2-1-2024

# Serological assays for differentiating natural COVID-19 infection from vaccine induced immunity

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ARTICLE INFO

ABSTRACT

*Background:* Natural SARS-CoV-2 infection may elicit antibodies to a range of viral proteins including non-structural protein ORF8. RNA, adenovirus vectored and sub-unit vaccines expressing SARS-CoV-2 spike would be only expected to elicit S-antibodies and antibodies to distinct domains of nucleocapsid (N) protein may reliably differentiate infection from vaccine-elicited antibody. However, inactivated whole virus vaccines may potentially elicit antibody to wider range of viral proteins, including N protein. We hypothesized that antibody to ORF8 protein will discriminate natural infection from vaccination irrespective of vaccine type.
*Methods:* We optimized and validated the anti-ORF8 and anti-N C-terminal domain (N—CTD) ELISA assays using sera from pre-pandemic, RT-PCR confirmed natural infection sera and BNT162b2 (BNT) or CoronaVac vaccinees. We then applied these optimized assays to a cohort of blood donor sera collected in April-July 2022 with known vaccination and self-reported infection status.
*Results:* We optimized cut-off values for the anti-ORF8 and anti-N-CTD IgG ELISA assays using receiver-operating-characteristic (ROC) curves. The sensitivity of the anti-ORF8 and anti-N-CTD ELISA for detecting past infection was 83.2% and 99.3%, respectively. Specificity of anti-ORF8 ELISA was 96.8 % vs. the pre-pandemic cohort or 93% considering the pre-pandemic and vaccine cohorts together. The anti-N-CTD ELISA specificity of 98.9% in the pre-pandemic cohort, 93% in BNT vaccinated and only 4 % in CoronaVac vaccinated cohorts. Anti-N-CTD antibody was longer-lived than anti-ORF8 antibody after natural infection.
*Conclusions:* Anti-N-CTD antibody assays provide good discrimination between natural infection and vaccination in BNT162b2 vaccinated individuals. Anti-ORF8 antibody can help discriminate infection from vaccination in either type of vaccine and help estimate infection attack rates (IAR) in communities.

## a. ORF8
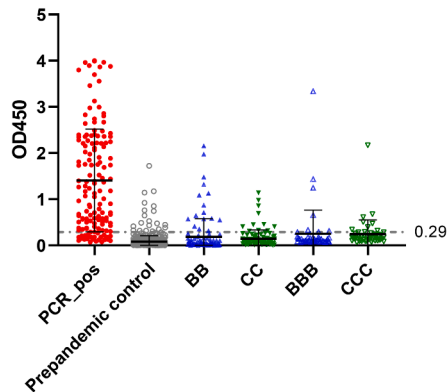


| | PCR positives | Prepandemic control | BB | CC | BBB | CCC |
|---|---|---|---|---|---|---|
| N | 143 | 626 | 100 | 100 | 50 | 50 |
| Mean (95% C.I.) | 1.41(1.22-1.59) | 0.08(0.07-0.09) | 0.19(0.11-0.26) | 0.14(0.11-0.18) | 0.25(0.10-0.40) | 0.24(0.16-0.33) |
| No. of Pos | 120 | 20 | 16 | 11 | 7 | 9 |
| % Pos | 83.9% | 3.2% | 16.0% | 11.0% | 14.0% | 18.0% |

## b. N-CTD
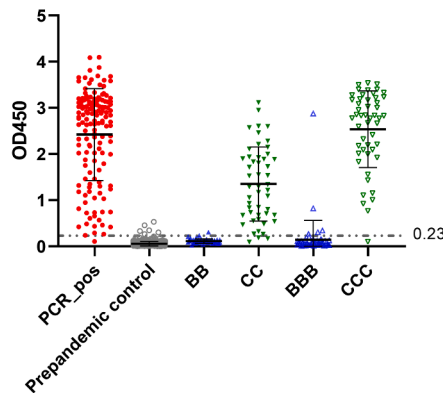


| | PCR_pos | Prepandemic control | BB | CC | BBB | CCC |
|---|---|---|---|---|---|---|
| N | 143 | 526 | 50 | 50 | 50 | 50 |
| Mean (95% C.I.) | 2.42(2.26-2.59) | 0.06(0.05-0.06) | 0.11(0.10-0.13) | 1.35(1.12-1.58) | 0.15(0.03-0.26) | 2.54(2.30-2.77) |
| No. of Pos | 142 | 6 | 2 | 47 | 5 | 49 |
| % Pos | 99.3% | 1.1% | 4.0% | 94.0% | 10.0% | 98.0% |

**Fig. 1.** SARS-CoV-2 anti-ORF8 and anti-N—CTD IgG antibody responses in individuals infected by ancestral SARS-CoV-2 (RT-PCR pos), healthy individuals before COVID-19 pandemic (prepandemic), individuals vaccinated with 2 (BB) or 3 (BBB) doses of BNT162b2, or 2 (CC) or 3 (CCC) doses of CoronaVac. a) ORF8 ELISA. b). N—CTD ELISA. Dotted line denotes the cutoff for each ELISA assay.

## 1. Introduction

SARS-CoV-2 is a single strand RNA virus and is the cause of the COVID-19 pandemic. The virion is composed of four structural proteins, spike protein (S), membrane protein (M), envelope protein (E) and nucleoprotein (N); and expresses during infection 16 nonstructural protein (nsp) nsp1–16; and 9 accessory proteins, ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF9c and ORF10 [1]. Among these non-structural proteins, ORF8 elicited the highest antibody response following natural infection and preliminary evidence suggested that it could discriminate between infection and vaccination [2,3].

Sero-epidemiology can be used to assess infection attack rates in the population but vaccination may confound such assessments because vaccines may elicit antibodies to different virus proteins. Thus, serological assays that can distinguish natural infection from vaccination are needed. Such an approach using antibody to NS1 antibody responses to differentiate vaccination from infection with influenza but was found to have low sensitivity [4]. The Hong Kong government launched its vaccination program in early 2021 using two types of vaccines, the whole virus inactivated vaccine CoronaVac (Sinovac Biotech) and the mRNA vaccine BNT162b2 (Corminaty). As of 14 June 2023, over 20 million doses of vaccines have been administered in Hong Kong. These two vaccines are also among the most widely used vaccines world-wide. Enzyme-linked immunosorbent assay (ELISA) is extensively used in detection of SARS-CoV-2 antibodies in sero-epidemiological assays. Usually, serology assays target the structural proteins S or N. Natural infection would elicit antibodies against both S and N while mRNA vaccines encoding S elicits and adenovirus vectored vaccines elicit antibody only to S. Whilst the N shares some similarity to endemic common cold coronaviruses, and testing of the different domains of the N proteins in various combinations (NTD, CTD, LKR, Carm and full length), showed the N—CTD to be the most specific and sensitive for discrimination for SARS-CoV-2 infection [5].

