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Zhu et al. studied healthcare workers who had received homologous or heterologous prime-boost COVID-19 vaccination to find that the inoculation order of heterologous vaccines was associated with serum neutralization breadth against SARS-CoV-2 variants. The findings suggested using heterologous booster vaccines with high potency as a cost-efficient strategy against future variants.
Clinical and Translational Report

Association of neutralizing breadth against SARS-CoV-2 with inoculation orders of heterologous prime-boost vaccines

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SUMMARY

Background: Emerging evidence suggests heterologous prime-boost COVID-19 vaccination as a superior strategy than homologous schedules. Animal experiments and clinical observations have shown enhanced antibody response against influenza variants after heterologous vaccination; however, whether the inoculation order of COVID-19 vaccines in a prime-boost schedule affects antibody response against SARS-CoV-2 variants is not clear.

Methods: We conducted immunological analyses in a cohort of health care workers (n = 486) recently vaccinated by three types of inactivated COVID-19 vaccines under homologous or heterologous prime-boost schedules. Antibody response against ancestral SARS-CoV-2 (Wuhan-Hu-1) was assessed by total antibody measurements, surrogate virus neutralization tests, and pseudovirus neutralization assays (PNA). Furthermore, serum neutralization activity against SARS-CoV-2 variants of concern was also measured by PNA.

Findings: We observed strongest serum neutralization activity against the widely circulating SARS-CoV-2 variant B.1.617.2 among recipients of heterologous BBIBP-CorV/CoronaVac and WIBP-CorV/CoronaVac. In contrast, recipients of CoronaVac/BBIBP-CorV and CoronaVac/WIBP-CorV showed significantly lower B.1.617.2 neutralization titers than recipients of reverse schedules. Laboratory tests revealed that neutralizing activity against common variants but not the ancestral SARS-CoV-2 was associated with the inoculation order of heterologous prime-boost vaccines. Multivariable regression analyses confirmed this association after adjusting for known confounders.

Conclusions: Our data provide clinical evidence of inoculation order-dependent expansion of neutralizing breadth against SARS-CoV-2 in recipients of heterologous prime-boost vaccination and call for further studies into its underlying mechanism.

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INTRODUCTION

The repeated emergence of severe acute respiratory distress syndrome coronavirus 2 (SARS-CoV-2) variants with varying levels of antigen drift limits the effectiveness of
global vaccination programs and prolongs the COVID-19 pandemic.1–3 Most existing COVID-19 vaccines are based on the spike protein of the ancestral SARS-CoV-2 strain, which have showed reduced protection against later variants such as B.1.351 (beta) and P.1 (gamma) with E484/K417 mutations of the spike.4 Recently, heterologous prime-boost vaccination with adenovirus-based ChAdOx1 nCoV-19 and one of the mRNA-based vaccines has become a popular proposal to overcome the immune evasion of SARS-CoV-2 by eliciting stronger antibody response.5–7 However, many countries without access to these vaccines rely on inactivated COVID-19 vaccines with weaker immunogenicity, which are at a natural disadvantage in protecting against immune evasive variants.8,9 Inactivated COVID-19 vaccines are usually inoculated in a homologous prime-boost schedule to improve their immunogenicity.10,11 Interestingly, heterologous prime-boost schedules of inactivated influenza vaccines have shown to elicit antibody response with increased neutralizing breadth against variants than homologous prime-boost schedules in both animal studies and clinical observations.12,13 We therefore sought to assess serum neutralization activity against SARS-CoV-2 and variants of concern in those who received homologous or heterologous prime-boost vaccination and investigate whether the inoculation order was associated with neutralizing capacity or breadth.

