on specific lineages or sub-variants, which may limit their applicability to other variants, leaving the overall evolutionary trajectory of real-world population immunity still not fully understood.

In response to this challenge, we have previously established and validated a high-throughput broad neutralizing-antibody (bNAb) assay based on fluorescence-encoded magnetic beads and a flow cytometer, which enables the simultaneous detection of NAbs against multiple SARS-CoV-2 variants with high reproducibility. The results obtained using the bNAb assay showed a strong correlation with the IgG serological assay (R = 0.89), the FDA-approved cPass sVNT assay (R = 0.93), as well as neutralizing assays based on pseudoviruses (R = 0.96 for D614G, R = 0.66 for BA.1) or live viruses (R = 0.79 for D614G, R = 0.64 for BA.1).[14] These indicate an association between our surrogate neutralization assay and humoral protective immunity.

In the present study, we examined 890 serum samples from individuals with SARS-CoV-2 primary infection or re-infection between January 2020 and September 2023 to delineate immune response profiles against a spectrum of variants (D614G, Alpha, Beta, Gamma, Kappa, Delta, BA.1, BA.2, BA.2.75, BA.3, BA.4, BA.5, BF.7, BQ.1.1, XBB, XBB.1.5, XBB.1.9.1, and EG.5.1). Additionally, we described the humoral immune characteristics induced by infections and/or vaccinations, elucidating the serological classification of SARS-CoV-2 under hybrid immune pressure.

Results

Serum samples with anti–SARS-CoV-2 NAbs from a longitudinal infection cohort

Of the 1034 individuals enrolled in this study, the SARS-CoV-2 infection cohort comprised 890 COVID-19 patients, including 405 females and 485 males, with a median age of 42.50 years. The demographic characteristics of the participants are detailed in Table 1. The remaining 144 serum samples had been collected before the COVID-19 pandemic and served as controls to minimize the impact of background noise. Participants were categorized into 13 infection sub-cohorts based on the prevalence of variants and the timing of individual infections. This categorization included 11 primary infection sub-cohorts and 2 re-infection sub-cohorts, spanning the following three major periods throughout the pandemic: the pre-vaccination period (January to December 2020), the vaccination roll-out period (January to December 2021), and the post-vaccination period (January 2022 to September 2023) (Fig. 1A).

Utilizing the bNAbs detection method established by our team, we assessed the protective immunity induced by infections or vaccinations against 18 different SARS-CoV-2 variants in a high-throughput system. These variants included D614G, Alpha, Beta, Gamma, Delta, Kappa, and Omicron sub-lineages BA.1, BA.2, BA.2.75, BA.3, BA.4, BA.5, BF.7, BQ.1.1, XBB, XBB.1.5, XBB.1.9.1, and EG.5.1 (Fig. 1B).

Before sample testing, we evaluated the reproducibility, specificity, and sensitivity of the technology. The average variations in NAbs detection within a single experiment and among different experiments were 0.19% and 0.89%, respectively (Fig. 1C). Receiver operating characteristic (ROC) curve analysis demonstrated the excellent performance of our assay platform in distinguishing the infected individuals from the uninfected individuals (area under the curve [AUC] = 0.8169) (Fig. 1D), with high discrimination capability for infections with specific variants (all AUCs > 0.8) (Fig. 1E). These findings confirm that our proteomic technology provides a reliable and effective method for detecting NAbs.

Synergistic evolution of SARS-CoV-2 variants and the protective immunity

To investigate changes in protective immunity, we assessed the neutralizing ability of each serum collected from primary infection cohorts (Fig. S1) and plotted cumulative distribution function (CDF) graphs using the NAbs data from all the individuals to depict the prevalence of anti-SARS-CoV-2 NAbs in the infected population (Fig. 2).

During the initial wave of the pandemic and before the availability of vaccines, > 75% of COVID-19 patients demonstrated neutralization against the D614G variant, with the neutralization rates exceeding 60% (Fig. 2A, C1, and C2). The spectrum of the infection-induced NAbs exhibited robust neutralization centered on the D614G variant and moderate neutralization against the Alpha and Delta variants, suggesting that these variants may not be the primary drivers of immune evasion but may instead influence viral adaptability and infectivity. However, the ability of the NAbs to neutralize Omicron lineages significantly declined, with few individuals exhibiting evidence of humoral immunity against the XBB lineages (Fig. S1, C1, and C2).

With the widespread implementation of vaccination, the seroprevalence of anti-SARS-CoV-2 NAbs among infected individuals increased steadily (Fig. 2A, C3). By the second half of 2021, the

Table 1
Demographic characteristics of COVID-19 patients observed between January 2020 and September 2023.

