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Distinctive serotypes of SARS-related coronaviruses defined by convalescent sera from unvaccinated individuals

Chee Wah Tan[1,#,*], **Feng Zhu**[1,#], **Wan Ni Chia**[1,#], **Barnaby E. Young**[2,3,4], **Aileen Ying Yan Yeoh**[1], **Thomas Althaus**[5,6], **Chee Fu Yung**[4,7,8], **Wee Chee Yap**[1], **Beng Lee Lim**[1], **Mark I-C Chen**[2,3], **Jinyan Zhang**[1], **Yun Yan Mah**[1], **Eric Voiglio**[6], **Alex Sigal**[9], **Jianxin Huo**[10], **Shengli Xu**[10,11], **Yee Joo Tan**[12,13], **Kong-Peng Lam**[10,12,14], **David Lye**[2,3,4,12], **Lin-Fa Wang**[1,15,*]

[1]Programme in Emerging Infectious Diseases, Duke-NUS Medical School, Singapore

[2]National Center of Infectious Diseases, Singapore

[3]Tan Tock Seng Hospital, Singapore

[4]Lee Kong Chian School of Medicine, Nanyang Technology University, Singapore

[5]Centre Scientifique de Monaco, Monaco

[6]Direction de l'Action Sanitaire de Monaco, Monaco

[7]Infectious Disease Service, Department of Pediatrics, KK Women's and Children's Hospital, Singapore

[8]Duke-NUS Medical School, Singapore

[9]African Health Research Institute, Durban, South Africa

[10]Singapore Immunology Network, Agency for Science, Technology and Research, Singapore

[11]Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

[12]Department of Microbiology and Immunology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

[13]Infectious Diseases Translational Research Programme, National University of Singapore, Singapore

[*]Correspondence: cheewah.tan@duke-nus.edu.sg (C.W.T.); linfa.wang@duke-nus.edu.sg (L.F.W.).
[#]These authors contributed equally to this work

AUTHOR CONTRIBUTIONS

C.W.T. and L.F.W. designed the study. C.W.T., F.Z., W.N.C., A.Y.Y.Y., W.C.Y., B.L.L., J.Z. and Y.Y.M. performed experiments. C.W.T., F.Z. and W.N.C. analyzed the data. W.N.C., B.E.Y., T.A., C.F.Y., M.I.C.C., E.V., A.S., J.H., S.X., Y.J.T., K.P.L. and D.L. coordinated cohorts and provided serum samples. C.W.T., L.F.W., F.Z. and W.N.C. wrote the manuscript with inputs from all authors.

ETHICS APPROVAL

Ethics statements, institutional review board approvals, and references to previous studies are listed in Table S1.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.hlife.2023.07.002.

DECLARATION OF COMPETING INTERESTS

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.

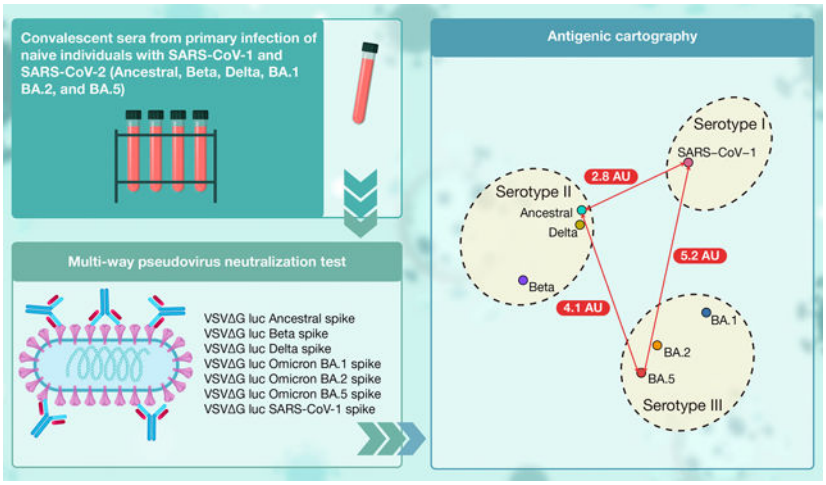
[14]School of Biological Sciences, Nanyang Technological University, Singapore

[15]Singhealth Duke-NUS Global Health Institute, Singapore

Abstract

Multiple Omicron sub-lineages have emerged, with Omicron XBB and XBB.1.5 subvariants becoming the dominant variants globally at the time of this study. The key feature of new variants is their ability to escape humoral immunity despite the fact that there are limited genetic changes from their preceding variants. This raises the question of whether Omicron should be regarded as a separate serotype from viruses serologically clustered with the ancestral severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus. Here, we present cross-neutralization data based on a pseudovirus neutralization test using convalescent sera from naïve individuals who had recovered from primary infection by SARS-CoV-1 and SARS-CoV-2 strains/variants including the ancestral virus and variants Beta, Delta, Omicron BA.1, Omicron BA.2 and Omicron BA.5. The results revealed no significant cross-neutralization in any of the three-way testing for SARS-CoV-1, ancestral SARS-CoV-2 and SARS-CoV-2 Omicron subvariants. The data argue for the assignment of three distinct serotypes for the currently known human-infecting SARS-related coronaviruses.