RESULTS

We enrolled healthcare workers of Xiangyang Central Hospital, a large regional medical center in central China with more than 3,900 full-time employees, who were vaccinated prior to or during the institution-wide campaign in March to May 2021 and had thus received the second dose 1 to 3 months before blood collection, an ideal period to evaluate immune responses. Since heterologous prime-boost schedules were only allowed using inactivated vaccines, 486 recipients of three types of inactivated COVID-19 vaccines, BBIBP-CorV (BB, based on SARS-CoV-2 strain HB01) and WIBP-CorV (WB, based on WIV04) manufactured by SinoPharm,14 and CoronaVac (CV, based on CN2) manufactured by Sinovac15 in homologous or heterologous prime-boost schedules, were included in this study (Figure 1). All three vaccines were designed to be inoculated in homologous prime-boost schedules. However, due to unforeseeable availability of specific vaccines, recipients were allowed to voluntarily receive different inactivated vaccines as the booster dose to avoid long wait times of the booster dose. In this cohort, median (interquartile range [IQR]) dosing interval and interval between second dose and sample collection were 35 (29–46) and 53 (38–78) days, respectively. Regarding vaccination schedules, 283 (58%) received homologous of BB (24%), WB (13%), or CV (22%), whereas 203 (42%) received heterologous schedules in one of the six possible combinations of BB/WB/CV. Sex assigned at birth, allergy history prevalence, and adverse event rates were comparable among three homologous schedules and between each pair of heterologous schedules in reverse order (Table 1). Recipients of homologous BB had slightly older ages (median [IQR], BB/WB/VC, 32 [25–40]/25 [22–30]/27.5 [24–38], p = 0.001) and longer dosing intervals (median [IQR], BB/WB/VC, 39 [29–61]/37 [31–44]/33 [29–43], p = 0.012) and between second dose and blood collection (median [IQR], BB/WB/VC, 55.5 [38–100]/54 [43–77]/51 [31–64], p = 0.017) than recipients of other homologous schedules (Table 1), since BB was the first vaccine available in this region and was in short supply later. Clinical qualitative tests showed that 95.7%, 94.3%, and 96.7% of participants were positive for total, immunoglobulin (Ig) G, and neutralizing antibodies targeting SARS-CoV-2 spike protein, respectively, and homologous WB was the only group with less than 90% seroconversion (Table 1).
Chemiluminescent microparticle immunoassays (CMIA)s were used to quantitatively measure serum levels of total antibodies targeting the receptor binding domain (RBD) of SARS-CoV-2 spike protein. The linear range of CMIA was assessed by serial dilutions of CR3022 monoclonal antibody targeting the RBD (signal/cutoff [S/CO] 0.13–880, $R^2 = 0.9984$) and validated by serial dilutions of serum samples with the highest readout in this study (signal/cutoff [S/CO] 0.05–612, $R^2 = 0.9980$) (Figures S1A and S1B). Among recipients of homologous schedules, CV elicited more total anti-RBD antibodies than BBIBP and WIBP (Figure 2A). Heterologous BB/CV could further increase total anti-RBD antibody levels compared with homologous CV, while the inoculation orders of heterologous vaccines were not associated with antibody levels (Figure 2A).

Serum neutralization activity against the ancestral SARS-CoV-2 strain Wuhan-Hu-1 was measured by both surrogate virus neutralization tests (sVNT) for all samples and pseudovirus neutralization assays (PNA) in receiving order for homologous schedule samples and for all samples of heterologous schedules. Serum inhibition percentages of surrogate virus binding were in line with qualitative results and anti-RBD antibody levels, which showed superior neutralizing activity among both homologous and heterologous CV recipients regardless of inoculation orders (Figure 2B). Neutralization titers of Wuhan-Hu-1 PNA were largely in agreement with the sVNT results with the exception of a better performing homologous WB group (Figure 2C), which may arise from the difference of SARS-CoV-2 strains used in the manufacturing of BB and WB. These data suggested that the immune response against the ancestral SARS-CoV-2 was not associated with the inoculation order of heterologous vaccines.