Thus, serology assays targeting N protein can differentiate antibodies elicited by infection and vaccination with mRNA or adenoviral vectored vaccines but not following whole virus inactivated vaccines which also carry the N protein. We hypothesized that anti non-structural protein ORF8 antibody will help distinguish vaccination from natural infection, irrespective of the type of vaccine used.

In this study, we optimize the anti-ORF8 ELISA and anti-N-CTD ELISA using sera collected from COVID-19 RT-PCR confirmed patients stratified by disease severity as positive controls, pre-pandemic sera to serve as negative controls and use these optimized assays in other cohorts with known vaccine and self-reported infection status. (VE).

## 2. Methods

**Sample collection.** Sera ($N = 143$) from SARS-CoV-2 RT-PCR confirmed patients were collected during 2020–2021 as positive controls for initial optimization of the anti-ORF8 ELISA and anti-N-CTD ELISA assays. Pre-pandemic sera ($N = 626$) collected before 2020 from blood donors served as negative controls. A subset of sera ($N = 145$) collected longitudinally from 47 RT-PCR confirmed COVID-19 patients with record of disease severity was used for evaluation of anti-ORF8 and anti-N-CTD antibody waning. Additionally, sera ($N = 70$) from 30 asymptomatic patients were included in the analysis to compare antibody responses from asymptomatic and symptomatic patients. Severity level was defined as asymptomatic, mild, moderate, severe, and critical illness [6]. Sera from BNT162b2 ($N = 150$) and CoronaVac ($N = 150$) vaccinees were collected in 2021, before the first major outbreak of COVID-19 that only occurred in Hong Kong in early 2022. Paired sera collected before vaccination ($N = 135$) and 1 month after vaccination ($N = 135$) were tested in parallel to investigate the effect of the two vaccines on anti-ORF8 and anti-N-CTD ELISA responses. Sera from blood donors ($N = 4423$) with documented vaccination history and self-reported infection history were collected during April to July 2022 for real-world evaluation of the two assays to assess their ability to discriminate between infection and vaccination. The sample collections was approved by the Institutional Review Board of The Hong Kong University and the Hong Kong Island West Cluster of Hospitals (approval numbers UW16–254; UW 20–169; UW 20–273 and UW 20–132.

**Anti-ORF8 and anti-N-CTD ELISA.** The ELISAs were performed as previously described [3,7]. The N—CTD protein (E Coli expressed) was obtained from Prof Gaya Amerasinghe, Washington University St Louis ([5,8]) and the ORF8 protein (Tobacco BY-2 epressed) from Dr Masashi
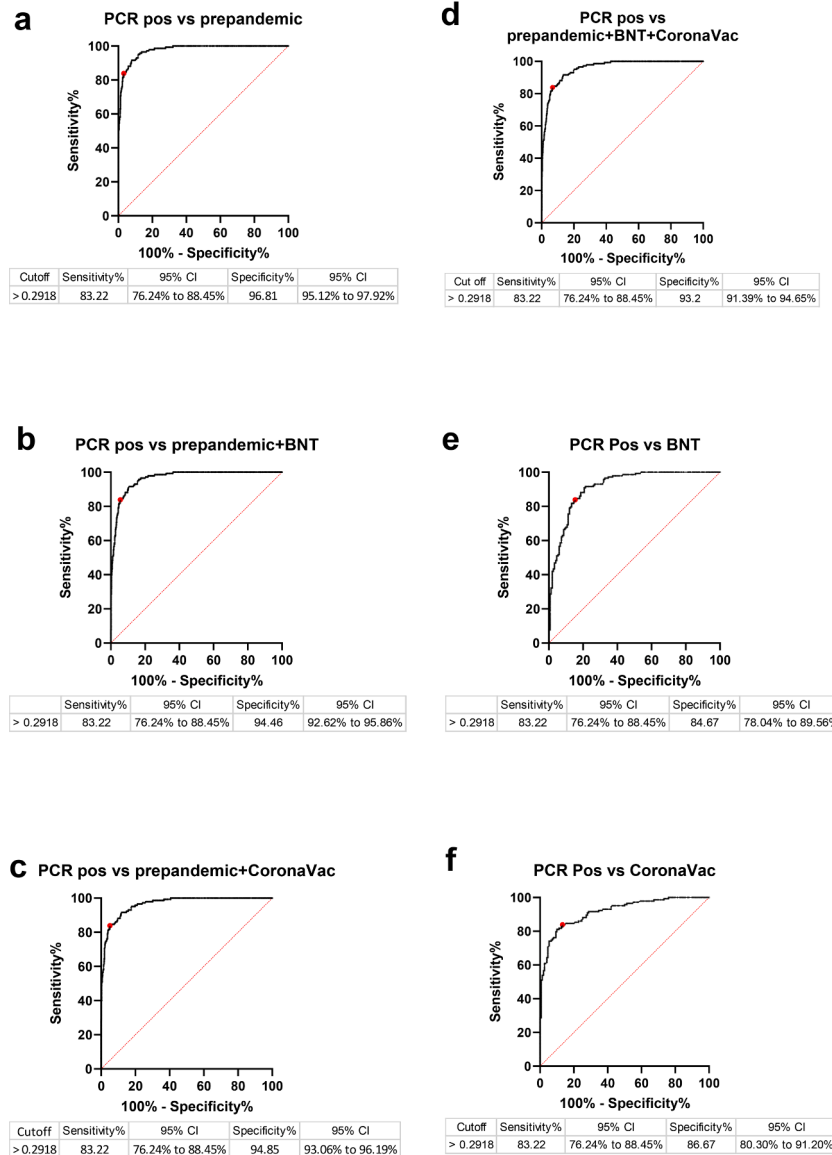
# ORF8