Characteristic		WT (n = 94)	D614G (n = 75)	Alpha (n = 100)	Delta (n = 97)	BA.1 (n = 82)	BA.2 (n = 119)	BA.2.76 (n = 33)	BA.5.2 (n = 40)	BF.7 (n = 106)	XBB (n = 38)	BA.5.2/ BF.7+XBB (n = 17)	EG.5 (n = 74)	BA.5.2/ BF.7+EG.5 (n = 15)
Sex														
	male	49 (52.13%)	41 (54.67%)	67 (67.00%)	59 (60.82%)	35 (42.68%)	54 (45.38%)	11 (33.33%)	27 (67.50%)	63 (59.43%)	22 (57.89%)	12 (70.59%)	35 (47.30%)	10 (66.67%)
	female	45 (47.87%)	34 (45.33%)	33 (33.00%)	38 (39.18%)	47 (57.32%)	65 (54.62%)	22 (66.67%)	13 (32.50%)	43 (40.57%)	16 (42.11%)	5 (29.41%)	39 (52.70%)	5 (33.33%)
Age [m(P25,P75)]		**34.00** (23.75, 45.50)	**47.00** (34.00, 54.00)	**38.00** (29.25, 49.00)	**44.00** (34.00, 53.00)	**40.00** (32.00, 52.25)	**36.00** (27.00, 49.00)	**19.00** (17.00, 33.50)	**29.00** (21.00, 36.75)	**65.00** (50.75, 72.00)	**55.00** (43.50, 60.00)	**40.00** (33.00, 70.00)	**69.00** (61.00, 75.25)	**46.00** (26.00, 74.00)
Age group	≤50 y	76 (80.85%)	50 (66.67%)	78 (78.00%)	62 (63.92%)	59 (71.95%)	94 (78.99%)	30 (90.91%)	36 (90.00%)	26 (24.53%)	14 (36.84%)	10 (58.82%)	12 (16.22%)	9 (60.00%)
	> 50 y	18 (19.15%)	25 (33.33%)	22 (22.00%)	35 (36.08%)	23 (28.05%)	25 (21.01%)	3 (9.09%)	4 (10.00%)	80 (75.47%)	24 (63.16%)	7 (41.18%)	62 (83.78%)	6 (40.00%)
Vaccination														
	0	94 (100.00%)	75 (100.00%)	33 (33.00%)	16 (16.49%)	7 (8.54%)	2 (1.68%)	0 (0.00%)	3 (7.50%)	26 (24.53%)	14 (36.84%)	2 (11.76%)	19 (25.68%)	4 (26.67%)
	1	0 (0.00%)	0 (0.00%)	3 (3.00%)	2 (2.06%)	1 (1.22%)	1 (0.84%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
	2	0 (0.00%)	0 (0.00%)	29 (29.00%)	30 (30.93%)	30 (36.59%)	24 (20.17%)	19 (57.58%)	6 (15.00%)	14 (13.21%)	9 (23.68%)	0 (0.00%)	10 (13.51%)	0 (0.00%)
	3	0 (0.00%)	0 (0.00%)	0 (0.00%)	48 (49.48%)	40 (48.78%)	83 (69.75%)	10 (30.30%)	29 (72.50%)	38 (35.85%)	14 (36.84%)	9 (52.94%)	17 (22.97%)	4 (26.67%)
	≥4	0 (0.00%)	0 (0.00%)	1 (1.00%)	0 (0.00%)	2 (2.44%)	5 (4.20%)	0 (0.00%)	1 (2.50%)	1 (0.94%)	1 (2.63%)	0 (0.00%)	10 (13.51%)	0 (0.00%)
	unknown	0 (0.00%)	0 (0.00%)	34 (34.00%)	1 (1.03%)	2 (2.44%)	4 (3.36%)	4 (12.12%)	1 (2.50%)	27 (25.47%)	0 (0.00%)	6 (35.29%)	18 (24.32%)	7 (46.67%)
Disease severity														
	mild	19 (20.21%)	12 (16.00%)	41 (41.00%)	41 (42.27%)	67 (81.71%)	111 (93.28%)	31 (93.94%)	38 (95.00%)	33 (31.13%)	4 (10.53%)	10 (58.82%)	25 (33.78%)	10 (66.67%)
	moderate	61 (64.89%)	53 (70.67%)	57 (57.00%)	56 (57.73%)	15 (18.29%)	8 (6.72%)	2 (6.06%)	2 (5.00%)	39 (36.79%)	30 (78.95%)	7 (41.18%)	36 (48.65%)	5 (33.33%)
	severe	11 (11.70%)	10 (13.33%)	1 (1.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	27 (25.47%)	3 (7.89%)	0 (0.00%)	11 (14.86%)	0 (0.00%)
	critical	3 (3.19%)	0 (0.00%)	1 (1.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	7 (6.60%)	1 (2.63%)	0 (0.00%)	2 (2.70%)	0 (0.00%)
Disease duration (Days)[a] [m (P25, P75)]		**21.00** (16.00, 24.25)	**18.00** (8.00, 22.00)	**16.50** (14.00, 24.00)	**13.00** (10.00, 20.00)	**3.00** (1.00, 10.00)	**3.00** (1.00, 11.00)	**9.00** (4.00, 10.50)	**10.00** (9.00, 11.00)	**13.00** (7.75, 18.00)	**14.00** (12.00, 19.00)	**7.00** (5.50, 9.00)	**12.50** (6.00, 17.00)	**9.00** (7.00, 14.00)

Bold values signify the medians for the corresponding data sets.
[a] The period from the symptom onset to the sample collection.

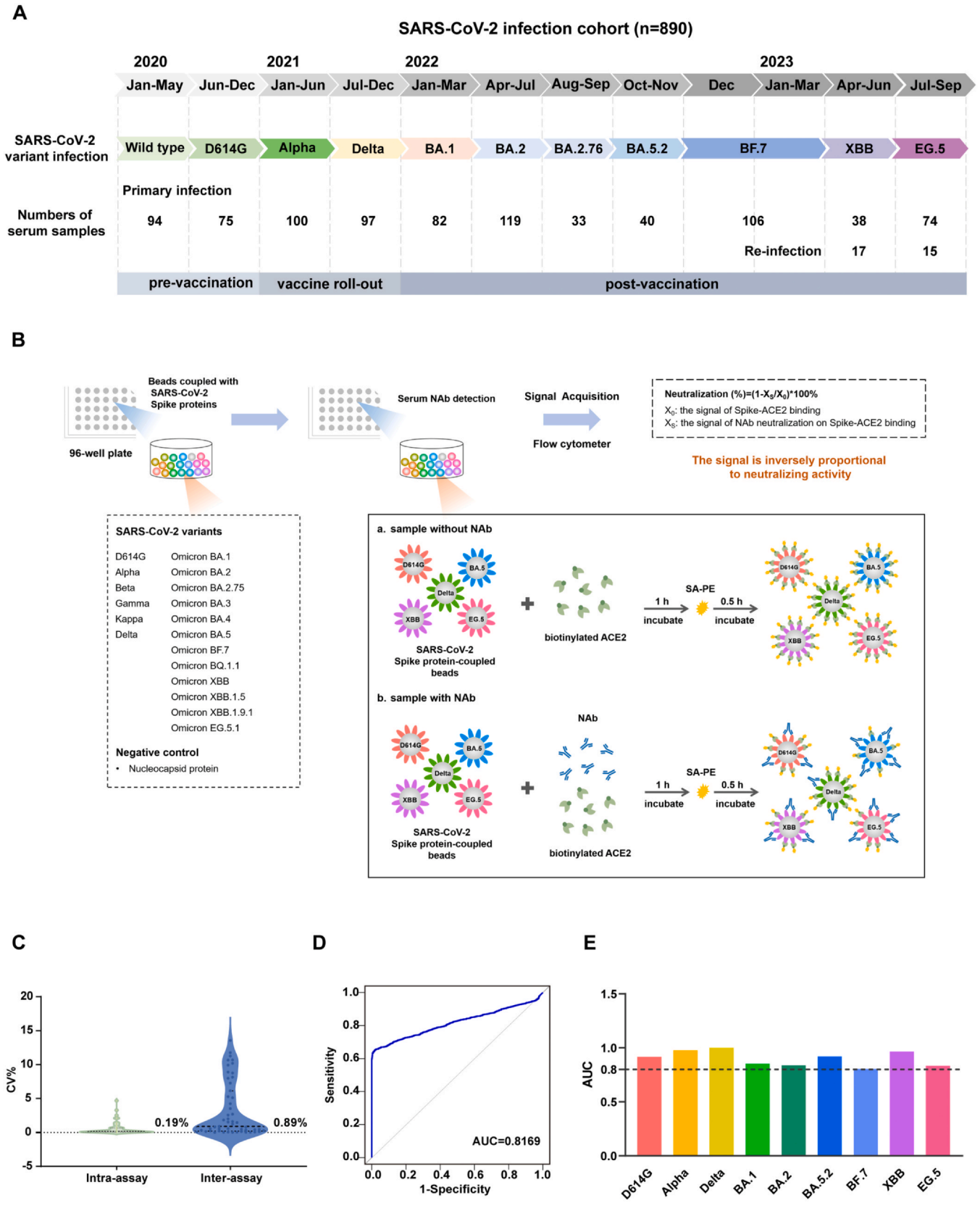

Fig. 1. Quantitation of the anti–SARS-CoV-2 neutralizing antibodies (NAbs) in serum samples from individuals infected in 2020–2023. (A) Timeline of sample collection from individuals infected with various SARS-CoV-2 variants. (B) Schematic of NAb detection via the broad neutralizing-antibody assay. (C) Intra- and inter-assay coefficients of variation for the NAb detection. (D and E) Receiver operating characteristic curve analysis of the NAbs for distinguishing between the infected and uninfected groups collectively across all the variants (D) and individually (E) (representative results). The AUC results are presented as box plots.