We next measured serum neutralization activity against circulating SARS-CoV-2 variants using PNA. Currently, the globally dominant variant B.1.617.2 (delta) has shown moderate resistance to neutralization by convalescent and vaccinated serum. In this cohort, neutralization titers of homologous schedule groups against B.1.617.2 were proportionally reduced compared with their titers against Wuhan-Hu-1 (Figure 2D). In contrast, neutralization titers of heterologous schedule groups against B.1.617.2 were strongly depending on the inoculation order of vaccines with up to 10-fold difference in neutralization titers between reverse schedules (Figure 2D). Multivariable regression models confirmed that the inoculation order of heterologous vaccines was associated with neutralization titers against B.1.617.2
### Table 1. Demographic and serological characteristics of participants

|                      | Overall (n = 486) | Homologous vaccination | Heterologous vaccination |
|----------------------|-------------------|------------------------|--------------------------|
|                      | BB (n = 116)      | WB (n = 61)            | CV (n = 106)             |
|                      | WB/BB (n = 25)    | BB/WB (n = 36)         | CV/BB (n = 30)           |
|                      | BB/CV (n = 48)    | CV/WB (n = 34)         | WB/CV (n = 34)           |
| **p**                |                   |                        |                          |
| Age, years (IQR)     | 30 (24–43)        | 32 (25–40)             | 25 (22–30)               |
|                      |                   | 27.5 (24–38)           | 27 (23–40)               |
|                      |                   | 0.001                  | 0.078                    |
|                      |                   |                        | 0.950                    |
|                      |                   |                        | 15 (50.0)                |
|                      |                   |                        | 28 (58.3)                |
|                      |                   |                        | 0.493                    |
|                      |                   |                        | 17 (56.7)                |
|                      |                   |                        | 20 (58.8)                |
|                      |                   |                        | 1.000                    |
| Female at birth, n (%)| 319 (65.6)       | 81 (69.8)              | 44 (72.1)                |
|                      |                   | 75 (70.8)              | 14 (56.0)                |
|                      |                   |                        | 25 (69.4)                |
|                      |                   |                        | 0.416                    |
|                      |                   |                        | 15 (64.0)                |
|                      |                   |                        | 28 (58.3)                |
|                      |                   |                        | 0.493                    |
|                      |                   |                        | 17 (56.7)                |
|                      |                   |                        | 20 (58.8)                |
|                      |                   |                        | 1.000                    |
| Allergy history, n (%)| 9 (1.9)           | 0 (0)                  | 3 (4.9)                  |
|                      |                   | 4 (3.8)                | 0 (0)                    |
|                      |                   |                        | /                        |
|                      |                   |                        | 0 (0)                    |
|                      |                   |                        | /                        |
|                      |                   |                        | 1 (3.3)                  |
|                      |                   |                        | 1 (2.9)                  |
|                      |                   |                        | 1.000                    |
| AE Grade 1, n (%)     | 24 (4.9)          | 5 (4.3)                | 3 (4.9)                  |
|                      |                   | 5 (4.7)                | 1 (4.0)                  |
|                      |                   |                        | 3 (8.8)                  |
|                      |                   |                        | 0.395                    |
|                      |                   |                        | 0 (0)                    |
|                      |                   |                        | 1 (2.1)                  |
|                      |                   |                        | 1.000                    |
|                      |                   |                        | 4 (13.3)                 |
|                      |                   |                        | 2 (5.9)                  |
|                      |                   |                        | 0.407                    |
| AE Grade 2, n (%)     | 2 (0.4)           | 0 (0)                  | 0 (0)                    |
|                      |                   | 1 (0.9)                | 1 (4.0)                  |
|                      |                   |                        | 0 (0)                    |
|                      |                   |                        | 0 (0)                    |
|                      |                   |                        | 0 (0)                    |
|                      |                   |                        | 1.000                    |
| Dosing interval, days, median (IQR) | 35 (29–46) | 39 (29–61) | 37 (31–44) | 33 (29–43) | 0.012 | 40 (30–51) | 36.5 (29.5–45.5) | 0.437 | 35.5 (31–42) | 35.5 (29–46.5) | 0.992 | 32 (23–44) | 34 (29–42) | 0.264 |
| Second dose to sample collection, days, median (IQR) | 53 (38–78) | 55.5 (38–100) | 54 (43–77) | 51 (31–64) | 0.017 | 68 (40–89) | 44 (40–50) | 0.060 | 65 (39–80) | 51 (40–81) | 0.267 | 57 (24–70) | 48 (42–64) | 0.716 |
| Positive anti-spike antibodies, n (%) | 465 (95.7) | 110 (94.8) | 50 (82.0) | 105 (99.1) | <0.001 | 24 (96.0) | 34 (94.4) | 1.000 | 30 (100) | 48 (100) | / | 30 (100) | 34 (100) | / |
| Positive anti-spike IgG, n (%) | 458 (94.2) | 102 (87.9) | 51 (83.6) | 103 (97.2) | 0.014 | 24 (96.0) | 36 (100) | 0.410 | 30 (100) | 48 (100) | / | 29 (96.7) | 34 (100) | 0.469 |
| Positive sVNT, n (%) | 470 (96.7) | 111 (95.7) | 53 (86.9) | 105 (99.1) | 0.002 | 24 (96.0) | 35 (97.2) | 1.000 | 30 (100) | 48 (100) | / | 30 (100) | 34 (100) | / |