**Fig. 2. Receiver Operating Curves (ROC) of anti-ORF8 IgG ELISA in different cohorts of control sera.** a) 143 convalescent sera from unvaccinated RT-PCR positive individuals (days of illness ranged from 30 to 401 days) tested against 626 prepandemic negative control sera; b) 143 convalescent sera from unvaccinated RT-PCR positive individuals tested against 626 prepandemic negative control sera and 100 sera from individuals who were non infected and received 2 doses of BNT162b2; c) 143 convalescent sera from unvaccinated RT-PCR positive individuals tested against 626 prepandemic negative control sera and 100 sera from individuals who were non infected and received 2 doses of CoronaVac; d) 143 convalescent sera from unvaccinated RT-PCR positive tested against 626 prepandemic negative control sera, 100 sera from individuals who were non infected and received 2 doses of BNT162b2 and 100 sera from individuals who were non infected and received 2 doses of CoronaVac; e) 143 convalescent sera from unvaccinated RT-PCR positive individuals were tested against 100 sera from individuals who were non infected and received 2 doses of BNT162b2; f) 143 convalescent sera from unvaccinated RT-PCR positive individuals tested against 100 sera from individuals who were non infected and received 2 doses of CoronaVac. All sera from non-infected and vaccinated with BNT161b2 or CoronaVac were collected during 2020–2021.

Mori, Ishikawa Prefectural University, Japan [9]. Immunosorb flat 96-well ELISA plates (Nunc MaxiSorp, Thermo Fisher Scientific) were coated overnight with a range of concentrations of N—CTD and ORF8 antigens in PBS with positive and negative control sera to define optimal antigen concentrations. Concentrations of 40 ng/well N—CTD protein or 30 ng/well ORF8 protein in PBS buffer were used in subsequent studies. After antigen coating, plates were blocked with 100 μl of Chonblock blocking buffer (Chondrex Inc, Redmond, US) per well and incubated at room temperature for 2 h. Each serum sample was tested at a dilution of 1:100 in Chonblock blocking buffer in duplicate. They were added and were incubated for 2 h at 37 °C. After extensive washing with PBS

containing 0.2 % Tween 20 in plate washer machine (HydroSpeed, TECAN) and tap drying, horseradish peroxidase (HRP)-conjugated goat anti-human IgG (1:5000, GE Healthcare) was added and incubated for 1 h at 37 °C. The ELISA plates were then washed again with PBS containing 0.2 % Tween 20. Subsequently, 100 μL of HRP substrate (Ncm TMB One; New Cell and Molecular Biotech Co. Ltd, Suzhou, China) was added into each well. After 15 min incubation, the reaction was stopped by adding 50 μL of 2 M H2SO4 solution and analyzed on a microplate reader at 450 nm wavelength.

    **Waning model.** We modelled waning over time in longitudinal anti-N-CTD and anti-ORF8 ELISA OD responses since date of diagnosis using
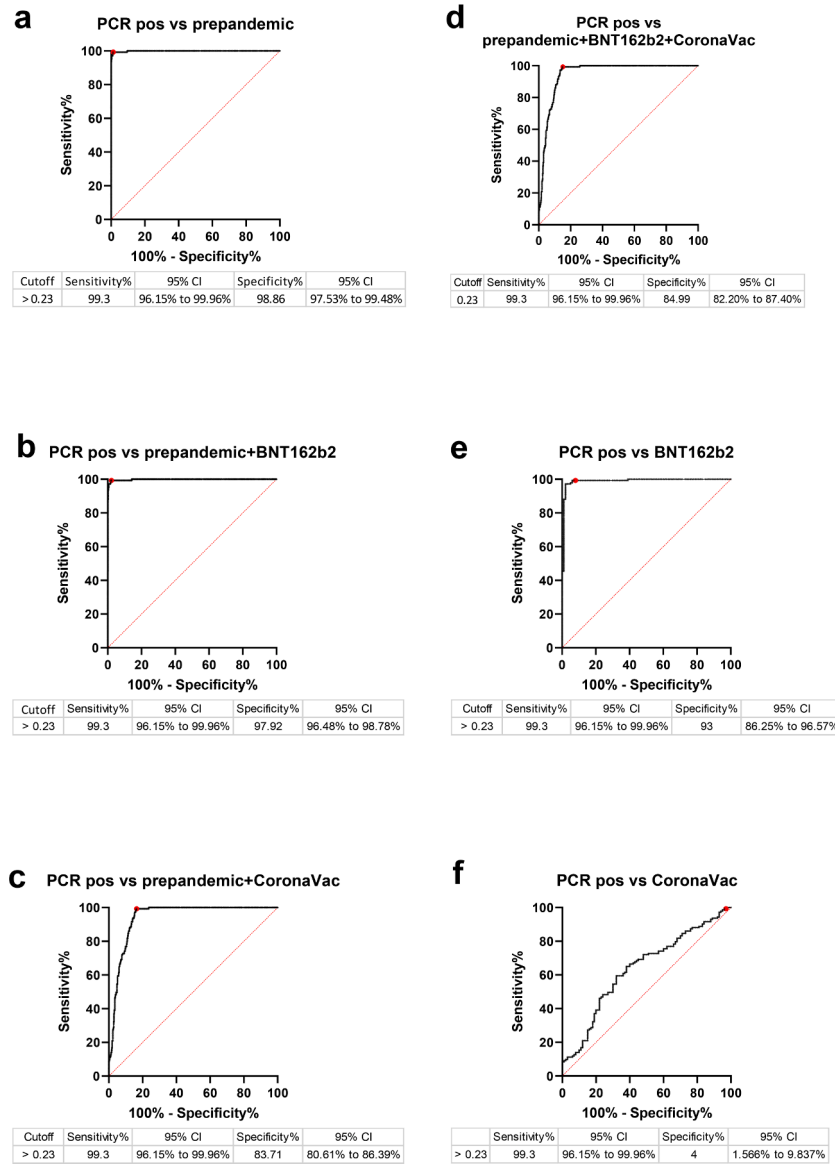
# N-CTD