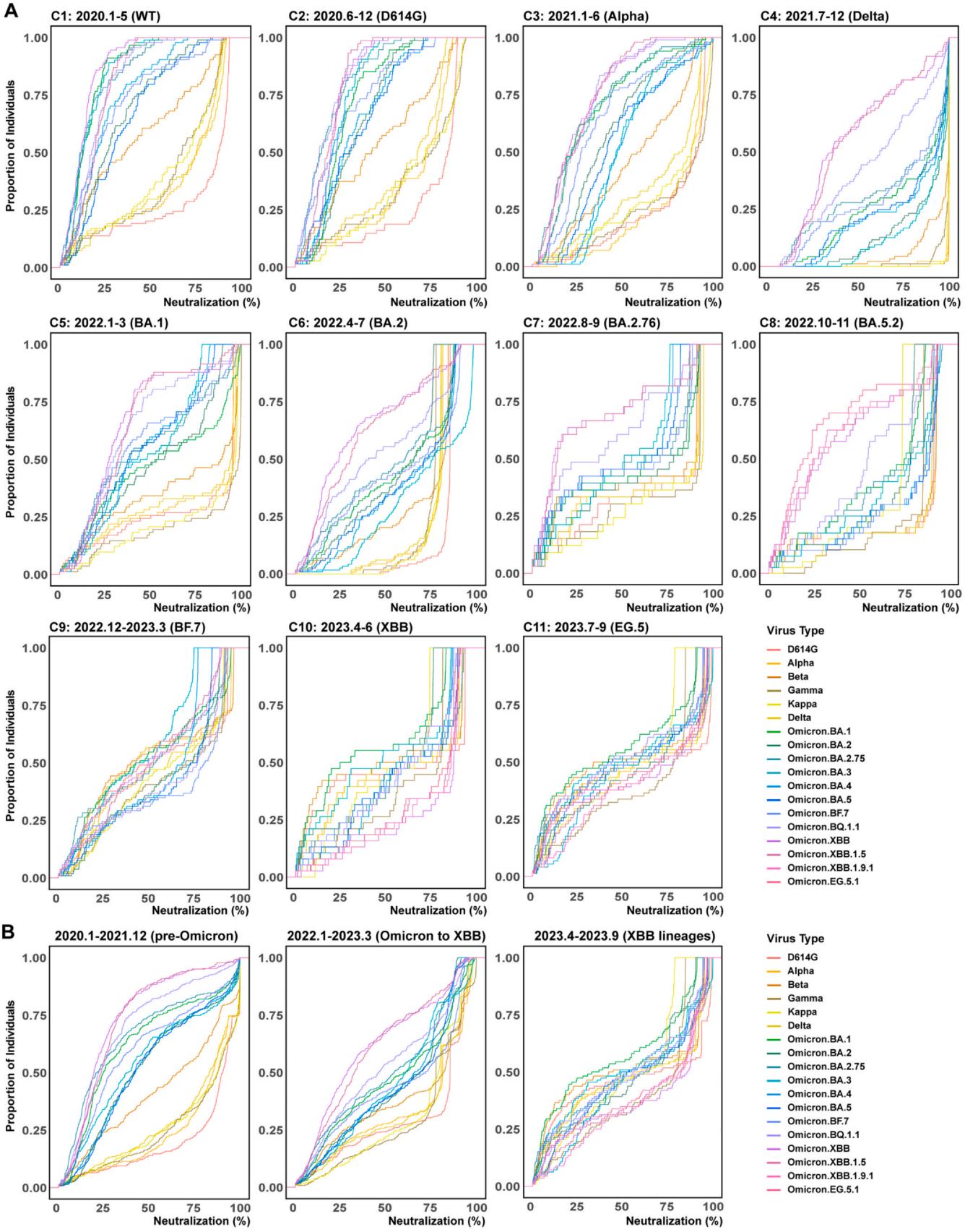

Fig. 2. The probability distribution of NAbs in COVID-19 patients. The cumulative distribution function is graphically presented where the x-axis shows the values of neutralization (%) against variants, and the y-axis indicates the proportion of patients with neutralization (%) ≤ a specific value on the x-axis. Each differently colored curve represents the probability distribution of NAbs against a specific variant. (A) The probability distributions of NAbs against different variants among the COVID-19 patients in 11 primary SARS-CoV-2 infection cohorts. (B) The 11 clinical cohorts were categorized into three groups based on the infection time—pre-Omicron, Omicron-to-XBB, and XBB. The probability distributions of NAbs among the COVID-19 patients in the three groups are shown in Figure B.

vaccination coverage rapidly escalated to 82.47% (80/97) (Table 1), resulting in a peak in the protective immunity against pre-Omicron variants (Fig. 2A, C4, and Fig. S1, C4). However, the global surge in infections with the Omicron variants did not appear to be mitigated by the immunity resulting from widespread vaccination and/or prior infections.

Infection with the BA.1 variant elicited immune responses that cross-reacted with the D614G, Alpha, and Delta variants (Fig. S1, C5), and the resulting NAbs were predominantly effective against pre-Omicron variants. Sera from COVID-19 patients who were at the acute phase of BA.1 infection demonstrated neutralizing activity against BA.2, BA.3, BA.4/5 and BF.7, though with limited potency. By mid-2022, following a 6-month circulation of Omicron lineages in China, the seroprevalence of anti-Omicron NAbs (neutralization rate > 50%) among COVID-19 patients had risen to 50% overall (Fig. 2A, C6).

In the BA.2 and BA.5.2 infection cohorts, the XBB lineages demonstrated significant resistance to neutralization. However, compared with the WT or D614G infection cohorts, there was a general and discernible increase in the levels of anti-SARS-CoV-2 NAbs (Fig. S1). Following a period of gradual increases in the fall of 2022, the seroprevalence of anti-Omicron NAbs (neutralization rate > 50%) rapidly increased to approximately 75% by the end of 2022 (Fig. 2A, C8). Overall, Omicron variants infections generated NAbs with increased cross-neutralization capabilities. Unfortunately, the XBB lineages have shown pronounced immune evasion against the NAbs induced by previous Omicron sub-variants infection. The serum neutralization rates against the XBB lineages were nearly half as effective as those against earlier Omicron variants (Fig. S1, C8).

The rise in the prevalence of anti-XBB NAbs was largely attributed to natural infections, as it happened after April 2023, when the XBB variant was predominant, and approximately 80% of infected individuals demonstrated neutralization rates > 50% against this variant and its descendants (XBB, XBB.1.5, XBB.1.9.1, and EG.5.1) (Fig. 2A, C10). EG.5, a descendant of the XBB.1.9.2 variant, has been designated as a VOI of SARS-CoV-2.[15,16] Sera from individuals with BA.5.2 infection did not confer protection against EG.5.1, and the neutralization efficacy against EG.5.1 was 2.3-fold lower than against BA.5 (Fig. S1, C8). XBB-infected and EG.5-infected individuals exhibited high levels of NAbs against the variants from the XBB lineage, with 69.15% and 55.94% neutralization rates against the EG.5.1 variant, respectively (Fig. S1, C10, and C11).