Graphical Abstract



Keywords

SARS-CoV-2; SARS-CoV-1; serotype; Omicron; primary infection; convalescent sera

INTRODUCTION

Over the last two decades, we have witnessed two major coronavirus outbreaks (SARS-CoV-1 and Middle East Respiratory Syndrome, MERS-CoV) that caused epidemics of concern in human and that preceded the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemics, resulting in inordinate human and economic losses worldwide and significantly disrupting global health. Since the first detection of SARS-CoV-2 in

December 2019, the virus has evolved extensively through mutations and/or recombinations predominantly in the spike protein, leading to multiple variants of concern that display either increased transmissibility or neutralizing antibody (NAb) escape. SARS-CoV-2 Omicron (B1.1.529) was first detected in South Africa in late November 2021, carrying more than 30 amino acid mutations in the spike protein. Since then, multiple Omicron subvariants have rapidly emerged at an unprecedented speed, most likely due to the selective pressure of pre-existing immunity against ancestral SARS-CoV-2 [1–4]. At the time of this study in early 2023, Omicron XBB (including its sublineage XBB.1.5) dominates SARS-CoV-2 infections worldwide (Figure 1A). Omicron subvariants are still evolving convergently from Omicron BA.2 or BA.5 by acquiring mutation(s) at key residues in the receptor-binding domain (RBD) (Figure 1B), leading to unprecedented escape of NAbs against the ancestral SARS-CoV-2 virus and growth advantage while maintaining sufficient ACE2-binding capability [1,5–12]. Additionally, probably due to immune imprinting, Omicron breakthrough infections caused significant reductions in NAb epitope diversity and increased proportion of non-NAbs, which promoted convergent RBD evolution [12]. Altogether, this poses serious challenges to the effectiveness of existing humoral immunity induced by natural infection and/or vaccination and the development of next-generation vaccines.

The unprecedented degree of immune evasion by Omicron variants raises the possibility that these viruses might be functionally behaving as a distinct serotype. It is becoming increasingly important to understand the antigenic relationships among newly emerged variants and the substitution that is responsible for the antigenic change. Serotyping of viruses has been a highly useful and proven approach for sub-species virus classification based on cross-neutralization by convalescent sera from infection of closely related viruses, which have been widely used to classify virus species such as dengue viruses [13] and polioviruses [14]. Defining SARS-CoV-2 serotypes will help to design better diagnostic tools, treatments and vaccines. Despite being phylogenetically closely related to ancestral SARS-CoV-2, significant NAb escape by Omicron subvariants highlights the need for serology-based viral classification [15]. Attempt to define the serotype of SARSr-CoV has been conducted using immunized animal sera [16], but there has been no report on serotyping using convalescent human sera from primary infection. To our knowledge, this study represents the first such investigation.

RESULTS

Limited Cross-Neutralization between SARS-CoV-1, Ancestral SARS-CoV-2 and Omicron

Previous attempts in defining antigenic cartography of SARS-CoV-2 strains/variants have relied on serum panels from infected animals [16] or vaccinees [2,17]. In contrast, in this study, we used sera from unvaccinated individuals who had recovered from primary infection by various SARS-related coronaviruses (SARSr-CoVs), which is much more accurate and relevant in the current research context of trying to better understand the antigenic relationship of different viruses during human infections. A total of 80 serum samples were used in this study, representing unvaccinated individuals with infection by ancestral SARS-CoV-2 (will be referred to as “Ancestral” from here on to avoid confusion with other SARS-CoV-2 variants), Delta, Beta, Omicron BA.1, Omicron BA.2, Omicron

BA.5 and SARS-CoV-1. Sera from naïve individuals infected with Ancestral failed to neutralize Omicron BA.1, Omicron BA.2, Omicron BA.5 or SARS-CoV-1, with 14.4- to 19.6-fold reduction in geometric mean titer 50% (GMT50) against Ancestral (Figure 2A). We observed the cross-neutralization of Delta viruses with serum samples derived from Beta-infected individuals (Figure 2B). It is interesting to note that there was some level of cross-neutralization to Omicron BA.1 and Omicron BA.2 in Beta-infected individuals, especially in samples with a high NAb titer (Figure 2B). Sera samples derived from Delta-infected individuals failed to neutralize Beta, Omicron subvariants or SARS-CoV-1 (Figure 2C). Similarly, SARS-CoV-1-infected patients produced significantly higher NAbs against SARS-CoV-1 with no cross-neutralization to Ancestral, Delta, Beta, Omicron BA.1, Omicron BA.2 or Omicron BA.5 (Figure 2D). For sera derived from naïve individuals infected with Omicron BA.1, Omicron BA.2 or Omicron BA.5, the overall NAb level was much lower than that in the other cohorts and with no cross-neutralization to Ancestral, Delta, Beta, or SARS-CoV-1. Notably, we observed limited cross-neutralization between Omicron subvariants (Figure 2E–2G). Sera derived from Omicron BA.1-infected individuals exhibited up to 4.8- and 27.8-fold reductions in GMT50 against Omicron BA.2 and Omicron BA.5, respectively (Figure 2E). There were up to 2.1- and 3.8-fold reductions in GMT50 against Omicron BA.1 and Omicron BA.5, respectively, with sera derived from Omicron BA.2-infected individuals (Figure 2F). Sera derived from Omicron BA.5-infected individuals exhibited a low level of cross-neutralization to Omicron BA.2, but not to Omicron BA.1, Ancestral and SARS-CoV-1 (Figure 2G).

