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An automated chemiluminescent immunoassay (CLIA) detects SARS-CoV-2 neutralizing antibody levels in COVID-19 patients and vaccinees

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ABSTRACT

Objectives: A specific and sensitive automated chemiluminescent immunoassay (CLIA) was developed to detect neutralizing antibody (NAb) levels. This assay can be used for the diagnosis of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection, treatment and vaccine evaluation.

Methods: The SARS-CoV-2 receptor-binding domain (RBD) and a stabilized version of the spike ectodomain as antigens were detected by CLIA. Sera NAb titers and concentrations from 860 SARS-CoV-2 vaccinees, 232 SARS-CoV-2 convalescent patients and 675 healthy individuals were tested by microneutralization test (MNT) and CLIA, respectively. Mathematical models were established to evaluate the relationship between two variables in different groups.

Conclusions: With the RBD-based CLIA protocol, CLIA can be used to replace MNT to test SARS-CoV-2 NAb. Vaccine effectiveness, protectiveness and durability can be evaluated effectively by mathematical models. It is

Results: Analysing the relationship between NAb titers and concentrations, R^2 for the decision-making tree was 0.870 and that of progressive linear fitting was 0.821. The receiver operating characteristic curve indicated specificity of 78.1%, sensitivity of 87.4%, cut-off value of 6.43 AU/mL and borderline range of 5.79–7.07 AU/mL for CLIA. Three-quarters (75.4%) of vaccinees were found to be NAb positive, and 5.35% vaccinees had NAb protective capability. The half-life of NAb in vaccinees was 10–11 weeks. for vaccinees to take a NAb assay periodically.

Introduction

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the causative agent for coronavirus disease 2019 (COVID-19), has resulted in a devastating global pandemic. Nucleic acid tests of SARS-CoV-2 represent the gold standard of clinical diagnostic methods for COVID-19 detection (World Health Organization, 2021). Serological assays can play a supplementary but indispensable role in: (1) diagnosis of suspected cases with negative viral RNA tests or previous COVID-19 infection; (2) monitoring immune responses to assess the course, degree and durability of immunity; (3) identifying potential convalescent plasma donors; (4) therapeutic antibody development and evaluation; and (5) vaccine development and evaluation (Liu and Rusling, 2021). Neutralizing antibody (NAb), a pivotal immune product for protection and treatment against SARS-CoV-2, is of particular interest to medical researchers. However, the internal mechanism between NAb titers and its protective and therapeutic functions remains unknown. The virus neutralizing assay, the gold standard test for NAb titers, has numerous restrictions, including complicated laboratory

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CLIA, chemiluminescent immunoassay; MNT, microneutralization test; NAb, Neutralizing antibody.

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requirements, long time period and rigorous operation standards (Gauger and Vincent, 2020). Most importantly, it has to be performed under biosafety level 3 (BSL-3) conditions. These restrictions make it more difficult to obtain data on NAb titers, and therefore make it difficult to apply in clinical routine inspection. Since the development of SARS-CoV-2 vaccines, the evaluation of vaccine protection has become crucial. The aim of this study was to develop a convenient method to evaluate sustained protective effects in vaccinees, and therapeutic effects in convalescent patients.

Several studies have reported the functions of NAb in convalescent plasma treatment. Wu et al. (2020) found that NAb titers were low in all patients for the first 10 days after symptom onset, and then increased 10–15 days after symptom onset, remaining stable thereafter. Thirty percent of convalescent patients had very low NAb titers. Ye et al. (2020) and Shen et al. (2020) reported the successful use of convalescent plasma in the treatment of patients with SARS-CoV-2 infection. With the spread of the epidemic, more and more studies have explored the mechanism and application of NAb.

This study aimed to develop an automated chemiluminescent immunoassay (CLIA) to measure NAb concentrations, and to use the microneutralization test (MNT) to measure NAb titers in convalescent patients and healthy vaccinees (Manenti et al., 2020). Mathematical models were built to explain the relationship between NAb titers and concentrations, and to compare NAb titer attribution between vaccinees and convalescent patients. The study results may provide significant information regarding vaccine effectiveness and protection in the plasma of convalescent patients.

Methods

Basic information

This retrospective study included 1767 participants: 232 convalescent patients, 675 healthy people, and 860 vaccinees who had received two doses of SARS-CoV-2 vaccine. Data for the convalescent group and some of the data for the vaccine group were collected between 1 February 2020 and 1 April 2021 from hospitals in China. Data for the healthy group and the rest of the data for the vaccine group were collected between 1 January 2020 and 1 April 2021 through volunteer recruitment.