We categorized the 11 primary infection cohorts into the following three groups based on infection time: pre-Omicron variants (C1–C4, January 2020–December 2021), Omicron to XBB variants (C5–C9, January 2022–March 2023), and XBB variants (C10 and C11, April 2023–September 2023). Significant trends in protective immunity were discerned within these groups, as illustrated by CDF in Fig. 2B.

In general, the neutralization in the first group was mostly against pre-Omicron variants. The seroprevalence of NAbs then gradually shifted toward Omicron-specific responses. Conversely, the neutralization spectra in the third group were predominantly directed against XBB lineages. In other words, under the immune pressure resulting from infections and/or vaccinations, the immune system underwent substantial remodeling, resulting in reduced susceptibility to Omicron lineages and increased NAb responses to XBB lineages.

Classification of protective immunity to SARS-CoV-2 variants

The ongoing evolution of SARS-CoV-2 has resulted in the emergence of multiple variants that exhibit strong immune evasion against previous variants infection induced or "hybrid" NAbs. Serotype, typically defined based on the immunogenic differences of viral surface antigens, is distinguished by the humoral immune response.[17] In this study, we classified SARS-CoV-2 serotypes based on the antigenicity of the Spike protein of variants.

During the pre-Omicron era, three different serotypes—serotypes I, II, and III—were identified (Fig. 3A). Each serotype comprised multiple genetically distinct SARS-CoV-2 variants. Specifically, all pre-Omicron variants, including Beta, which had previously been identified as the most resistant to neutralization,[18] were classified under serotype I. In accordance with that, the Beta variant demonstrated a correlation of 0.7–0.8 in eliciting NAb responses compared with earlier Omicron variants. Additionally, Omicron BA.1–5 and BF.7 were designated as serotype II, whereas Omicron BQ.1.1 was grouped with XBB and XBB.1.5 under serotype III, distinguishing it from earlier Omicron variants. Notably, aside from the Beta variant, serotypes I and II exhibited relatively low correlation (0.6–0.7), and the correlation between serotypes I and III was even lower (< 0.6). In contrast, the correlation between serotypes II and III was moderate (0.6–0.8). During the evolutionary transition from Omicron lineages to XBB lineages, the serological classification remained consistent (Fig. 3B). With the onset of the XBB era, BQ.1.1 was classified in serotype II alongside other Omicron variants (BA.1–5 and BF.7), and XBB lineages were categorized as serotype III (Fig. 3C). The correlation between serotypes I and II increased to 0.6–0.9, but the association between serotypes I and III remained low (< 0.6). Concurrently, the correlation between serotypes II and III was also enhanced, reaching 0.7–0.9.

Based on the serotype classification, we categorized real-world infections into three groups and observed clear changes in humoral immunity from the radar charts (Fig. 3D). Generally, NAbs resulting from infections with pre-Omicron variants had restricted neutralization spectra, solely against pre-Omicron variants, and failed to neutralize Omicron lineages. In contrast, the second group displayed neutralization spectra primarily against pre-XBB variants. Additionally, the NAbs in the third group were more specialized in neutralizing XBB lineages, while still preserving cross-neutralization ability against pre-XBB variants.

An intriguing finding regarding certain SARS-CoV-2 variants was observed. Delta demonstrated potent immunogenicity within the pre-Omicron variants group, capable of eliciting neutralization against Omicron variants, excluding the XBB lineages.[19] However, since Delta infection occurred during the period of vaccine roll-out, the impact of vaccination coverage on the production of NAbs should be considered.

Vaccination reshapes immune responses

In China, with the expansion of vaccination coverage and implementation of the "dynamic zero-COVID" policy, the proportion of severe cases decreased (Table 1, C1–C8).[20] However, following relaxation of the COVID-19 response strategies, a large wave of infection occurred in China, resulting in increased hospitalizations among elderly patients and a rise in moderate to severe cases (Table 1, C9).[21] Older adults frequently suffer from one or more chronic diseases, which may weaken the immune tolerance to SARS-CoV-2 infection. The concurrent processes of immunosenescence and inflammaging are believed to exacerbate the clinical severity of COVID-19.[22] Guidance for this age group should focus on measures to prevent SARS-CoV-2 infection and encourage vaccination.[23]

Sera from unvaccinated individuals infected with XBB lineages (XBB and EG.5) exhibited XBB-specific neutralization and minimal NAb responses to WT variant (Fig. 4A). In contrast, SARS-CoV-2 breakthrough infections, particularly those involving the XBB and EG.5 variants following three-dose vaccination, have been shown to enhance the cross-neutralization activity of NAbs, as evidenced by stronger and broader immune responses (Fig. 4A and Fig. S2). In XBB or EG.5 breakthrough infection, samples from participants who received three-dose vaccine showed a higher proportion of high