**Fig. 3. Receiver Operating Curves (ROC) of anti-N—CTD IgG ELISA in different control sera.** a) 143 convalescent sera from unvaccinated RT-PCR positive individuals (days of illness ranged from 30 to 401 days) tested against 626 prepandemic negative control sera; b) 143 convalescent sera from unvaccinated RT-PCR positive individuals tested against 626 prepandemic negative control sera and 100 sera from individuals who were non infected and received 2 doses of BNT162b2; c) 143 convalescent sera from unvaccinated RT-PCR positive individuals tested against 626 prepandemic negative control sera and 100 sera from individuals who were non infected and received 2 doses of CoronaVac; d) 143 convalescent sera from unvaccinated RT-PCR positive tested against 626 prepandemic negative control sera, 100 sera from individuals who were non infected and received 2 doses of BNT162b2 and 100 sera from individuals who were non infected and received 2 doses of CoronaVac; e) 143 convalescent sera from unvaccinated RT-PCR positive individuals were tested against 100 sera from individuals who were non infected and received 2 doses of BNT162b2; f) 143 convalescent sera from unvaccinated RT-PCR positive individuals tested against 100 sera from individuals who were non infected and received 2 doses of CoronaVac. All sera from non-infected and vaccinated with BNT161b2 or CoronaVac were collected during 2020–2021.

a Bayesian Generalised Additive Mixed Model, with individual-level random intercepts and severity group-level smoothers for time since date of COVID-19 diagnosis. All parameters were assigned mildly informative priors equal to the standardised normal distribution. Posterior distributions were estimated via Hamiltonian Markov Chain Monte Carlo using the brms package [10]. All estimated parameters had Gelman-Rubin Statistics R $\approx$1 indicating convergence.

## 3. Results

Anti-ORF8 (Fig. 1a) and anti-N-CTD (Fig. 1b) antibody responses in individuals infected with the original "Wuhan-like" SARS-CoV-2, pre-

pandemic controls and uninfected vaccinees receiving two or three doses of BNT or CoronaVac vaccines are shown. We then derived receiver operating characteristic (ROC) curves for the anti-ORF8 (Fig. 2) and anti-N-CTD (Fig. 3) assays using sera collected from PCR confirmed COVID-19 patients (positive controls), prepandemic sera (negative controls) and vaccination cohorts to define optimal cut-offs for as OD 0.29 for the anti-ORF8 ELISA assay and OD 0.23 for the anti-N-CTD ELISA assay, respectively. At an OD cutoff of 0.29, anti-ORF8 ELISA achieved sensitivity 83.2 %, and specificity 96.8 % vs. prepandemic cohort (Fig. 2a). Specificity, when comparing infected sera vs. prepandemic and BNT vaccinated sera considered together as the uninfected population was 94. 5% (Fig. 2b); 94.9 % when prepandemic and
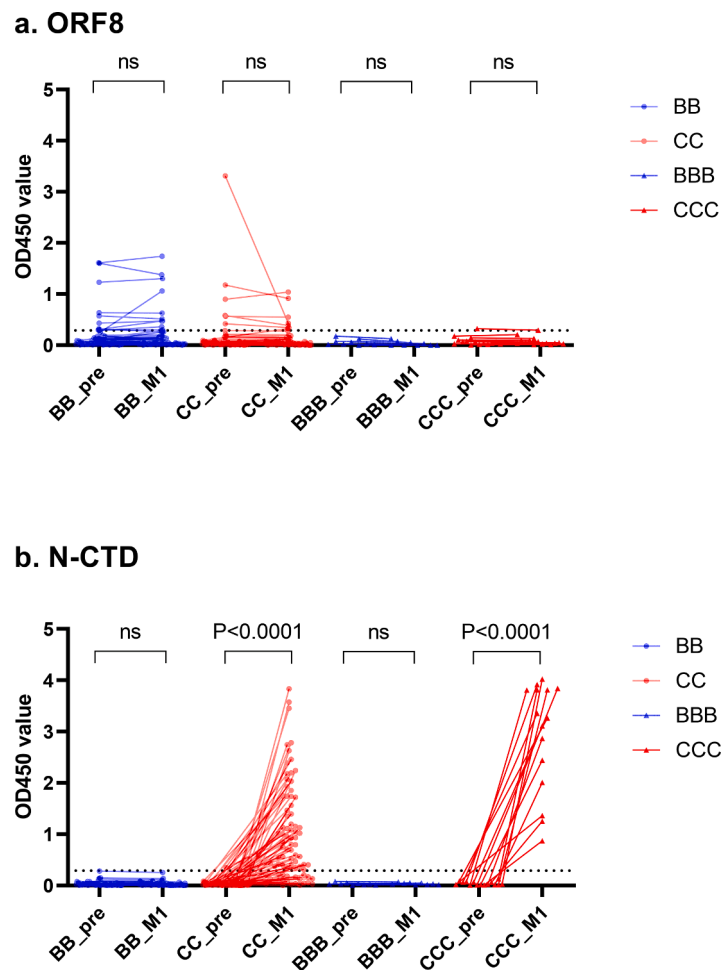
**Fig. 4.** SARS-CoV-2 anti-ORF8 (a) and anti-N—CTD (b) IgG antibody responses in individuals vaccinated with 2 (BB)($N = 100$) or 3 (BBB)($N = 50$) doses of BNT162b2, or 2 (CC)($N = 100$) or 3 (CCC)($N = 50$) doses of CoronaVac. Paired sera were collected before (pre) and 1 month(M1) after vaccination.

CoronaVac vaccinated sera were considered together (Fig. 2c); 93.2 % when prepandemic sera and both vaccinated cohorts were considered together (Fig. 2d); 84.7 % vs. BNT vaccinated sera (Fig. 2e) and 86.7 % vs. CoronaVac vaccinated sera (Fig. 2f). Using cutoff 0.23, anti-N-CTD ELISA achieved sensitivity 99.3 %, and specificity 98.9 % vs. the prepandemic cohort (Fig. 3a). Specificity was 97.9 % vs. prepandemic and BNT vaccinated sera together (Fig. 3b); 83.7 % vs. prepandemic and CoronaVac vaccinated sera together (Fig. 3c); 85.0 % vs. prepandemic and both vaccine cohorts together (Fig. 3d) and 93.0 % vs. BNT vaccinated sera (Fig. 3e). Specificity of anti-N-CTD vs. CoronaVac vaccinated sera was only 4.0 % (Fig. 3f), because CoronaVac vaccine includes multiple viral proteins including the N protein of the virus.