All convalescent patients and vaccinees signed informed consent forms. The convalescent patients had been diagnosed with COVID-19 and their plasma was collected after recovery. The vaccinees had received two doses of SARS-CoV-2 vaccine, and their plasma were collected dozens of days after vaccination.

In order to establish the cut-off value and borderline range for the NAb CLIA test, 675 SARS-CoV-2-negative serum samples collected before the current SARS-CoV-2 pandemic were measured. The mean NAb arbitrary unit plus six standard deviations was used to define the cut-off value, and the borderline range was ±10% cut-off (Peterhoff et al., 2021). The cut-off value and borderline range were validated by receiver operating characteristic (ROC) curves. Therefore, the NAb baselines for SARS-CoV-2-negative subjects, vaccinated subjects and symptom-onset subjects could be determined.

Microneutralization test

Virus and cells

SARS-CoV-2 virus 20SF014/vero-E6/3 was isolated from a patient with SARS-CoV-2 in Shenzhen in February 2020. SARS-CoV-2 virus 20SF18530/vero-E6/3 was isolated from a patient with SARS-CoV-2 in South Africa in December 2020. Stock virus was provided and propagated at a BSL-3 facility at China Guangdong Provincial

Centre for Disease Control and Prevention. Stock virus was amplified by growth in Vero E6 cells. The infected cells were incubated in Dulbecco's Modified Eagle Medium with L-glutamine (GIBCO, Life Technologies Corporation, Eugene, OR, USA), containing 10% heat-inactivated fetal bovine serum (GIBCO) in a humidified atmosphere at 37 °C with 5% carbon dioxide.

Virus and titration

The virus stock was titrated by 10-fold serial dilution. A cytopathic effect (CPE) was observed by microscope 7 days after inoculation. The endpoint dilution leading to CPE in 50% of inoculated wells was designated as one 50% tissue culture infecting dose (TCID50) (Reed and Muench, 1938). Serial four-fold dilutions of heat-inactivated sera were made. Serum dilutions of 240 μL were mixed with equal volumes of 120 μL TCID50 of SARS-CoV-2 as indicated. After 2 h of incubation, 100 μL of virus–serum mixture was added in quadruplicate to Vero E6 cell mono layers in 96-well microtiter plates. Next, an additional 100 μL of culture medium was added to each well and the plates were incubated for 7 days. A serial 10-fold dilution of the virus – 100 μL TCID50/50 μL, 10 μL TCID50/50 μL, 1 μL TCID50/50 μL and 0.1 TCID50/50 μL – was made as a control and loaded into eight wells of 96-well microtiter plates with an additional 100 μL of culture medium.

CPE read out

CPE was read 7 days after infection. The highest serum dilution that completely protected the cells from CPE in half of the wells was taken as the NAb titer. More than 4 times dilution could protect the cells from virus infection, and was set as the cut-off of the positive.

Automated chemiluminescent immunoassay

An automated CLIA method was developed to detect SAR-CoV-2 NAb concentrations using an Immu F6 Automatic Chemiluminescent Immunoassay Analyzer (Shenzhen Medcaptain Medical Technology Co., Ltd, Shenzen, China). The assay took the format of a competitive CLIA. The RBD domain in the spike (S) protein of SARS-CoV-2 was combined with magnetic microparticles. Upon mixing the sample with reaction diluent, NAb combined with the RBD domain in the S protein. The remaining uncombined RBD domain in the S protein of SAR-CoV-2 was collected and reacted with angiotensin-converting enzyme-2 (ACE2), labelled by acridinium ester. NAb concentration was inversely proportional to the chemiluminescence data. The reagents were manufactured by Shenzhen Medcaptain Medical Technology Co., Ltd. Calibration and control testing, and test operation procedures were performed in accordance with the manufacturer's instructions.

Mathematical model

NAb titers and concentrations were non-linearly related. There is a strong need for appropriate methods for parameter simulation to identify the relationship between NAb titers and concentrations (Parmar et al., 2015). Mathematical models were established according to Figure S1 (see online supplementary material).

Statistical analysis

Statistical analysis was conducted using MedCalc Version 18.2 and Orange Data Mining Version 2.8.2. Medians were used in general descriptions. Different statistical methods were used as needed.

Table 1

Characteristics of study subjects.