Abbreviations: AE, adverse events; BB, BBIBP-CorV; CV, CoronaVac; IQR, interquartile range; sVNT, surrogate virus neutralization test; WB, WIBP-CorV.

*p values were calculated by Kruskal-Wallis tests (between BB, WB, and CV) or Mann-Whitney U tests (between heterologous vaccination pairs) for continuous variables and chi-square tests (between BB, WB, and CV) or Fisher’s exact tests (between heterologous vaccination pairs) for categorical variables. ‘/’ indicates that tests were not performed due to categorical variables with only one value.*
but not Wuhan-Hu-1 after adjusting for known confounders including age, sex assigned at birth, \(^{17}\) dosing interval, \(^{18}\) and interval between second vaccination and sample collection (Table S1). \(^{19}\) These findings suggested that the inoculation order of heterologous vaccines was associated with vaccine-elicited neutralizing antibodies against variant B.1.617.2 but not the ancestral strain.

To investigate whether such variant-specific neutralization boost was due to an overall increased neutralizing breadth or sporadic cases of “super-immunity,” \(^{20}\) we calculated B.1.617.2 to Wuhan-Hu-1 ratios of neutralization titers for heterologous vaccinated samples and found few outliers but rather uniformed swing of ratios between groups of reverse schedules (Figure 3A). Moreover, the data indicated that the choice of booster vaccine alone in heterologous schedule was associated with neutralizing breadth (Figure 3C), and there was an apparent correlation of neutralizing breadth with the immunogenicity of booster vaccines (Figures 2A–2C). To further validate the neutralizing breadth against other variants, we chose 12 age- (\(\pm 5\) years), gender-, and second vaccination-to-sample-collection interval-matched...
10 days) samples from each group of homologous or heterologous schedules of the stronger CV and less potent BB vaccines to undergo PNA of other variants of concern, B.1.1.7 (Alpha), B.1.351, and P.1 (Figures S2A–S2D). Of note, CV/BB and CV/WB groups were combined due to fewer than 12 matched samples in the CV/BB group alone and their similar neutralization profiles against Wuhan-Hu-1 and B.1.617.2 (Figures 2C and 2D). The variant-to-ancestral ratios showed that while homologous BB and CV showed comparable neutralizing breadth against all four variants (Figure 3C), BB/CV exhibited significantly enhanced neutralizing breadth than CV/BB, with only the ratios of P.1 not reaching statistical significance due to higher intra-group variation and limited sample size (Figure 3D). In addition, PNA of the recently emerged B.1.1.529.1 variant (omicron BA.1) showed enhanced serum neutralizing activity after heterologous BB/CV vaccination compared with CV/BB vaccination (Figure S3), which was largely in agreement with recent studies of heterologous CV and BNT162b2 (Pfizer-BioNTech) vaccines.21–23 These results together suggested that the inoculation order of heterologous prime-boost vaccines was associated with neutralizing breadth against SARS-CoV-2.