Paired pre- and post-vaccine sera with CoronaVac or BNT vaccines were tested in anti-ORF8 and anti-N-CTD assays. None of the vacinees reported SARS-CoV-2 infections prior to collecting these blood samples. Furthermore, all of them were tested for anti-N-CTD antibody at the time of the first vaccine dose and the study completed on or before October 2021, a period of time when infection attack rate in Hong Kong was very low (<1 % of population). There was no change in anti-ORF8 antibody levels, except for one BNT vaccinated individual and one CoronaVac vaccinated individual (Fig. 4a). On the other hand, anti-N-CTD antibody significantly increased one month after CoronaVac injection, while no significant change was observed in BNT vaccinees (Fig. 4b). anti-ORF8 antibody and anti-N-CTD antibody level waned over time following

natural infection (Fig. 5). ORF8 seroprevalence at 3 months compared to 12 months post infection decreased from 93.6 % to 61.8 % while that for N—CTD decreased from 100 % to 90.8 % over the same time period. Both anti-ORF8 and anti-N-CTD antibodies decreased after 3 months of SARS-CoV-2 infection (Fig. 5). anti-ORF8 and anti-N-CTD antibodies responses following infection were further stratified according to disease severities, as asymptomatic, mild, moderate, severe and critical. Mean OD of anti-ORF8 ELISA dropped by 2.58 (2.51) (asymptomatic), 1.91 (1.25) (mild), 1.52 (0.49) (moderate), 2.21(0.96) (severe) and 2.04 (0.72) (critical) folds(standard deviation) (Fig S1 g), respectively from around half year to one year after confirmation by SARS-CoV-2 real time qPCR (Figure S1). On the other hand, mean OD of anti-N-CTD ELISA dropped by 3.76(1.46), 2.39(1.39), 1.22(1.04), 1.16(0.53) and 1.15 (0.22) folds (standard deviation), respectively (Figure S2). Thus, anti-ORF8 and anti-N-CTD antibodies waned faster in asymptomatic patients compared to symptomatic patients. While asymptomatic patients have weaker responses in the anti-N-CTD ELISA in the early convalescent stage, responses to anti-ORF8 ELISA were more robust (Figs. 6 and 7). Children have a higher response to anti-ORF8 and anti-N-CTD ELISA compared to adults (Figs. 6a-b, and 7a-b).

In the 4423 blood donor samples collected during April to July 2022, a period after a major Omicron BA.2 outbreak in Hong Kong, significantly higher seroprevalence of anti-ORF8 and anti-N-CTD antibody was observed in those self-reporting infection than in non-infected

## a. ORF8
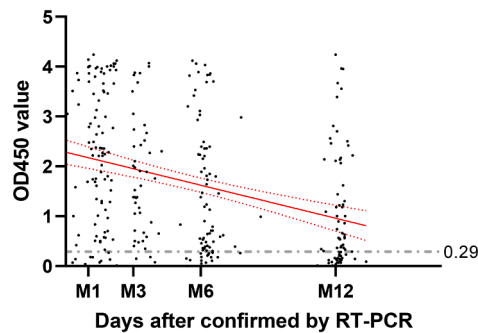


| Days | <21 | 30-60 | 90-120 | 180-210 | 360-400 |
|---|---|---|---|---|---|
| N | 11 | 88 | 47 | 83 | 76 |
| No. of Positive | 9 | 80 | 44 | 67 | 47 |
| % Positive | 81.8% | 90.9% | 93.6% | 80.7% | 61.8% |
| Mean OD (95% C.I.) | 1.93 (0.89-2.97) | 2.27 (1.98-2.55) | 1.76 (1.42-2.11) | 1.49 (1.21-1.78) | 1.01 (0.75-1.27) |

## b. N-CTD
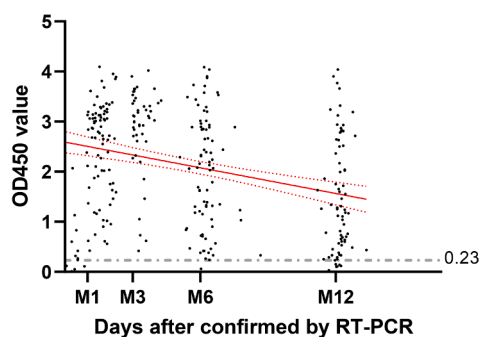


| Days | <21 | 30-60 | 90-120 | 180-210 | 360-400 |
|---|---|---|---|---|---|
| N | 11 | 88 | 47 | 83 | 76 |
| No. of Positive | 8 | 87 | 47 | 82 | 69 |
| % Positive | 72.7% | 98.9% | 100.0% | 98.8% | 90.8% |
| Mean OD (95% C.I.) | 0.55 (0.14-0.96) | 2.5 (2.30-2.70) | 2.88 (2.63-3.12) | 2.02 (1.77-2.27) | 1.48 (1.22-1.73) |

**Fig. 5.** SARS-CoV-2 anti-ORF8 (a) and anti-N—CTD (b) IgG antibody responses in unvaccinated individual convalescent from ancestral SARS-CoV-2 infection at different times post RT-PCR confirmation (ranged from day 2–401) (N = 305).

individuals (Fig. 8). Among unvaccinated individuals without self-reported history of infection, 39.8 % and 17.9 % were seropositive to ORF8 and N—CTD respectively (Fig. 8). Sero-positivity to ORF8 in those with two to four doses of BNT162b2 vaccine without self-reported infection ranged from 9.4 % to 17.5 % while in those with self-reported infection was 70.4 % to 77.8 % (Fig. 8). Sero-positivity in those with two to four doses of CoronaVac in those without self-reported infection was 10.8 % to 11.0 % and with self-reported infection ranged from 76.1 % to 80.4 %. Sero-positivity to N—CTD in those with two to four doses of BNT162b2 vaccine without self-reported infection ranged from 20.9 % to 30.1 % while in those with self-reported infection was 91.8 % to 96.5 %. Sero-positivity in those without self-reported infection with two doses of CoronaVac was 59.5 % and those with three or four doses of CoronaVac was 97.6 %. In those without self-reported infection with two to four doses of CoronaVac ranged from 93.5 % to 97.4 % (Fig. 8). Some of the sero-prevalence observed in those without self-

reported infection was likely to be unsuspected infection but some of the anti-N-CTD antibody response in CoronaVac vaccinated individuals may be attributed the N protein in the vaccine.