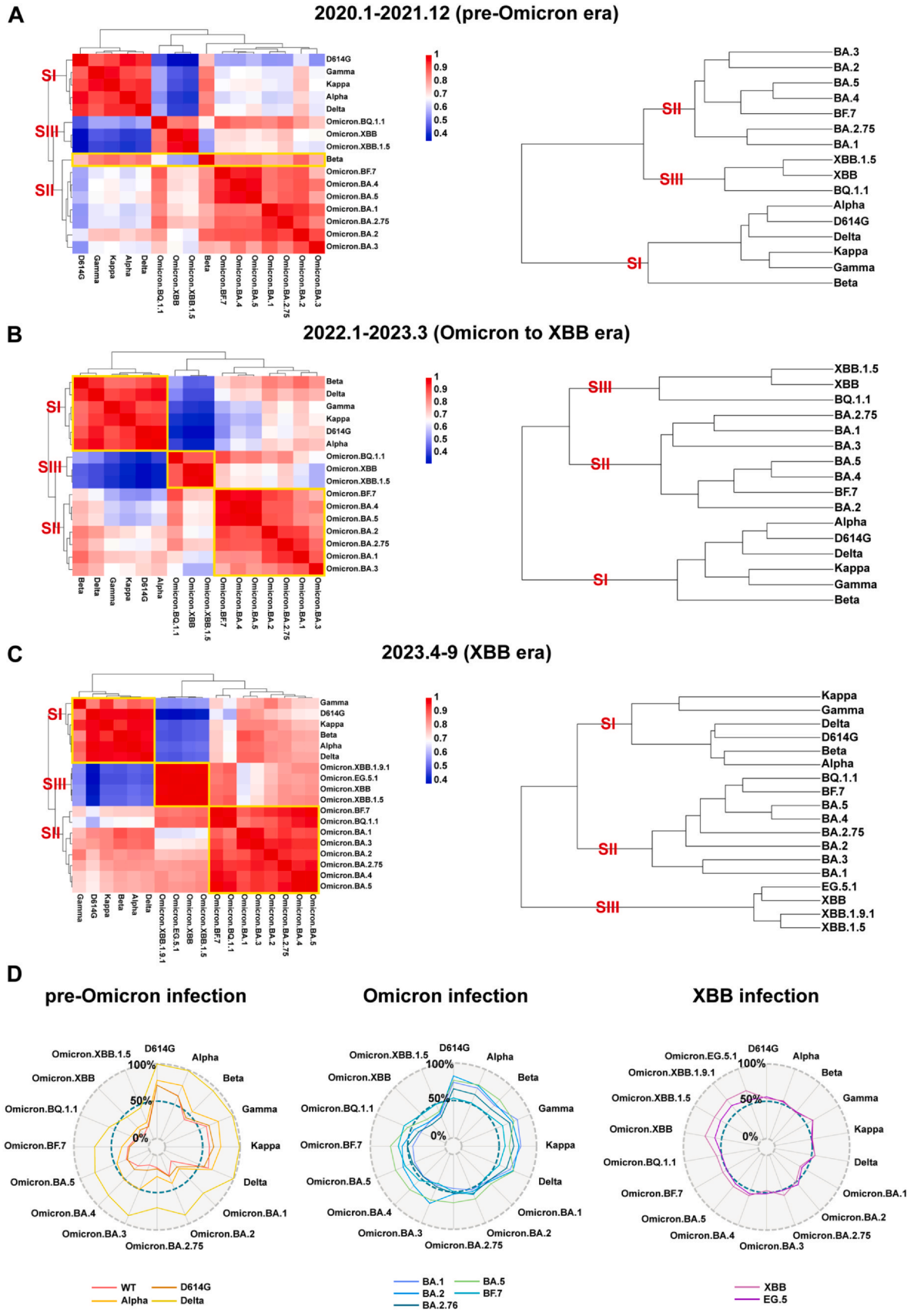

Fig. 3. Immunological classification of SARS-CoV-2 variants. (A–C) Classification of SARS-CoV-2 serotypes based on NAb responses against the tested variants. The correlation analysis was visualized using a heatmap, and clustering analysis was conducted using the UPGMA algorithm. Red and blue colors in the heatmap indicate high and low correlation, respectively. (D) Evaluation of the immunogenicity of SARS-CoV-2 variants in real-world settings. Based on the classification of serotypes, real-world infections were categorized into three groups–pre-Omicron, Omicron, and XBB infections. The radar charts display the mean neutralization (%) of NAbs against the tested variants.

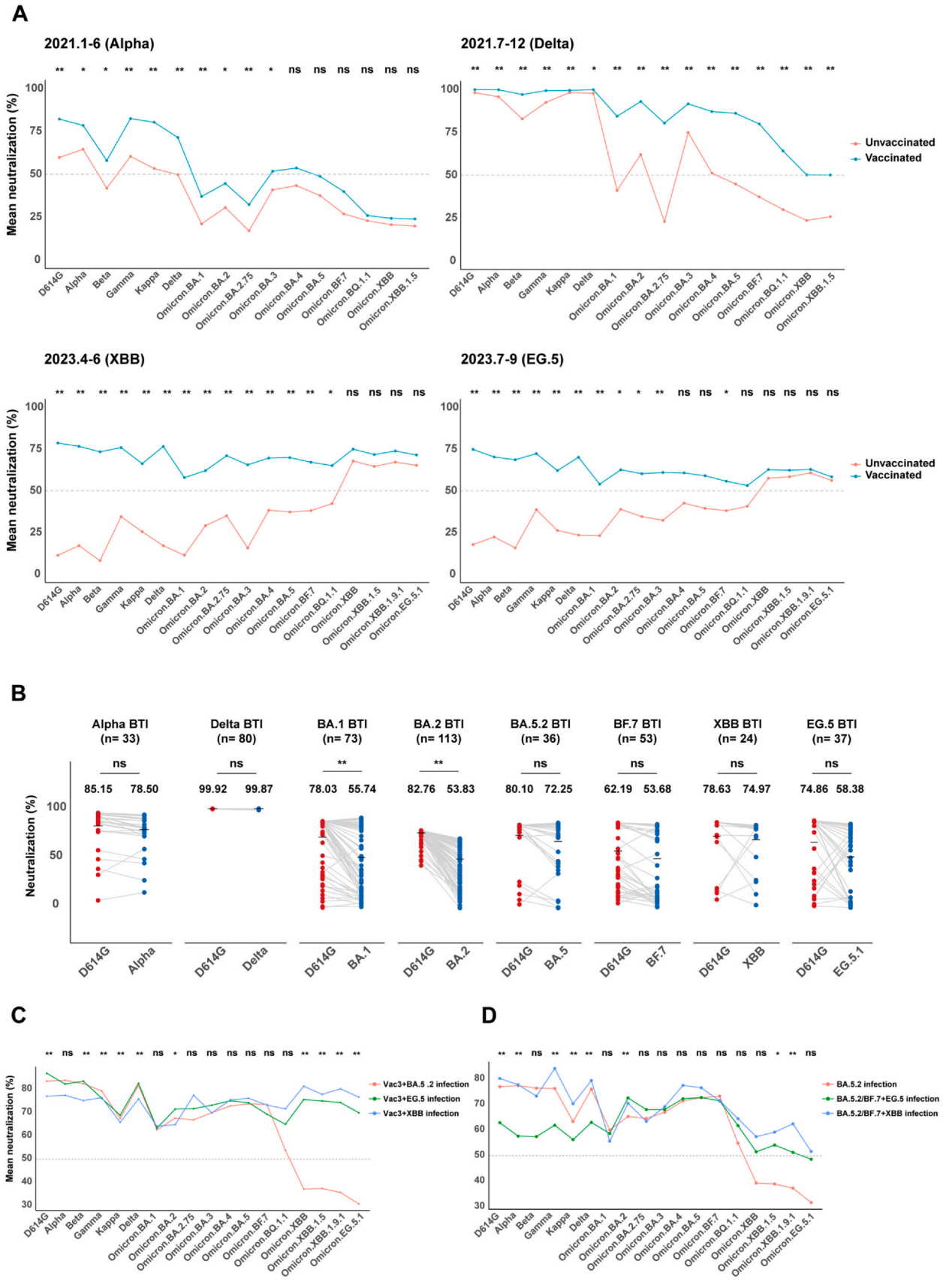

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Fig. 4. NAb responses in COVID-19 patients with breakthrough infections (BTIs) or re-infections. (A) The participants were divided into two groups based on their vaccination status. The figure shows the comparison of NAb responses between COVID-19 patients with prior vaccination and those without. (B) Immune imprinting after BTIs. The neutralization capacities of sera from patients with Alpha, Delta, BA.1, BA.2, BA.5.2, BF.7, XBB, and EG.5 breakthrough infections against the Spike protein of the Alpha, Delta, BA.1, BA.2, BA.5, BF.7, XBB, and EG.5.1 variants, compared with that against D614G. The mean neutralization rates against D614G and the infecting variants are shown below the line. (C) NAb responses were evaluated in sera from individuals who had received three vaccine doses and experienced a single Omicron breakthrough infection. The blue, green, and red curves represent XBB, EG.5, and BA.5.2 breakthrough infections, respectively. (D) Comparison of NAb responses between individuals with a single Omicron infection and those with an Omicron re-infection.

neutralizing activity (neutralization rate > 50%) against the XBB lineages compared to those who only received two-dose vaccine (Fig. S3). However, no statistical differences in neutralization against the XBB lineages were observed between infection groups that received different vaccine doses (Fig. S2), indicating that the XBB lineages might compromise the immunity established by preexisting vaccination.