Characteristics	Vaccinees (860) Experiment (760)	Validation (100)	Convalescent patients (232) Experiment (134)	Validation (39)	Observation (59)	Healthy people (675)
Sex Male	362	26	39	22	28	348
Female	398	74	95	17	31	327
Age Male	38.30±10.08	35.16±8.83	47.16±16.66	35.97±9.54	45.28±9.35	41.16±18.24
(years) Female	38.51±10.00	35.09±9.41	47.49±15.97	40.15±11.65	43.12±9.26	42.20±19.39

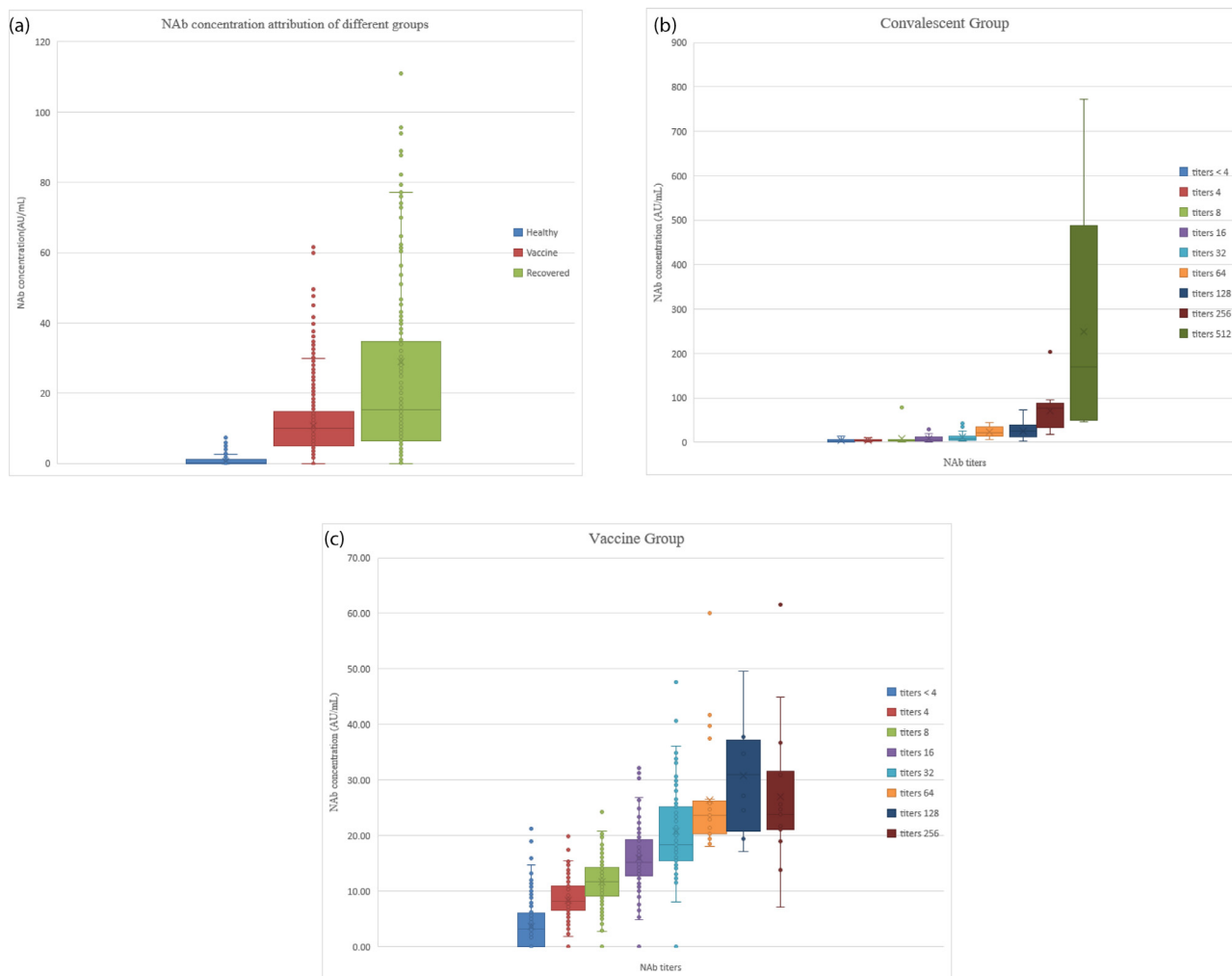Age is expressed as mean±standard deviation.

Figure 1. **(a)** Neutralizing antibody (NAb) concentration attribution in different groups.
(b) Box plot of NAb titers and concentrations in the convalescent group.
(c) Box plot of NAb titers and concentrations in the vaccine group.

Results

Clinical characteristics

In total, 232 convalescent patients, 860 vaccinees and 675 healthy people were included in this study (Table 1). Fifty-nine participants in the convalescent group were used to observe changes in NAb from symptom onset to 6 months later.

Outcome of models

Concentration distribution of different titers in different groups

The distribution of NAb concentrations differed significantly between the healthy group, the vaccine group and the convalescent group ($P<0.01$) (Figure 1a). NAb concentrations and titers were strongly positively related in the vaccine group and the convalescent group ($P<0.001$) (Figure 1b,c).