Antigenic Distance Defined by Convalescent Antisera from Unvaccinated Individuals

Based on the antigenic map generated with serum samples derived from unvaccinated individuals who had recovered from primary infection, our data supported that Omicron BA.1, Omicron BA.2 and Omicron BA.5 were antigenically distinct (> 2 antigenic unit, AU) from SARS-CoV-1 or Ancestral (Figure 2H and 2I). This observation is consistent with data generated using hamster antisera [16]. From the antigenic map, we observed that Omicron BA.5 was antigenically closely related to Omicron BA.2. This finding is significant as serotyping of viruses is largely based on convalescent sera from natural infection instead of highly immune vaccinated sera. Considering the antigenic similarities between Ancestral, Alpha, Delta and Beta as observed in our study (Figure 2H and 2I) and available in the published data [15,18,19], these variants should be considered within the same serotype using commonly accepted serotype classification criteria [13,15]. On the other hand, lack of cross-neutralization (with GMT50 < 1:100 cutoff and/or antigenic distance > 2) between Ancestral, Omicron BA.1/BA.2/BA.5 and SARS-CoV-1 (Figure 2) supports the classification of Omicron BA.1/BA.2/BA.5 as a distinct serotype. Our current data hence indicate that distinctive serotypes exist for different SARSr-CoVs which are capable of infecting and causing diseases in humans.

Antigenic Distance Defined by Antisera from Vaccinated Individuals

Bearing these limitations in mind, we expanded our study using sera from vaccinees to further access the antigenic distance and relationship of different variants, including the latest variant Omicron XBB and Omicron BQ.1.1, albeit only possible for a one-way analysis. Using the pseudovirus neutralization test (pVNT), we demonstrated that

individuals who had received 2–3 doses of the Pfizer BNT162b2 mRNA vaccine produced high levels of NAbs against Ancestral. The third mRNA dose resulted in a 4.2-fold higher GMT50 against Ancestral and induced a higher level of cross-NAbs against Omicron subvariants. Despite that, we observed limited cross-neutralization against Omicron XBB and Omicron BQ.1.1 (Figure 3A and 3B), with up to 59.8- to 61.8-fold reduction in GMT50 compared with the Ancestral. It is important to note that the NAb-evasion capability of Omicron XBB and Omicron BQ.1.1 has exceeded that of SARS-CoV-1 despite both Omicron XBB and Omicron BQ.1.1 being phylogenetically much more closely related to Ancestral and amino acid sequence similarity identified between Ancestral and Omicron XBB/BQ.1.1 spike proteins at 96.7%/97.1% and between SARS-CoV-1 and Omicron XBB/BQ.1.1 at 75.0%/75.2%. This indicates extensive antigenic drift by the latest Omicron subvariants (Figure 3A and 3B). Our data indicate that Omicron XBB and Omicron BQ.1.1 were more antigenically distinct relative to Ancestral than SARS-CoV-1 (Figure 3C and 3D). For those receiving two doses of BNT162b2, the antigenic distance was 6.4–7 AU (XBB/BQ.1.1 to Ancestral) *vs* 4.9 AU (SARS-CoV-1 to Ancestral); for the three-dose group, it was 6 AU (XBB/BQ.1.1 to Ancestral) *vs* 4.1 AU (SARS-CoV-1 to Ancestral) (Figures 3C and 3D, 4A and 4B). Three doses of BNT162b2 reduced the antigenic distance between Ancestral and Omicron BA.1/BA.2, but to a lesser extent for Omicron XBB and Omicron BQ.1.1 (Figure 4A and 4B). However, with serum panel derived from individuals who had received three doses of mRNA vaccine, SARS-CoV-1 was clustered together with Omicron BA.4.6 and Omicron BA.2.75.2 (Figure 3D) and was antigenically closer to Omicron XBB (2.2 AU) than to Ancestral (4.1 AU) (Figure 4B).

DISCUSSION

The strong selective pressure driven by population immunity favors the emergence of new antigenic distinct variants, and therefore, it is becoming increasingly important to understand the antigenic relationships between variants. Unlike viruses such as measles and polioviruses that have little to no change in their sensitivity to vaccine-induced immunity for decades, the high structure plasticity of the coronavirus spike protein and the vast diversity of animal coronaviruses make the complete eradication an impossible task with current vaccines. Antigenic maps of vaccinated sera showed a greater extent of antigenic differences between the circulating Omicron variants and SARS-CoV-2, implying that pre-existing SARS-CoV-2 immunity is insufficient to prevent current and future infections. In addition, because of the original antigenic sin, breakthrough infections do not increase NAb epitope diversity but instead further promote the RBD to evolve convergently.