Since most individuals have received WT-based vaccines, immune imprinting from WT vaccination may limit the development of NAbs against new variants. Consistent with previous findings,[24] NAb responses induced by a single breakthrough infection to the corresponding variant were lower than those to D614G, and neutralization against D614G decreased as the antigenic distance between the corresponding variant and WT variant increased (Fig. 4B). In cases of one-time breakthrough infection, although a higher proportion of individuals who received three-dose vaccines had high-level Omicron-specific NAbs (neutralization rate > 50%) than those who received two doses, the proportion of individuals with high NAb responses against newly emerging variants in a distinct serotype was lower or comparable in the three-dose group compared with that of the two-dose group (Fig. S3).

Therefore, it is crucial to develop next-generation vaccines that can elicit strong immune responses against the latest variants. XBB lineages have shown extraordinary immune evasion capabilities, significantly weakening the neutralizing efficacy induced by BA.5.2 breakthrough infections following three-dose vaccination, with the neutralization (%) decreasing to approximately 30% (Fig. 4C). In other words, the immune response elicited by primary immunization with WT vaccination, followed by a booster immunogen with BA.5.2 infection, was insufficient to neutralize XBB lineages, posing a significant challenge to currently available BA.5-containing bivalent booster vaccines.

Then, we evaluated the neutralizing profiles following first infection with a BA.5.2/BF.7 variant and subsequent re-infection with a variant of XBB lineages. As expected, the levels of NAbs against Omicron variants were higher in cases of repeated Omicron infections (irrespective of COVID-19 vaccination) than in those with a single infection (Fig. 4D).[24–26] XBB or EG.5 re-infection led to approximately a 2-fold increase in neutralization (%) against the highly immune-evasive XBB, XBB.1.5, XBB.1.9.1, and EG.5.1 variants, suggesting that re-infection with a different variant from a distinct serotype may broaden the spectrum of NAb responses and induce more robust humoral immunity compared to the immunity induced by a single Omicron infection.

NAb serve as a reliable biomarker for monitoring COVID-19 severity

COVID-19 patients exhibit differential disease severity after SARS-CoV-2 infection. Current evidence suggests that the host immune response's dysregulation may play a role in the onset and progression of COVID-19. Elevated neutrophil-to-lymphocyte ratio (NLR), cytokine IL-6, and C-reactive protein (CRP) levels help identify severe COVID-19 cases.[27–29] Although these traditional indicators reflect inflammation, they lack specificity since they can occur in many other diseases. NAbs, which are virus-specific, can effectively curtail infection. Therefore, we analyzed the relationship between NAb responses and the disease severity in patients with acute COVID-19 to explore the potential of NAb as a biomarker for COVID-19 severity.

COVID-19 patients manifest with a wide range of clinical symptoms, from asymptomatic cases to critical ones. We observed that disease severity positively correlates with NAb responses. Severe symptomatic patients mounted the most robust NAb responses, while mild patients exhibited lower abundances of NAbs (Fig. S4A). Strikingly, the severe illness was associated with an increase in the magnitude and breadth of the ensuing humoral immune response. Next, we evaluated the diagnostic value of these immunological indicators for differentiating between non-severe and severe COVID-19 cases (Fig. S4B). For assessing COVID-19 severity, the AUC value of anti-SARS-CoV-2 NAbs was not the highest among those of the other clinical indices (0.725 vs. 0.792, 0.767, and 0.648, for NLR, CRP, and IL-6, respectively). The combined diagnostic model using logistic regression analysis and incorporating these four risk factors yielded an AUC of 0.856. These results showed that NAb, serving as a reliable biomarker for COVID-19 severity, can be effectively combined with NLR and serum levels of CRP and IL-6 to enhance diagnostic assessment during the acute phase of the disease.

Discussion

Although > 3 years have passed since the emergence and global transmission of SARS-CoV-2, our understanding of the evolution of protective immune responses to the virus is still limited. In this study, we constructed a comprehensive protective immune landscape and examined the cross-neutralizing responses to various SARS-CoV-2 variants in individuals who contracted the virus between the beginning of the pandemic and the end of 2023.

The spectrum and strength of NAb responses varied, with the level of cross-protection depending on the specific infecting variant. Before the availability of vaccines, most COVID-19 patients exhibited robust NAb responses to pre-Omicron variants but showed little evidence of humoral immunity against Omicron variants. As COVID-19 vaccines were introduced, the prevalence of anti-SARS-CoV-2 NAbs increased, leading to a transition in protective immunity from purely infection-induced immunity to hybrid immunity. Following the emergence of Omicron variants, the immune system underwent substantial remodeling, resulting in a gradual shift in NAbs seroprevalence from WT-specific to Omicron-specific. When the XBB lineages became predominant, the protective immunity exhibited reduced susceptibility to Omicron and increased NAb responses to XBB lineages.

Therefore, we propose that the human protective immunity co-evolved with SARS-CoV-2 under the immune pressure induced by natural infections and/or vaccinations, this co-evolution occurred in three distinct periods: the pre-Omicron era, the Omicron to XBB era, and the XBB era. Furthermore, we classified three SARS-CoV-2 serotypes based on the antigenicity of the Spike protein of variants, including the pre-Omicron variants, Omicron lineages, and XBB lineages. Serotype I encompasses all tested pre-Omicron variants, while Omicron and its sub-lineages are distributed across serotypes II and III based on their evolutionary stages.[30] Specifically, in the pre-XBB era, Omicron BA.1–5 and BF.7 were classified as serotype II. Meanwhile, Omicron BQ.1.1, XBB, and XBB.1.5 were categorized as serotype III, aligning with the evolutionary trend of SARS-CoV-2, where BQ.1.1 demonstrated stronger immune evasion than earlier

Omicron variants.[31] With the onset of the XBB era, XBB lineages were distinctly clustered in a separate branch, diverging from other Omicron variants, symbolizing a new epoch of human protective immunity. Consequently, due to the pressure of the protective immunity, SARS-CoV-2 has undergone two significant adaptations involving immune-evasive mutations that enhance its transmission advantage.[32] Moreover, NAbs generated by a previous serotype could not typically effectively protect against another serotype that emerges later in the evolutionary stages.