## 4. Discussion

RT-PCR confirmed SARS-CoV-2 infection elicited anti-N-CTD and anti-ORF8 antibody in 99.3 % and 83.9 % of patients, respectively. The ROC curves of the N—CTD IgG and ORF8 IgG ELISA with the pre-pandemic sera showed a higher proportion of non-specific reactions in the ORF8 IgG ELISA. To optimize both sensitivity and specificity, the anti-ORF8 IgG ELISA OD cut-off had to be set at a level which led to 3.2 % of positives being false positives. Seasonal coronaviruses do not have an ORF8 protein and thus cross-reaction as a result of seasonal coronavirus infection is unlikely to explain this result. The false-positive rates ranged from 11 %–18 % in different vaccine cohorts with no reported natural infection before 2022, a period with very low infection attack rates (<1 %) in the population due to aggressive COVID-19 containment measures (Peiris – unpublished data) (Fig. 1a). With two exceptions which may be due to non-specific binding, neither vaccine induced change in levels of anti-ORF8 antibody (Fig. 4a). ORF8 is a non-structural protein and is not expected to be present in high concentrations in either vaccine. However, it is known that ORF7a integrates into the viral membrane and since ORF8 has membrane glycoprotein domains [11], it is possible that it may also integrate into the virion at low levels during viral assembly. This may explain the higher ORF8 sero-positivity observed in a proportion of CoronaVac vaccine, which is a whole-virus inactivated vaccine. While this reduced the specificity of the anti-ORF8 ELISA in discriminating natural infection from vaccination irrespective of vaccine type, it still can be used to assess infection attack rates at population level, provided this imperfect specificity is recognized; less than 9.4% for infection naïve BBB/B and 11.0% for naïve CCC/C were anti-ORF8 IgG positive.

SARS-CoV-2 infection and vaccination with CoronaVac elicit antibodies to N protein (Figs. 1b and 4b). In contrast, only 1.1 % of pre-pandemic controls and 4 % of those with two doses of BNT had detectable anti-N-CTD antibodies and this can be used to differentiate natural infection from BNT vaccination. Inactivated whole virus vaccine CoronaVac elicited N antibody in 94–98 % of vaccinees, and thus the anti-N-CTD ELISA was not suitable for differentiating natural infection from CoronaVac vaccination (Fig. 1b). Applying a cutoff 0.23 to CoronaVac vaccinees, the anti-N-CTD has negligible specificity.

Asymptomatic patients have lower anti-ORF8 and anti-N antibodies at around 6 months after infection compared to symptomatic patients (Figs. 6 and 7). Anti-ORF8 antibody level was high in early stage of infection irrespective of symptom severity while anti-N antibody levels were higher in those with increased disease severity. Thus, anti-ORF8 antibody may be an option for early diagnosis of infection, irrespective of symptom severity. Asymptomatic or mildly diseased children had higher anti-ORF8 antibody level compared to adults. This was also noted in our previous study [3]. anti-ORF8 and anti-N antibody kinetics differed with disease severities. Other studies have also shown the impact of milder disease on reduced S antibody responses in general [12–15].

The utility of anti-ORF8 and anti-N-CTD serology was examined in blood donor sera collected in 2022 (after the first major peak of SARS-CoV-2 infections by Omicron BA.2 in Hong Kong). As expected, higher anti-ORF8 (72.4 %) and anti-N (96.6 %) antibody sero-prevalence was noted in individuals who self-reported history of infection than those who did not, in unvaccinated and vaccinated cohorts. However, higher ORF8 sero-prevalence was also observed in unvaccinated (39.3 %) or two dose-vaccinated (10.8 %−17.5 %) individuals who believed they had no past COVID-19 infection, compared to pre-pandemic controls
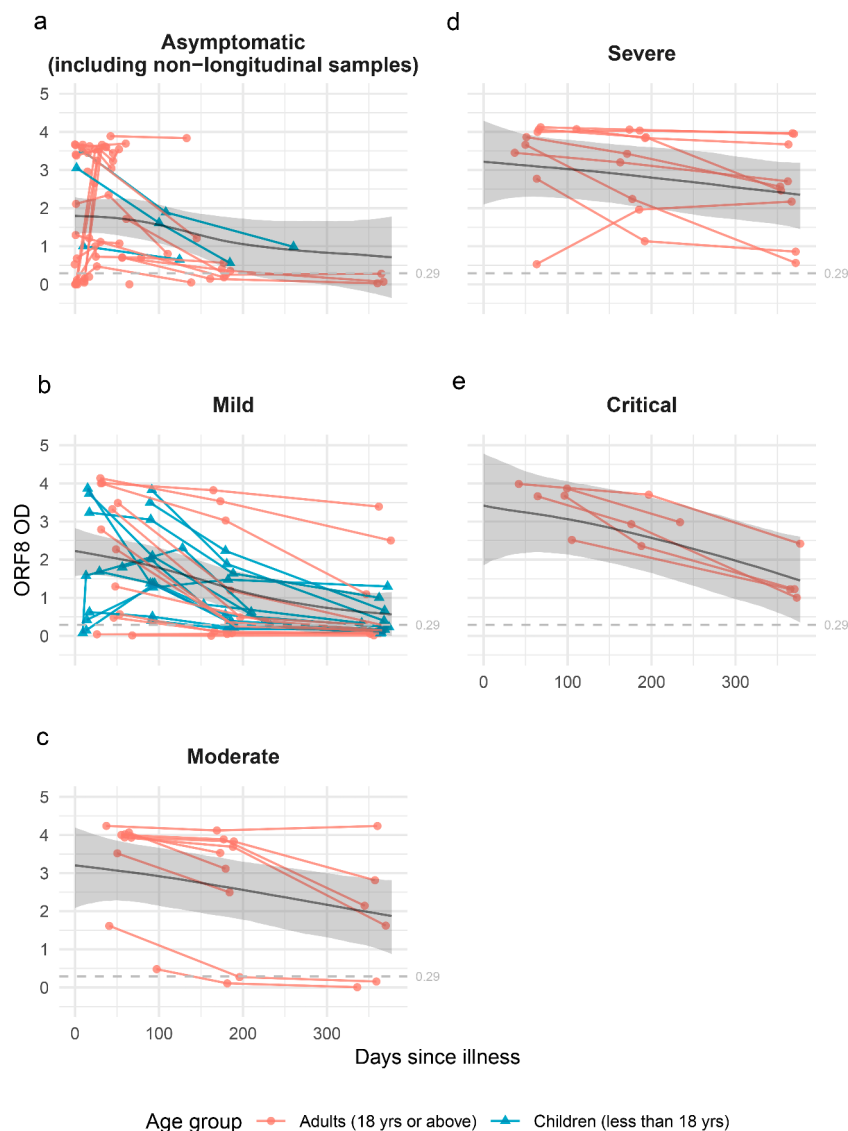
**Fig. 6.** Anti ORF8 IgG ELISA OD responses after RT-PCR confirmation of infection stratified by severity ($N = 215$). Dots represent individual data points, solid lines represent the posterior means of a Generalised Additive Mixed Model fitted against the data, and shaded areas represent the 95% credible intervals. Small random noises were added to individual data points for better presentation. Dotted line represents the OD cutoff denoting seropositivity (0.29). a) asymptomatic patients including non-longitudinal samples. b) symptomatic mild disease. c) moderate disease. d) severe disease. e) critically ill patients.