The data based on vaccine serum highlighted two important findings. First, antigenic cartography generated using serum samples derived from individuals who had hyperimmunity from multiple vaccinations against Ancestral is not meaningful in the context of serotyping analysis. Second, the latest Omicron XBB and Omicron BQ.1.1 have evolved to such a degree that antigenically they are more related to SARS-CoV-1 than Ancestral, which is highly important in our future development of broad-spectrum vaccine or monoclonal antibody candidates.

Our study indicates the importance of using convalescent sera from primary infection of naïve individuals as vaccinated or hybrid immune sera tend to give a masked profile. It is worth to note that the current study suffers from three major limitations due to the difficulty in accessing convalescent sera from unvaccinated individuals. First, the sample size for Omicron BA.5 cohort is small. Second, there was no serum available for those infected by the newest Omicron sub-lineages post the Omicron BA.5 wave due to high vaccination and/or infection rates. Third, as the serum collections were taken without a standardized protocol or consent, we were unable to normalize the samples using demographic data. Lastly, this study could be limited by the usage of a pseudovirus system as the distribution conformation and density pattern of the spike protein on pseudoviruses might not reflect the nature state of the surface of the authentic virus.

In conclusion, the data in this study support the assignment of distinct serotypes for SARS-CoV-1, Ancestral, and Omicron subvariants among the known human-infecting SARSr-CoVs. Furthermore, the lack of cross-NAbs in unvaccinated Omicron-infected individuals to Ancestral or between Omicron subvariants emphasizes the importance of vaccination for unvaccinated individuals who have recovered from natural infection to strengthen the population's immunity and preparedness against future variants. The unexpected antigenic relationship of Ancestral, SARS-CoV-1 and other Omicron subvariants further highlights the risk of future variants or sarbecovirus emergence as their evolutionary paths can be highly complicated and unpredictable based on genomic sequences alone.

MATERIALS AND METHODS

Human Serum Panels

Serum samples were collected from unvaccinated individuals with infection by Ancestral (n = 20), Delta (n = 10), Beta (n = 10), Omicron BA.1 (n = 11), Omicron BA.2 (n = 10), Omicron BA.5 (n = 5) or SARS-CoV-1 (n = 14). The vaccination or prior infection statuses were self-reported by these unvaccinated individuals and were validated by our multiplex surrogate neutralization test. The strain of SARS-CoV-2 was validated by either qPCR or sequencing. The vaccinated panels contain sera from individuals who received two (n = 20) or three (n = 18) shots of BNT162b2 vaccine, respectively. All vaccinated sera were collected at 14 days after the second or third dose of vaccine.

Cell Lines

Lung carcinoma epithelial (A549, ATCC CRM CCL-185) cells were grown and maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS). Human embryo kidney (HEK293T, ATCC CRL-3216) cells were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. The human ACE2 gene in the pFUGW vector was introduced into A549 cells by lentivirus transduction. The A549-ACE2 cells were maintained in Roswell Park Memorial Institute, RPMI-1640 supplemented with 10% FBS and 15 μg/mL of blasticidin.

Pseudovirus Production

Ancestral, Delta, Beta, Omicron BA.1, Omicron BA.2, Omicron BA.5, Omicron BA.2 mutants (L452R, F486V), Omicron BA.5, Omicron BA.2.75.2, Omicron BA.4.6.1, Omicron XBB, Omicron BQ.1.1, and SARS-CoV-1 full-length spike pseudotyped viruses were produced by transfecting 20 μg of pCAGGS spike plasmid into 5 million HEK293T cells using FuGENE 6 (Promega, Madison, WI, USA). At 24 h post transfection, the transfected cells were infected with VSVΔG luc seed virus at multiplicity of infection (MOI) of 5 for 2 h. After two washes with phosphate buffered saline (PBS), infected cells were replenished with DMEM supplemented with 10% FBS and 1:5000 diluted anti-VSV-G mAb (Clone 8GF11, Kerafast, Boston, MA, USA). Upon 80% cytopathic effect, pseudoviruses were harvested by centrifugation at 2000 g for 5 min.

Pseudovirus Neutralization Test

pVNT was performed as previously described [20,21]. In brief, pseudoviruses (~ 3 million relative light units (RLU)) were pre-incubated with four-fold serial diluted test serum in a final volume of 50 μL for 1 h at 37 °C, followed by infection of A549-ACE2 cells. At 20–24 h post infection, an equal volume of ONE-Glo luciferase substrate (Promega, Madison, WI, USA) was added, and the luminescence signal was measured using the citation 5 microplate reader (BioTek, Winooski, VT, USA) with Gen5 software version 3.10.