DISCUSSION

Heterologous vaccination has immense potential not only thanks to its superior immunogenicity but also due to the ongoing logistic burden of vaccine distribution that frequently delays the vital booster dose of homologous schedules.7,24 To our knowledge, our analyses are the first report of inoculation order-dependent
expansion of neutralizing breadth against SARS-CoV-2 after heterologous vaccination, which could be translated into a promising strategy of using potent booster vaccines in heterologous prime-boost schedules to counter the antigen drift of SARS-CoV-2 variants. As we learned from seasonal influenza vaccines, mass production of variant-specific vaccines may never catch up the rate of viral mutations, and heterologous vaccination might represent a more efficient strategy using existing stockpiles to protect against future variants. Furthermore, our data provide preliminary evidence that using less immunogenic vaccines as primer might not compromise the neutralization activity against variants elicited by more potent booster vaccines because of enhanced neutralizing breadth. Therefore, using abundantly available vaccines as the primer in tandem with a more potent booster vaccine may achieve the same coverage as single-dose strategy of the potent vaccine but with protection comparable with the two-dose schedule.

A number of factors including antigen sequences, vaccine platforms, and adjuvants may contribute to the inoculation sequence-dependent expansion of neutralizing breadth. While our data could not provide a definitive answer regarding these possibilities, recent progress in the field suggested that vaccine platforms or antigen sequences were unlikely to play a major role here. Reference sequences of SARS-CoV-2 HB02 (used in BB), WIV04 (WB), and CN2 (CV) strains are available in GenBank. A detailed analysis of those sequences found only minimal sequence variations between these strains, which was as expected due to their close timing of isolation and geographic localization. These sequence variations were unlikely to alter the immunogenic profile compared with the larger number of mutations in known variants. Furthermore, the results from ChAd(Oxford–AstraZeneca)/BNT(Pfizer–BioNTech) heterologous prime-boost studies showed similar expansion of variant-reactivity breadth of neutralizing antibodies as higher beta/alpha or beta/D614G ratios of neutralization titers after heterologous ChAd/BNT vaccination compared with homologous prime-boost vaccination of either vaccine on similar schedules, suggesting that our findings may not be platform-specific. Similar with inactivated vaccines in our study, ChAd and BNT were also based on the ancestral spike sequence with minor optimizations that should not alter their antigenicity. In contrast, delta breakthrough patients did not gain such expanded breadth of neutralizing antibodies against the beta variant, despite a significant increase of neutralization titers against D614G and delta variants than those who received three doses of homologous mRNA vaccines. Considering delta infection as an immunization event, these findings suggest that a booster based on a novel variant spike could induce stronger neutralization against this specific variant only, which is different from heterologous prime-boost vaccination that could expand neutralizing breadth not limited to specific variants. Of note, the adjuvants of BB, WB, and CV are the same according to manufacturers’ instructions. Together, our and others’ data strongly suggest that antigen sequence or platform variation between each vaccination is not associated with the inoculation order-dependent expansion of neutralizing breadth.

Nonetheless, our data did not rule out the association of heterologous vaccine antigens, regardless of inoculation order, with the neutralizing breadth, since prior studies have shown that immunization using different variants of the same antigen could further expand antibody cross-reactivity against other variants in an animal model. Therefore, development of variant-specific vaccines would still be of high value in containing future variants, while our findings would offer an anti-variant strategy based on existing vaccine stockpile, which is currently more feasible given the shortage of potent vaccines in many regions.
We are also curious about the underlying molecular mechanism of our findings. Increasing the neutralizing breadth of vaccine-induced antibodies is a fundamental goal in vaccine development, and heterologous vaccination has been tested in this aim before. Despite the long-known advantage of heterologous vaccination, the effect of inoculation order was often overlooked. One of the few relevant studies examined DNA/Ad heterologous vaccination against HCV. Homologous Ad vaccination was more potent than homologous DNA vaccination. Similar to our findings, among all four schedules (DNA/DNA, Ad/Ad, DNA/Ad, and Ad/DNA), the DNA/Ad schedule induced not only the highest T cell response but also T cells binding strongly with more E2 epitopes. On the contrary, Ad/DNA schedule induced T cells binding most weakly with E2 epitopes, despite a decent T cell response. These findings resonate with ours that the breadth of immune response may be associated with the immunogenicity of the booster vaccine in a heterologous prime-boost schedule. Mechanistically, it was found that DNA/Ad induced strikingly high CD4+ T cells against target antigens. Intriguingly, a recent report of heterologous ChAd/BNT vaccination found that spike-specific CD8+, not CD4+, T cells were highly enriched after ChAd/BNT vaccination compared with ChAd/ChAd or BNT/BNT vaccination. We therefore speculate that certain adaptive immune pathways might be involved in this expansion of immune responses. In fact, recent studies have revealed that prolonged or repeated exposure to SARS-CoV-2 antigens could enhance neutralizing potency and breadth via affinity maturation. We have also shown that unvaccinated survivors of severe COVID-19 underwent long-term post-recovery expansion of neutralizing breadth, a feature that was not found among mild to moderate COVID-19 survivors or vaccine recipients. Since our data have shown that CV was more immunogenic than the other two vaccines, it is possible that this superior immunogenicity led to enhanced or prolonged affinity maturation only after the inoculation of a booster dose.