The classification of serotypes may enhance our comprehensive understanding of the diversity and dynamic evolution of SARS-CoV-2, guiding the development of broad-spectrum vaccines and diagnostic reagents. Multiple rapid antigenic tests have demonstrated lower sensitivity to Omicron lineages,[33] probably because the antigens used for detection were from different immunological classifications. Serological testing utilizing antigens sensitive to homologous antibodies may improve the accuracy of detecting the Omicron-induced antibodies. Moreover, immunological classification of SARS-CoV-2 helps quickly identify the serotype of new variants, enabling the use of reference anti-sera to rapidly assess the risks for immune evasion.[34] Ideally, updated vaccines will be required to provide protection against these emerging variants. Repeated WT-vaccination could recall previous immune memory, potentially dampening immune responses to new variants. This phenomenon, known as "original antigenic sin",[35] refers to the preference of the immune system to target ancestral variants over the new variants. However, hybrid immunity resulting from WT-vaccination and subsequent breakthrough infection could provide broader and longer-lasting protection than immunity solely from natural infection.[36] Re-exposure to XBB or other Omicron variants either through infection or vaccination after WT-vaccination could also induce broad immunity against SARS-CoV-2.[37] Deciphering the differences in the strength and spectrum of NAb responses arising from diverse SARS-CoV-2 infections will help determine which variant spike sequences are optimal for incorporation into next-generation vaccines.[38]

Despite the potential imprinting of the immune response by previous antigen exposure, the established B cell memory pool can be refocused and significantly reshaped by re-exposure to heterologous Spike glycoproteins to allow the neutralization of variants that evade a previously conferred NAb responses.[39] We explore how "original antigenic sin" affected the production of antibody profile from repeated vaccination,[37] breakthrough infection after vaccination (Fig. 4A), and SARS-CoV-2 re-infection (Fig. 4D). These results suggested that the presence of "original antigenic sin" may promote the production of broad-spectrum antibodies. The antibody repertoires induced by Omicron breakthrough infections differ from those stimulated by WT infection or vaccination.[12,24,40] Repeated Omicron infections could induce a large proportion of Omicron-specific NAbs targeting neutralizing epitopes compared to one-time Omicron breakthrough infection. These Omicron-specific mAbs primarily target new RBD epitopes, demonstrating exceptional broad-spectrum neutralizing efficacy.[24] A class of immunogens represented by the XBB lineages following 3-dose WT vaccine elicited the most robust NAb responses in our study. Additionally, repeated Omicron infections could mitigate immune imprinting from previous vaccination and broaden the neutralization spectrum against Omicron sub-variants.[26] Reviewing the evolution of the host immunity, incorporating XBB-based spike sequences into vaccines appears to be an effective way to achieve broad-spectrum protection. Patients infected with XBB lineages may have acquired cross-immunity, potentially offering protection against subsequent infections caused by related variants of the same lineage. Our findings support updating vaccines to align with newly prevalent SARS-CoV-2 variants and underscore the importance of administering updated COVID-19 vaccines even to those previously infected with Omicron.[25] Before adopting this approach, we recommend prioritizing the broad distribution of the primary series of COVID-19 vaccines to establish robust and comprehensive immunity in the population.

In addition, although NAbs are generally considered to be associated with virus clearance and protection, robust NAb responses did not seem to mitigate COVID-19 progression. Disease severity may serve as a clinical predictor of the magnitude of the NAb responses mounted against SARS-CoV-2.[41] Humoral responses in COVID-19 patients are associated with cytokine levels, which play a pivotal role in severe systemic inflammatory responses known as "cytokine storms".[42] Intense NAb responses might suggest strong immune activation in severe SARS-CoV-2 cases. Notably, combining NAbs with other markers, such as the NLR and serum levels of CRP and IL-6, can help identify severe and critical COVID-19 patients, guiding the treatment course.

In summary, we employed a flexible high-throughput proteomics technology platform that can be adjusted in real time according to pathogen variations. This platform enables the simultaneous detection of NAbs against different variants, allowing for rapid assessment of individual and population immunity under complex conditions such as natural infections and vaccinations. It can serve as a backup technology for responding to other emerging infectious diseases, offering theoretical support for the immune prevention and control of infectious diseases. To our knowledge, this is the most extensive study on the evolution of protective immunity in a real-world population since the onset of the pandemic to date.

Our study showcases several strengths, but we acknowledge certain limitations. Firstly, in some infection cohorts, the period from the symptom onset to the sample collection was relatively short, which may result in incomplete development of NAbs. Nonetheless, the results still provide an appropriate immunological picture of the different stages of SARS-CoV-2 infection in the real world. Secondly, since May 2023, most infected individuals sought outpatient care, and some with mild symptoms did not undergo chest computed tomography, leading to these individuals frequently being classified as mild COVID-19 cases. Thirdly, based on the vaccination timeline, we speculated that the vaccines administered in this study were primarily original COVID-19 vaccines. In our real-world population study, we encountered participants who, due to memory decline and communication barriers, were unable to furnish a comprehensive vaccination record. To maintain transparency, we have designated the vaccination history of these individuals as "unknown" in Table 1. Our results demonstrate that the NAbs effectively distinguish between participants with and without infection, irrespective of their vaccination status (Figs. 1D and E). Moreover, as part of the synergistic evolution between our host immunity and SARS-CoV-2 mutations, vaccination does not influence the shifts in serotypes associated with the emergence of new SARS-CoV-2 variants (Fig. 3). Lastly, the method used in our study is primarily designed to detect antibodies that can block the interaction between the Spike protein and ACE2, potentially missing antibodies that target other receptors, such as ASGR1 and KREMEN1.[43] It is imperative to recognize that other antibody-dependent manner may also play an indispensable role in immune protection and may contribute to the overall immune response against SARS-CoV-2, and should be investigated in future.

Materials and methods

Samples and patients

We collected a total of 1034 serum samples from the Ditan Hospital Affiliated with Capital Medical University. The sample set comprised 890 serum samples from individuals infected with SARS-CoV-2 between January 2020 and September 2023, and 144 serum samples from individuals before the pandemic for comparison. We

obtained demographic information, COVID-19 vaccination history, and clinical illness symptoms from medical records acquired from the hospital. Most vaccine recipients had received inactivated virus vaccines (CoronaVac or BBIBP-CorV), which are widely used in China. This dataset allowed us to analyze the immune responses in a diverse population across different stages of the pandemic and under different conditions of infection and vaccination.

Samples collected during the "Dynamic Zero-COVID Policy" (DZCP) period in China were all COVID-19 patients with primary SARS-CoV-2 infection. Based on the infection waves driven by different variants in Beijing (https://ngdc.cncb.ac.cn/ncov/monitoring/country/China), individuals were separated into 11 sub-cohorts.

Cohort 1 (C1): 94 samples collected in January–May 2020, during which ancestral variant (WT) was dominant during the early stage of the epidemic.[44]

C2: 75 samples collected in June–December 2020, D614G was prevalent during this period.[45]

C3: 100 samples collected during the Alpha wave in January–June 2021.[46]

C4: 97 samples collected during the Delta wave in July–December 2021.[47]

C5: 82 samples collected during the BA.1 wave in January–March 2022.[48]

C6: 119 samples collected during the BA.2 wave in April–July 2022.[21,48]

C7: 33 samples collected during the BA.2.76 wave in August–September 2022.[48]

C8: 40 samples collected during the BA.5.2 wave in October–November 2022.[21,48]

C9: 106 samples collected in December 2022–March 2023, when BF.7 was the predominant variant. BA.5.2/BF.7 variants caused the first wave of infections after the "DZCP" in China was lifted.[21]

C10: 38 samples collected in April–June 2023, when the XBB variant was predominant, causing the second wave post-DZCP.[49,50]

C11: 74 samples collected during the EG.5 wave in July–September 2023. EG.5 and its descendants accounted for 45% of the infections in July 2023 and were on course to supplant the XBB variant.[15]

In addition, we included serum samples from 2 re-infection sub-cohorts. These samples were collected after the "adjusted COVID-19 response strategies" period.