Prevalence of Omicron Variants over Time

Prevalence of Omicron (BA.1, BA.2, BA.2.75, BA.5, BQ.1.1, XBB and XBB.1.5) in different countries and worldwide was retrieved from GISAID (https://www.gisaid.org/, last accessed on 28 March 2023) and processed in R (version 4.2.3) with the R package “outbreak info” (version 0.2.0). The code is available online (https://github.com/Lelouchzhu/OmicronPrevalence).

Antigenic Cartography

The antigenic map was generated using the R package “Racmacs” (version 1.1.35) in R (version 4.2.3) based on the matrix of neutralization titer 50% of serum in the pseudovirus neutralization assay. The number of optimizations was set to 1000. Each unit of antigenic distance (length of one grid-square side, measured in any direction) is equivalent to a two-fold dilution in the neutralization assay. A four-fold difference in NT50 titer (2 AU in the antigenic map), between emerging variants, is generally considered a sufficient antigenic difference [22].

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9 software. The differences between paired groups were analyzed using paired two-sided Wilcoxon rank-sum test. A P value less than 0.05 is considered statistically significant. The threshold at 1:100 was determined by testing pre-pandemic sera against these pseudoviruses. The NT50 value below this threshold was regarded as negative.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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HIGHLIGHTS

- Convalescent sera from severe acute respiratory syndrome (SARS) patients are unable to neutralize SARS coronavirus 2.
- Neutralization escape by Omicron variants indicates significant antigenic change.
- Distinctive serotypes of SARS-related coronaviruses (SARSr-CoVs) revealed using convalescent sera of primary infection.
- Defining serotypes of SARSr-CoVs is important for future vaccine development.