In summary, our data provide clinical evidence of inoculation order-dependent expansion of neutralizing breadth against SARS-CoV-2. Pending further validation of our conclusions, heterologous vaccination in a weak/potent order may represent an efficient strategy to achieve optimal protection against SARS-CoV-2 variants with existing vaccine stockpiles.

Limitations of the study
This study has several limitations. First, the non-randomized and observational nature made the conclusions prone to confounding effects and precluded any causal interpretation of the findings. Since heterologous vaccination was approved later than homologous schedules due to lengthy waiting for a matched vaccine, time intervals between the second vaccination and sample collection were shorter in contrast to longer dosing intervals among recipients of heterologous schedules in this cohort. Furthermore, since heterologous vaccination in the prime-boost schedule with 1-month interval between shots was not a standard practice for the public at the time of this study, the participants were limited to healthcare workers and in limited quantity. Of note, we have used multivariable regression models to adjust for these potential confounders in relevant analyses. Nonetheless, we would caution against interpreting our data as the indicator of immunogenicity or efficacy of homologous vaccination schedules. Also, it is worth noting that previous evidence of heterologous vaccination-induced enhancement of variant-reactivity was found among recipients of inactivated vaccines and all three vaccines used in this study are also inactivated vaccines, which may limit the generalization of the above conclusions to vaccines developed on other platforms.
Other limitations of this study include the lack of neutralization assays based on live viruses. The use of PNA instead of live virus neutralization assays limited the interpretation to the inhibition of first-round viral entry, while the inhibition of viral replication and secondary infection was not assessed in this study. Last, our sample size was not sufficient to explore potential adverse events associated with heterologous vaccination, which were reported to be more frequent than those associated with homologous vaccination. We thus caution proactive implementation of heterologous vaccination before more evidence on its adverse effects emerge.

STAR METHODS

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

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.medj.2022.05.003.

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AUTHOR CONTRIBUTIONS

Y. Zhu and P. H. conceived and designed the study; Y. Zhu, C. Z., T. G., Y. W., R. L., Y. Li, L. W., Y. Zhan, J. C., J. G., J. W. and Linyu Zhu recruited participants and collected specimens; Y. Zhu, C. Z., Y. W., R. L., Y. Li, T. G., L. W., and Z. C. conducted clinical laboratory tests; Y. Lu, G. T., M. G., W. Z., J. Q., Longchao Zhu, and F. X. conducted PNA; Y. Zhu, Y. Lu, and P. H. had unrestricted access to all data and conducted statistical analyses; M. C., Y. X., Z. Zheng, Z. Zhou, and Z. C. interpreted clinical and statistical findings; Y. Lu and P. H. wrote the original draft; Y. Zhu, Y. Lu, Z. Zhou, Z. C., and P. H. reviewed and revised the manuscript. All authors agreed to submit the manuscript, read and approved the final draft, and take full responsibility for its content, including the accuracy of the data and the fidelity of the study to the registered protocol and its statistical analysis.
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**STAR METHODS**