(3.2 %), suggesting unsuspected infection in some of these blood donors. A Study of Meta-analysis on 350 studies showed asymptomatic infection rate 35.1 % (95 % CI: 30.7 to 39.9 %) [16]. Meta-analysis from another study covering 29,776,306 individuals also demonstrated high percentage of asymptomatic infections in confirmed population 40.5 % (95 %CI: 33.5 %−47.5 %) [17]. N—CTD sero-prevalence in those receiving three or more doses of BNT (29.3 %) was significantly lower than in those who were unvaccinated (57.9 %) (Chi square 21.7; $p<0.00001$) or in those who only had two doses of BNT vaccine (69 %) (Chi square 452; $p<0.00001$). Similarly, ORF8 seroprevalence in those receiving three or more doses of CoronaVac (23.3 %) was significantly lower than those who were unvaccinated (56.1 %)(Chi Square 293; $p<0.00001$) or had two doses (49.4 %)(Chi Square 43.6; $p<0.00001$) of CoronaVac vaccine. This implied that better protection from infection (symptomatic or asymptomatic) was given by booster doses of either vaccine. A limitation of this study was that we have not assessed whether anti-ORF8 antibody responses will also be elicited by more recent SARS-CoV-2 virus variants such as the Omicron BA.5 descendent lineages which

have a CT transition in the transcription regulatory sequence (TRS) located upstream of ORF8 gene. This could affect the expression of ORF8 protein and so as the sensitivity of anti-ORF8 ELISA. The effect of CT transition in TRS region on ORF8 expression and antibody responses should be further studied.

**5. Conclusions**

We optimized and evaluated anti-N-CTD and anti-ORF8 IgG ELISA assays for differentiation of infection from vaccination. While the anti-N-CTD IgG ELISA effectively discriminated infection from vaccination in BNT vaccinated individuals, it was not useful in those vaccinated with CoronaVac, because this whole virus inactivated vaccine strongly elicited antibodies to N—CTD. In contrast, IgG antibody to ORF8 was able to differentiate vaccination from infection in those vaccinated with either vaccine, through mid-2022, prior to the emergence of BA.5 variants.
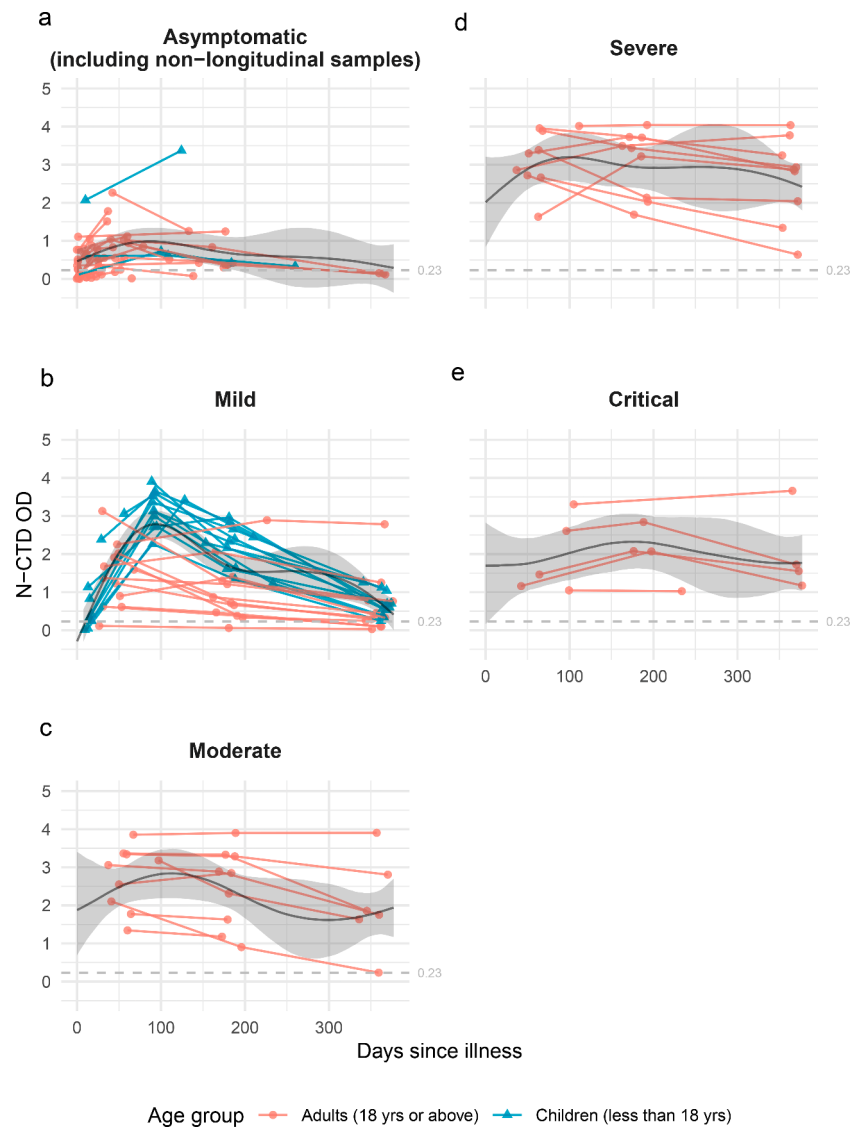
**Fig. 7.** Anti N—CTD ELISA OD responses after RT-PCR confirmation of infection stratified by severity (*N* = 215). Dots represent individual data points, solid lines represent the posterior means of a Generalised Additive Mixed Model fitted against the data, and shaded areas represent the 95% credible intervals. Small random noises were added to individual data points for better presentation. Dotted line represents the OD cutoff denoting seropositivity (0.23). a) asymptomatic patients including non-longitudinal samples. b) symptomatic mild disease. c) moderate disease. d) severe disease. e) critically ill patients.