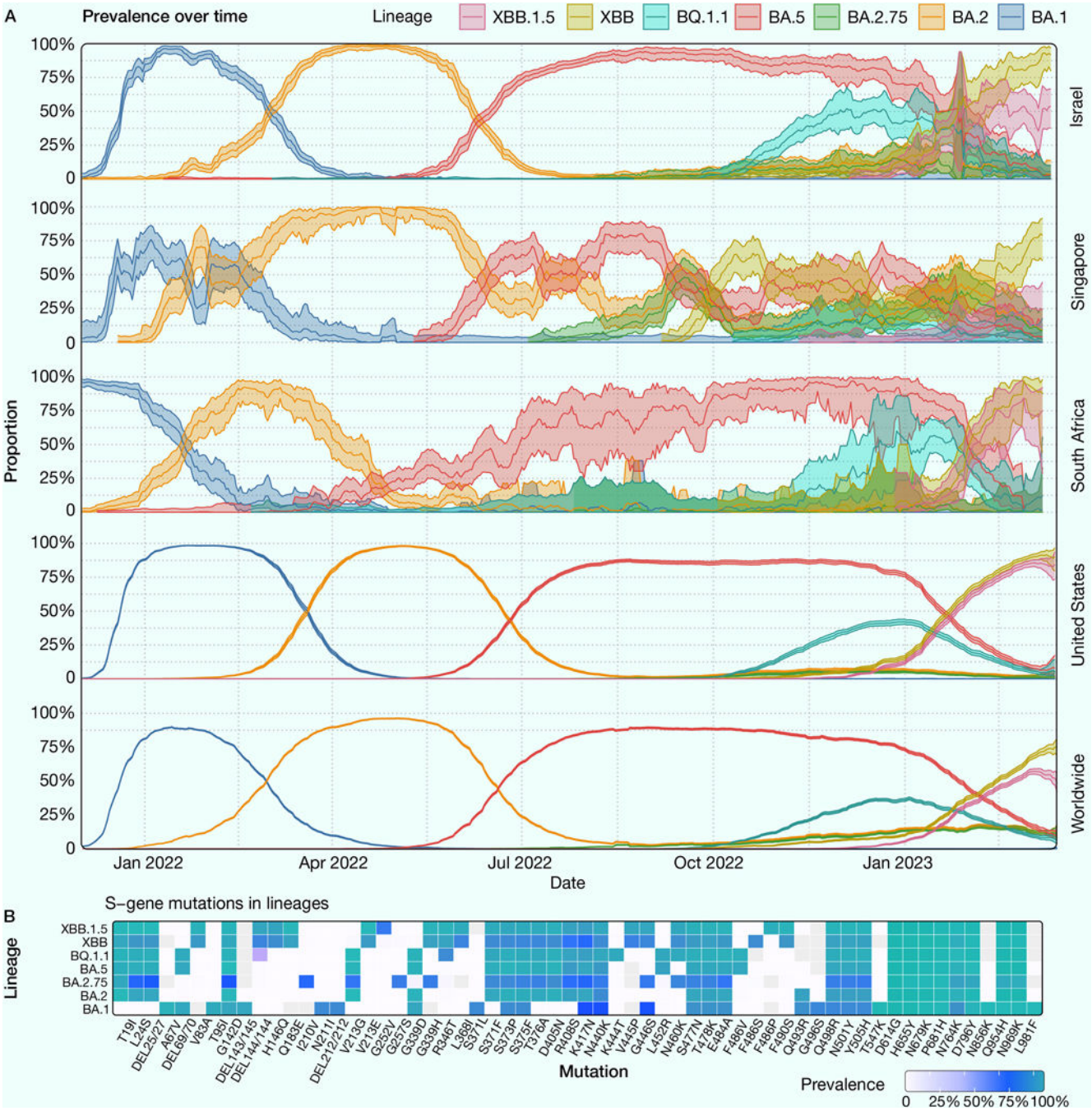

Figure 1. Prevalence of major Omicron variants over time

(A) Prevalence of Omicron (BA.1, BA.2, BA.2.75, BA.5, BQ.1.1, XBB and XBB.1.5) in Israel, Singapore, South Africa, the United States and worldwide over the last 15 months (since December 2021). (B) Mutations on the spike genes in Omicron (BA.1, BA.2, BA.2.75, BA.5, BQ.1.1, XBB and XBB.1.5). Data were retrieved from GISAID (https://www.gisaid.org/, 28 March 2023) *via* the outbreak.info API.

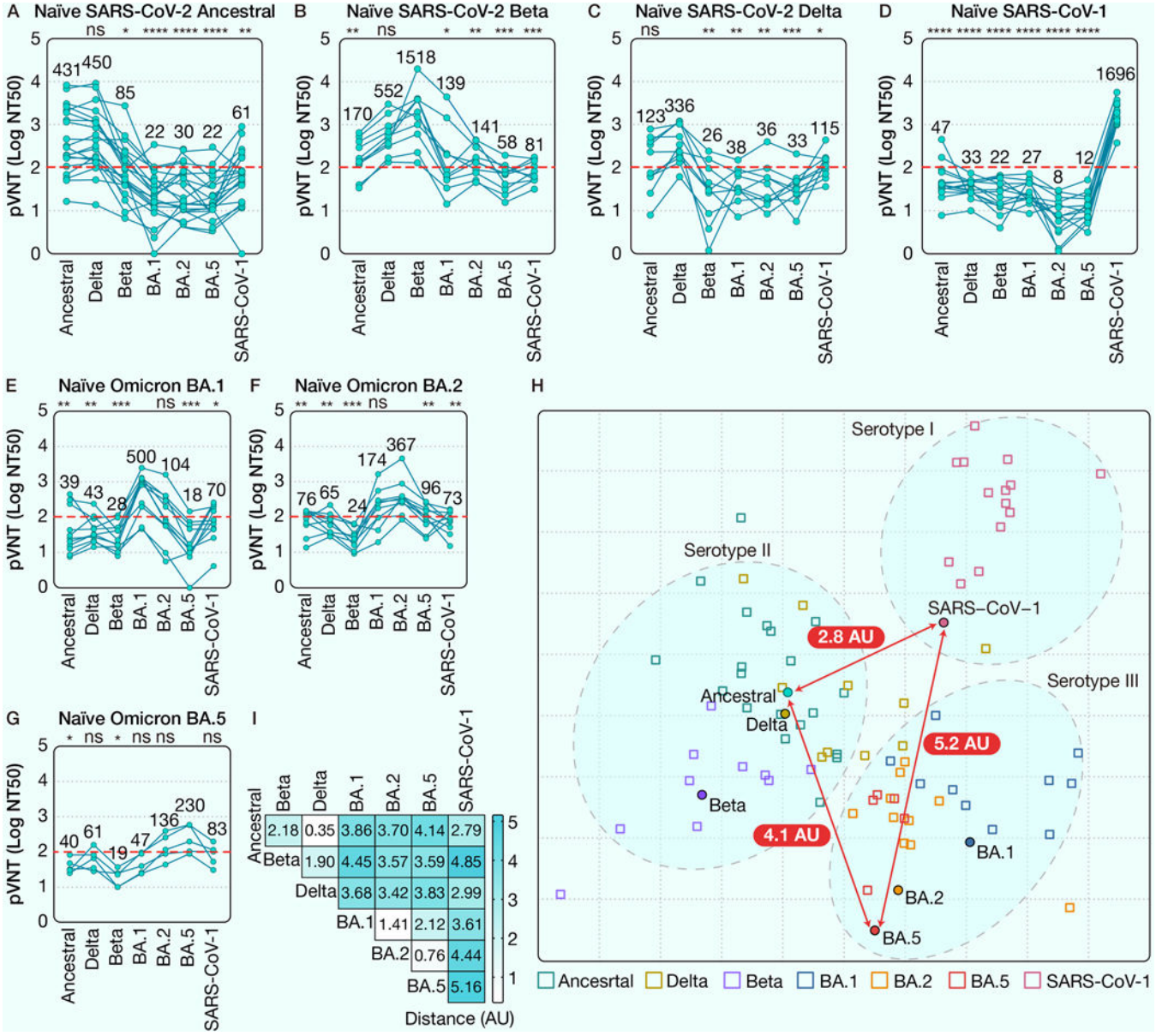

Figure 2. Neutralization titers against 11 different pseudoviruses with spike proteins derived from ancestral SARS-CoV-2, Beta, Delta, Omicron BA.1, BA.2, BA.5, and SARS-CoV-1