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Recombinant Anti-SARS-CoV-2 Spike Glycoprotein S1 antibody [CR3022] - Chimeric | Abcam | AB_2847846 |
| Critical commercial assays |        |            |
| 2019-nCoV antibody detection kit [ELISA] | InnoDx | 2019-nCoV-Ab |
| 2019-nCoV IgG antibody detection kit [ELISA] | InnoDx | 2019-nCoV-IgG |
| 2019-nCoV neutralizing antibody detection kit | InnoDx | 2019-nCoV-NAb |
| 2019-nCoV antibody detection kit [CMIA] | InnoDx | 2019-nCoV-CMIA |
| Experimental models: Cell lines |        |            |
| HEK293/ACE2 cells, replication-defective | GenScript | M00770 |
| Experimental models: Organisms/strains |        |            |
| SARS-CoV-2 Wuhan-Hu-1 pseudovirus, Luc reporter | GenScript | SC2087A |
| SARS-CoV-2 B.1.1.7 pseudovirus, Luc reporter | GenScript | SC2087K |
| SARS-CoV-2 B.1.351 pseudovirus, Luc reporter | GenScript | SC2087L |
| SARS-CoV-2 P.1 pseudovirus, Luc reporter | Vazyme Biotech | DD1546 |
| SARS-CoV-2 B.1.617.2 pseudovirus, Luc reporter | GenScript | SC2087V |
| SARS-CoV-2 B.1.1.529.1 pseudovirus, Luc reporter | Vazyme Biotech | DD1768 |
| Software and algorithms |        |            |
| SPSS 26 | IBM | https://www.ibm.com/analytics/spss-statistics-software |
| Prism 9 | GraphPad | https://www.graphpad.com |
| Other |        |            |
| Caris 200 CMIA analyzer | UMIC Medical Instrument | Caris200 |

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Peng Hong (peng.hong@downstate.edu).

**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**
- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Human sample**
This study was based on a prospective observational cohort of hospital staff located in Xiangyang city of Hubei, China, where eligible healthcare workers were required to receive prime-boost COVID-19 vaccines. The cohort included participants 18 years of age or older who had received at least two doses of COVID-19 vaccines at least 7 days before the date of blood collection. Participants with the following conditions were excluded: (a) prior COVID-19 diagnosis or positive serological tests, (b) under immune-modulatory medication, (c) active infection or any inflammatory...
disease, (d) coagulation disorder or other conditions precluding safe blood
collection, (e) physician assessment, laboratory examination, or any other conditions
making the subject unsuitable for the study. For an effect size of 0.15 at 5%
significance level and 90% power for the neutralization titers, the cohort requires
459 subjects that complete the study with equal distribution between two groups.
A maximum of 1,000 subjects will be enrolled. The study protocol (#2021-034)
was approved by the Medical Ethics Committee of Xiangyang Central Hospital.

Participants information on sex, age, and race was self-reported. Information on
socioeconomic status was not collected. Dates of vaccination and manufacturers
of vaccines were provided by participants after signing the informed consent. The
participants were asked for the history of allergy and chronic disease and medication
taken since the first vaccine dose. Adverse effects potentially related to vaccination
were also recorded. After confirming the eligibility of participation, 5 mL of venous
blood was collected from the participant and proceeded to serum isolation immedi-
ately. Serum samples were aliquoted for each assay to minimize freeze-thaw cycles.

METHOD DETAILS
Serological assays
SARS-CoV-2 serology was determined by both qualitative and quantitative assays.
Anti-spike total and IgG antibodies were separately assessed by qualitative ELISA
kits (2019-nCoV antibody detection kit [ELISA], InnoDx; 2019-nCoV IgG antibody
detection kit [ELISA], InnoDx) according to manufacturer’s instructions. Cutoff value
was calculated as 0.16 + the average of negative control readouts. Samples with
readouts equal to or higher than the cutoff value were deemed positive.