## Author contributions statement

MP, SAV, JTW, JJL and SMSC planned and coordinated the study, MM, CW and GKA provided ORF8 and N—CTD antigens, SMSC and MP wrote first draft of manuscript, SMSC, JJL, LCHT, AH, NK, SC, RWKW, JKMY, ZYHC and LLMP coordinated the laboratory testing and laboratory data analysis, JJL, KL, CKL, KY and DSCH coordinated clinical studies and data management. All authors commented on the manuscript draft and agreed with its submission.

**Supplementary Figure S1.** Anti-ORF8 antibody waning in RT-PCR confirmed patients who were not vaccinated stratified by symptom severity. Sequential samples from same individual at different time points were connected by lines. Red dots indicate samples from children. a) asymptomatic infections with longitudinal sera. b) asymptomatic infections with both longitudinal and single sera. c) mild disease. d) moderate disease. e) severe disease. f) critically ill. g) Mean ODs stratified by severity from around 6–7 months to 12–13 months after SARS-CoV-2 RT-PCR confirmation.

**Supplementary Figure S2.** Anti-N—CTD antibody waning in RT-PCR confirmed patients who were not vaccinated stratified by symptom severity. Samples from same individual in different time points were connected by lines. Red dots indicate samples from children. a) asymptomatic infections with longitudinal sera. b) asymptomatic patients with both longitudinal and single sera. c) mild disease. d) moderate disease. e) severe disease. f) critically ill. g) Mean ODs stratified by severity from around 6–7 months to 12–13 months after SARS-CoV-2 RT-PCR confirmation.

**Supplementary Table 1.** Demographics of the sample cohorts used in the study.

## Declaration of Competing Interest

AH, NK, LLMP, MP and SAV have filed an IDF (US 63/016,898) for the use of ORF8 and ORF3d as diagnostics of SARS-CoV-2 infection. MM produced ORF8 by patent process based on US Patents 8507,220 and 8586,826. Other authors have no declaration of interest.
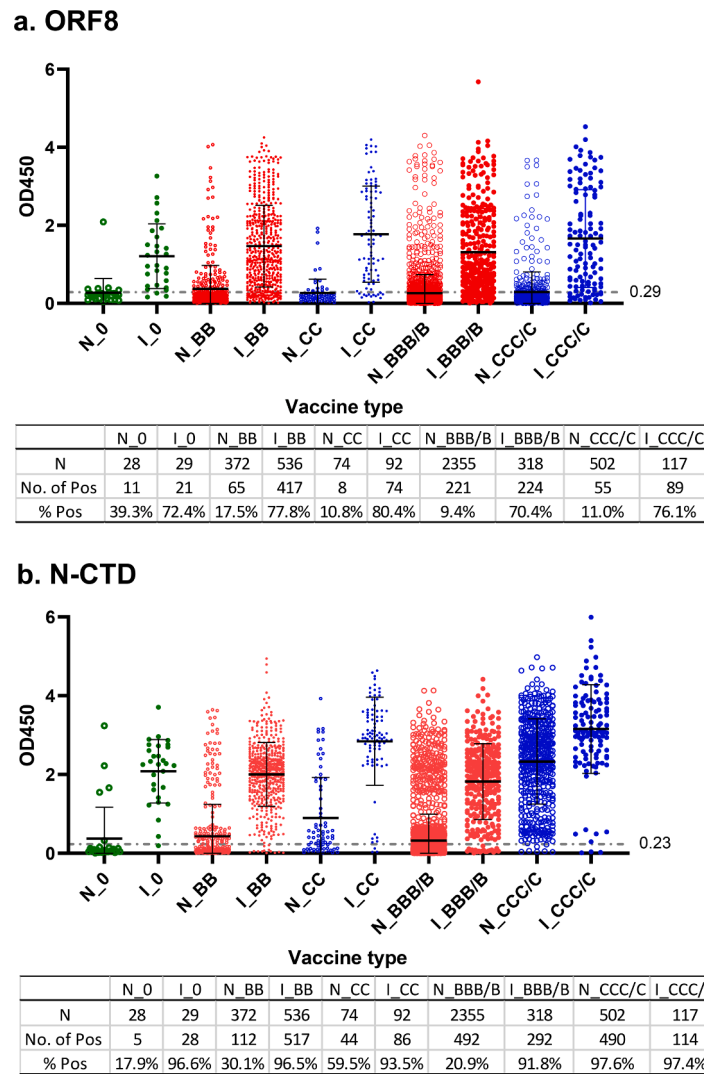
## a. ORF8



| | N_0 | I_0 | N_BB | I_BB | N_CC | I_CC | N_BBB/B | I_BBB/B | N_CCC/C | I_CCC/C |
|---|---|---|---|---|---|---|---|---|---|---|
| N | 28 | 29 | 372 | 536 | 74 | 92 | 2355 | 318 | 502 | 117 |
| No. of Pos | 11 | 21 | 65 | 417 | 8 | 74 | 221 | 224 | 55 | 89 |
| % Pos | 39.3% | 72.4% | 17.5% | 77.8% | 10.8% | 80.4% | 9.4% | 70.4% | 11.0% | 76.1% |

## b. N-CTD



| | N_0 | I_0 | N_BB | I_BB | N_CC | I_CC | N_BBB/B | I_BBB/B | N_CCC/C | I_CCC/C |
|---|---|---|---|---|---|---|---|---|---|---|
| N | 28 | 29 | 372 | 536 | 74 | 92 | 2355 | 318 | 502 | 117 |
| No. of Pos | 5 | 28 | 112 | 517 | 44 | 86 | 492 | 292 | 490 | 114 |
| % Pos | 17.9% | 96.6% | 30.1% | 96.5% | 59.5% | 93.5% | 20.9% | 91.8% | 97.6% | 97.4% |

**Fig. 8. SARS-CoV-2 anti-ORF8 and anti-N—CTD IgG antibody responses in healthy blood donors (*N* = 4423) with known vaccination history and self-reported infection history.** Vaccination history denoted as non-vaccinated (0)(green); 2 doses of BNT162b2 (BB)(red), 2 doses of CoronaVac (CC)(blue), 3 or 4 doses of BNT162b2(BBB/B)(red) and 3 or 4 doses of CoronaVac (CCC/C)(blue). Self-reported infection history denoted as: Non-infected (N) (open dots) or infected (I) (solid dots). Sera were collected during the period 28 April 2022 and 30 July 2022, soon after a major wave of Omicron BA.2 infection.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2023.105621.

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