The neutralization titer 50% (NT50) values were determined using a pseudovirus neutralization test in plasma samples derived from naïve-infected individuals who had recovered from (A) Ancestral, (B) Beta, (C) Delta, (D) SARS-CoV-1, (E) Omicron BA.1, (F) Omicron BA.2 and (G) Omicron BA.5. Geometric mean NT50 was indicated on each of the dot plot. The red line indicates threshold of neutralization for each virus, and the samples under this threshold were regarded as negative. Paired two-sided Wilcoxon rank-sum tests were used. For each comparison, the infected virus strain was set as the reference group. All statistical analyses were performed using GraphPad Prism 9. A *P* value less than 0.05 indicates statistical significance. The *, **, ***, and **** indicate *P* values less than 0.05, 0.01, 0.001 and 0.0001, respectively. (H) Antigenic cartography was generated by the NT50

in pseudoviruses (color circles) from all serum panels examined (naïve Ancestral, $n = 20$; naïve Beta, $n = 10$; naïve delta, $n = 10$; naïve Omicron BA.1, $n = 11$; naïve Omicron BA.2, $n = 10$; naïve Omicron BA.5, $n = 5$; naïve SARS-CoV-1, $n = 14$). The x and y axes represent the antigenic distance, with the space of the grey grid lines showing 1 antigenic unit (two-fold dilution in titer). (I) The antigenic distances between viruses were shown in the heatmap.