Neutralizing antibodies were assessed by a surrogate virus neutralization assay kit
(2019-nCoV neutralizing antibody detection kit, InnoDx) according to manufac-
turer’s instructions. Results were expressed as inhibition percentages calculated
by the following formula: inhibition percentage = (negative control value – sample
value) × 100%/negative control value. Samples with inhibition percentages equal
to or higher than 50% were deemed positive.

Quantitative CMIA for total anti-RBD antibodies (2019-nCoV antibody detection kit
[CMIA], InnoDx) was performed on a Caris200 analyzer (UMIC Medical Instrument)
following manufacturer’s instructions. Cutoff value was calculated according to
manufacturer’s instructions. The assay sensitivity and specificity were 94.8 and
99.7% according to the manufacturer, and 90.8 and 98.9% in an independent
study, respectively. S/CO values less than 0.01 were recorded as 0.01 for all
analyses.

Quality control checks of all serological assays were conducted according to
manufacturer’s instructions. Samples failing any checks were re-tested after neces-
ary procedures to improve quality. Samples repeatedly failing checks or without
sufficient volume for further re-test were excluded from the analysis.

Pseudovirus neutralization assays
Seum neutralization titers against Wuhan-Hu-1 (GenScript), B.1.1.7 with Δ69-70,
Δ144, N501Y, A570D, D614G, P681H, T716I, S982A and D1118H mutations
(GenScript), B.1.351 with L18F, D80A, D242–244, R246I, K417N, E484K,
N501Y, D614G and A701V mutations (GenScript), P.1 with L18F, T20N, P26S,
D138Y, R190S, K417T, E484K, N501Y, D614G, H655Y and T1027I mutations
(Vazyme Biotech), B.1.617.2 with T19R, Δ156–158, L452R, T478K, D614G, P681R
and D950N mutations (GenScript), and B.1.1.529.1 pseudoviruses with A67V, Δ69–70, T95I, Δ142-144, Y145D, Δ211, L212I, ins214EPE, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F mutations (Vazyme Biotech) were measured by PNA. All PNA procedures were performed in a Biosafety Level 2 laboratory. In brief, HIV pseudoviruses carrying a luciferase reporter and encapsulated in ancestral or variant spike proteins were incubated with eight 4-fold serial dilutions of the serum sample by Opti-MEM (Gibco) for 1 h at room temperature. The mixture was then added to the culture of replication-deficient HEK293/ACE2 cells in 96-well plates with DMEM (Gibco)/10% FBS (Gibco)/1× antibiotics (Gibco) and incubated in a humidified cell culture chamber at 37 °C with 5% CO2 for 48 h. Medium was removed at the end of incubation, and 50 μL one-step luciferase detection reagent (GenScript) was added to each well. Luminescence in relative light units (RLUs) was measured by a luminometer (Synergy H1, BioTek Instruments) after 3 min of incubation at room temperature. Serum samples may be diluted to meet the initial volume requirement. Samples without maximum RLUs equal to 100 times of cell-only controls were tested again with dilution of the initial sample when necessary. Samples failed to yield meaningful results due to quality issues or limited volumes were excluded from analyses. RLUs of sample wells were normalized with positive control wells and pNT50 was calculated as EC50 by normalized four-parameter sigmoid curve fit in Prism 9.0 (GraphPad). pNT50 was arbitrarily set to 0.39, the limit of detection (LOD) of PNA, when EC50 was lower than the LOD or not computable due to low neutralization activity. The LOD was calculated as mean +1.96 × SD of the Wuhan-Hu-1 pNT50 of 12 serologically negative samples from healthy donors.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Participant characteristics and serological data were assessed with Chi-square tests for categorical variables or Kruskal-Wallis tests for continuous variables. Post hoc comparison methods were detailed in figure legends. Serological data were log-transformed before being analyzed by regression models. Confounders in multivariable regression models were selected based on documented associations and listed in Table S1. Analyses were performed using SPSS 26 (IBM) or Prism 9 (GraphPad). Missing data were excluded pairwise from analyses. Significance was evaluated at α = 0.05 and all tests were 2-sided.