C12: 17 samples with prior BA.5.2/BF.7 infection (subtype not identified) from individuals with XBB infection in April–June 2023, referred to as the BA.5.2/BF.7 + XBB infection cohort.

C13: 15 samples with prior BA.5.2/BF.7 infection (subtype not identified) from individuals with EG.5 infection in July–September 2023, referred to as the BA.5.2/BF.7 + EG.5 infection cohort.

Disease severity classification was evaluated according to China National Health Commission Guidelines for Diagnosis and Treatment of SARS-CoV-2 infection (Trial Version 10). The classification included:

1) Mild: cardinal symptoms of upper respiratory tract infections, for instance dry and sore throat, cough, fever, etc.;
2) Moderate: (i) persistent high fever for > 3 days, or (and) other symptoms such as cough and shortness of breath, etc., with respiratory rate (RR) < 30 breaths per minute, and oxygen saturation > 93% at rest. (ii) radiological imaging reveals characteristic presentations of COVID-19 pneumonia infection;
3) Severe: Adults who meet any of the following criteria, which cannot be attributed to causes other than SARS-CoV-2 infection: (i) dyspnea, with RR ≥30 breaths per minute; (ii) oxygen saturation ≤93% at rest; (iii) arterial partial pressure of oxygen (PaO_2)/fraction of inspired oxygen (FiO_2) ≤300 mmHg (1 mmHg = 0.133 kPa); (iv) progressive clinical symptoms, with radiological imaging showing a significant progression of the lung lesions by > 50% within 24-48 h.
4) Critical: (i) respiratory failure requiring mechanical ventilation; or (ii) shock; or (iii) multi-organ dysfunction requiring intensive care unit (ICU) monitoring and treatment.

All the infection samples were confirmed via polymerase chain reaction (PCR) or antigen testing. Re-infection was defined as a positive test occurring ≥90 days after the last positive test in the same individual.

The disease duration was defined as the period from symptom onset to sample collection. Symptom onset was determined via one of the following strategies:

1) A positive PCR or antigen test.
2) At least one of the following self-reported symptoms: fever, cough, headache, muscle soreness, fatigue, difficulty breathing, taste/smell impairment, pharyngeal discomfort, and diarrhea.

This study was approved by the Institutional Review Board of the Ethics Committee of Ditan Hospital (NO.DTEC-KY2022–052-02 and NO.DTEC-KY2021–010-01). All the procedures were conducted in accordance with the Declaration of Helsinki.

Detection of anti–SARS-CoV-2 NAbs via the bNAb assay

The bNAb assay has been described previously.[14] In this study, trimerized Spike protein from six non-Omicron variants (D614G, Alpha, Beta, Gamma, Kappa, and Delta) and twelve Omicron variants (BA.1, BA.2, BA2.75, BA.3, BA.4, BA.5, BF.7, BQ.1.1, XBB, XBB.1.5, XBB.1.9.1, and EG.5.1) were coupled to fluorescence-encoded magnetic beads. Briefly, 3 μg of Spike trimer proteins (Sino Biological and ACROBiosystems, China) were coupled to 1×10^6 magnetic fluorescent beads (Shenzhen Wellgrow Technology Co., Ltd., Beijing, China).

Before the assay, 50 μL of a mixed solution containing various SARS-CoV-2 Spike trimer protein-coupled beads (2500 beads per variant) was added to a 96-well plate. Simultaneously, 50 μL of serum samples diluted to a 1:20 ratio with the buffer PBST-B (phosphate-buffered saline pH 7.4, 0.05% Tween-20%, and 1% BSA) were added to each well. After incubating for 2 h at room temperature on a shaker, the beads were thoroughly washed three times with PBST-B. Then, they were incubated for 1 h at room temperature with 50 μL of biotinylated ACE2 (Sino Biological) (0.5 μg/mL).

The binding interaction between ACE2 and Spike trimer proteins was assessed by adding 50 μL of streptavidin-phycoerythrin (SA-PE, Thermo Scientific, USA, 2 μg/mL) after the incubation period, followed by a 30-minute incubation at room temperature. After two rounds of washing with PBST-B, the beads were resuspended in 200 μL PBST-B. An EasyCell flow cytometer (Wellgrow Technology Co., Ltd.) was used for detecting the fluorescent signal at 200 beads per region at the excitation wavelengths of 532 nm and 635 nm. For the analysis, median fluorescence intensity was used.

Notably, if the sample contains NAbs, the attachment of NAbs to the Spike protein obstructs its binding to the biotinylated ACE2 receptor, which is coupled to SA-PE. Magnetic beads with the SARS-CoV-2 nucleocapsid protein were used as a negative control. The percentage of neutralization was calculated using the following equation:

$$\text{Neutralization } (\%) = [1-(X_S/X_0)] \times 100\%$$

where X_0 and X_S are the signals of Spike-ACE2 binding and NAb neutralization on Spike-ACE2 binding, respectively.

Statistical analysis

To evaluate the neutralization (%) of NAbs against diverse SARS-CoV-2 variants, we first eliminated background noise by analyzing

the 144 serum samples collected before the COVID-19 pandemic. We subtracted the mean neutralization (%) of this population from the neutralization (%) of COVID-19 patients. The raw neutralization rates of each variant were then standardized using minimum-maximum normalization to ensure that values ranged from 0 to 1. Outliers with absolute Z-scores of neutralization rates > 3 were excluded.

Given the non-normal distribution of the data, differences in the neutralization (%) were compared using the Wilcoxon test, and $p < 0.05$ was considered to indicate statistical significance. CDF graphs using NAbs data were plotted to illustrate the prevalence of anti-SARS-CoV-2 NAbs in the infected population. The CDF was defined as the probability that the neutralization rate (%) of NAb was less than or equal to a specific value, expressed as $P(X \leq x)$, and mathematically represented as $F(x) = P(X \leq x)$.

To identify variations in NAb production against different variants among COVID-19 patients, we consolidated the data on NAb responses and used the UPGMA algorithm to construct a dendrogram and cluster SARS-CoV-2 into distinct serotypes. Additionally, correlation analysis of NAb responses was performed based on the neutralization rates against various SARS-CoV-2 variants. All the data analyses were conducted in R (v4.2.3).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the National Key Research and Development Program of China (2022YFE0210400 and 2023YFC0872400), Beijing Municipal Natural Science Foundation (M21003, M23010), Beijing Municipal International Cooperation and Exchange Project (Z231100002723004).

Appendix A. Supporting material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinf.2024.106310.

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