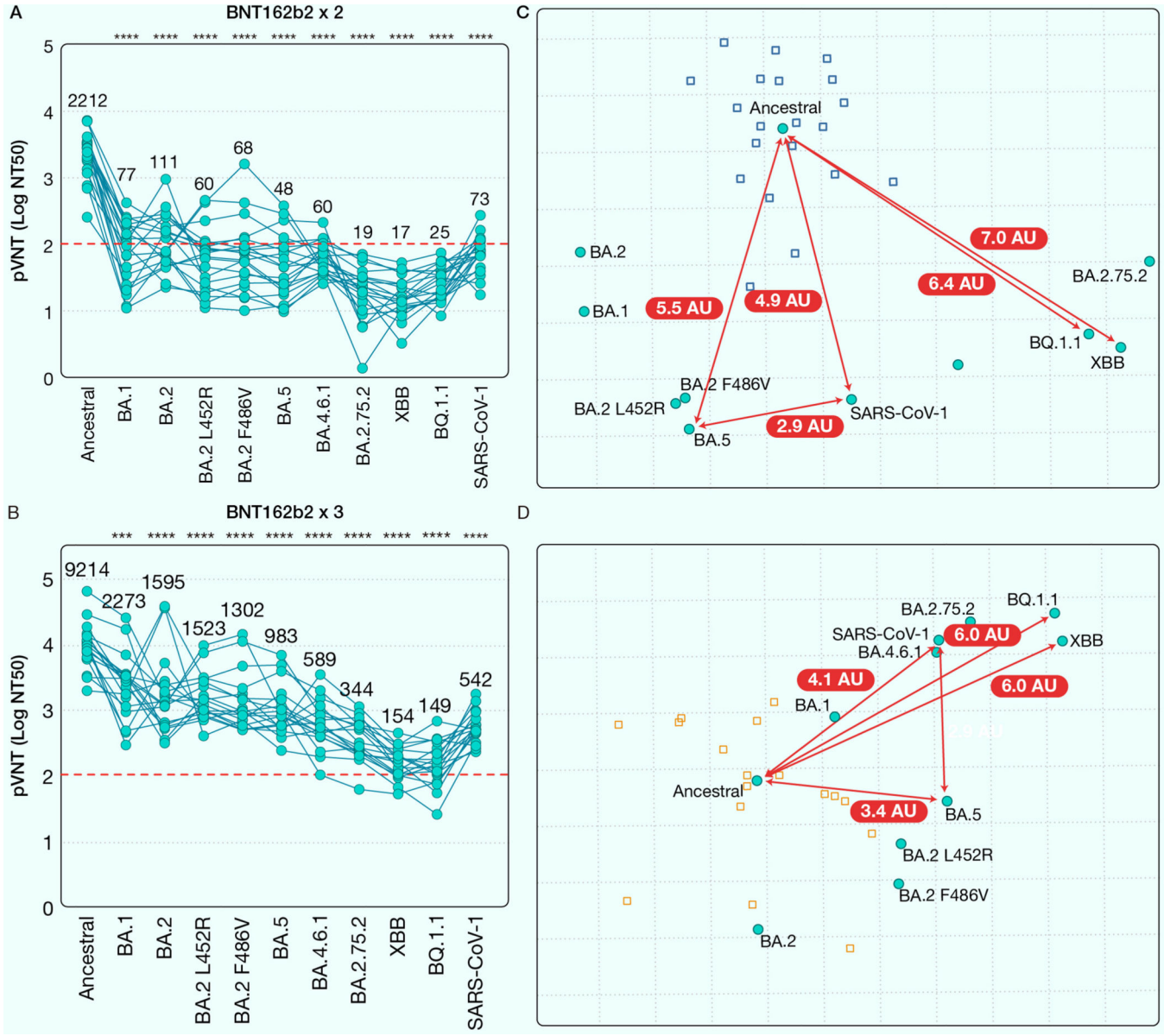

Figure 3. Neutralization titers against 11 different pseudoviruses with spike proteins derived from ancestral SARS-CoV-2, Omicron (BA.1, BA.2, BA.2 L452R, BA.2 F486V, BA.5, BA.4.6.1, BA.2.75.1, XBB, and BQ.1.1) and SARS-CoV-1

The neutralization titer 50% (NT50) values were determined using pseudovirus neutralization test in plasma samples derived from individuals who had received two (A) or three (B) doses of BNT162b2. A geometric mean NT50 value was indicated on each plot. The red line indicates threshold of neutralization for each virus, and the samples under this threshold were regarded as negative. Paired two-sided Wilcoxon rank-sum tests were used. The ancestral virus was set as the reference group. All statistical analyses were performed using GraphPad Prism 9. A *P* value less than 0.05 indicates statistical significance. The *, **, ***, and **** indicate *P* values less than 0.05, 0.01, 0.001 and 0.0001, respectively. The antigenic map was generated using the NT50 in pseudoviruses from serum samples derived from individuals who received two (C) and three (D) doses of BNT162b2. The *x* and *y* axes

represent the antigenic distance, with the space of the grey grid lines showing 1 antigenic unit (two-fold dilution in titer).

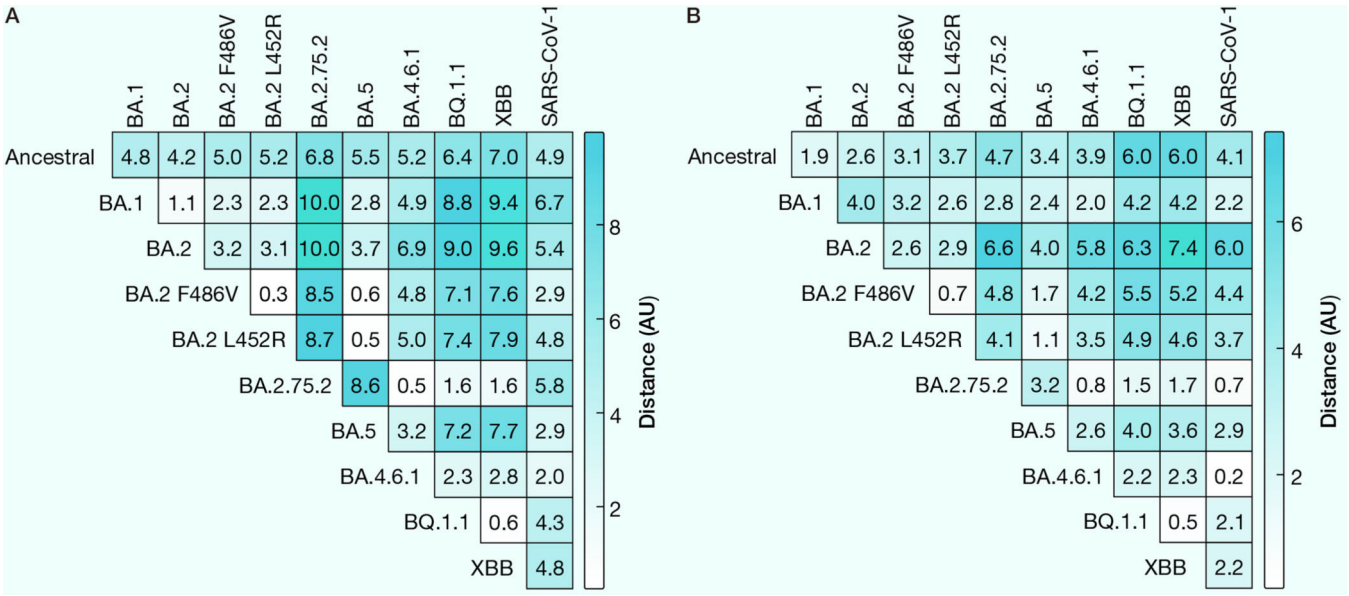

Figure 4. Antigenic characterization of ancestral SARS-CoV-2, Omicron (BA.1, BA.2, BA.2 F486V, BA.2 L452R, BA.5, BA.4.6.1, BQ.1.1, XBB), and SARS-CoV-1 using BNT162b2 vaccinated sera

The heatmap indicates antigenic unit (AU) distance between viruses determined using serum samples derived from individuals who had been receiving (A) two doses of BNT162b2 and (B) three doses of BNT162b2.