Cut-off value and borderline range

According to Peterhoff et al. (2021), the cut-off Nab concentration for healthy people was 6.277 AU/mL and the borderline range was 5.73–7.22 AU/mL. ROC curves for NAb concentrations for vaccinees and convalescent patients were used to validate this. The ROC curve showed that the cut-off value was 6.43 AU/mL and the borderline range was 5.79–7.07 AU/mL (Table 2 and Figure 2).

Table 2

Cut-off value and borderline range (AU/mL).

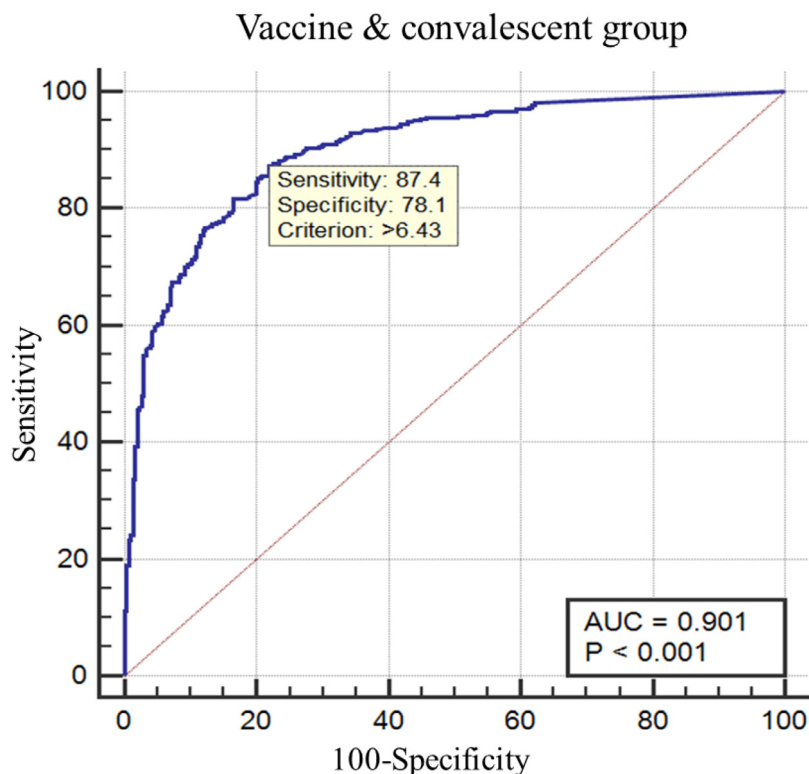| Methods	Cut-off value	Upper line	Bottom line
Vaccinees and convalescent patients (<1:4 titers) (1033)	6.43	7.07	5.79
Healthy people (675)	6.277	7.22	5.73

Figure 2. Receiver operating characteristic curve of neutralizing antibody concentrations in the vaccine and convalescent groups.

Table 3

Outcomes of different models.

Models	Absolute error	Correlation	R^2	R^2 (optimize)
Progressive linear fitting	**0.795**	**0.801**	**0.657**	**0.821**
Deep learning	0.864	0.782	0.596	0.631
Decision tree	**0.788**	**0.795**	**0.617**	**0.870**
Random forest	0.901	0.783	0.568	0.568
Gradient boosted trees	0.919	0.712	0.572	0.619
Support vector machine	4.521	0.564	-10.683	0.556

Absolute error represents the difference between model predicted value and true value. Correlation represents the inner relationship between neutralizing antibody (NAb) titers and concentrations. R^2 (optimize) represents the goodness of model fitting after parameter tuning. The progressive linear fitting and decision tree models performed best in the model fitting.

Mathematical model

Mathematical model selection

Several methods were applied to find mathematical relationships between NAb titers and concentrations. Decision-making tree and progressive linear fitting models performed better than other methods (Table 3). The decision-making tree model was propitious to describe an interval function, while progressive linear fitting was adept for describing a numerical function.

Decision-making tree model

The outcome using decision-making tree modelling is shown in Figure 3.

Progressive linear fitting model

The outcome using regression modelling is shown in Figure 4.

Outcome of model verification

Verification of cut-off value and borderline range

Using two different methods to determine the cut-off value and borderline range, as described above, the specificity of this assay was determined by measuring 675 independent SARS-CoV-2-negative serum samples. The false-positive rate was two out of 675 sera, corresponding to specificity of 99.7%.

Verification of two models

Both the progressive linear fitting model and the decision-making tree model showed the same results for verification. There
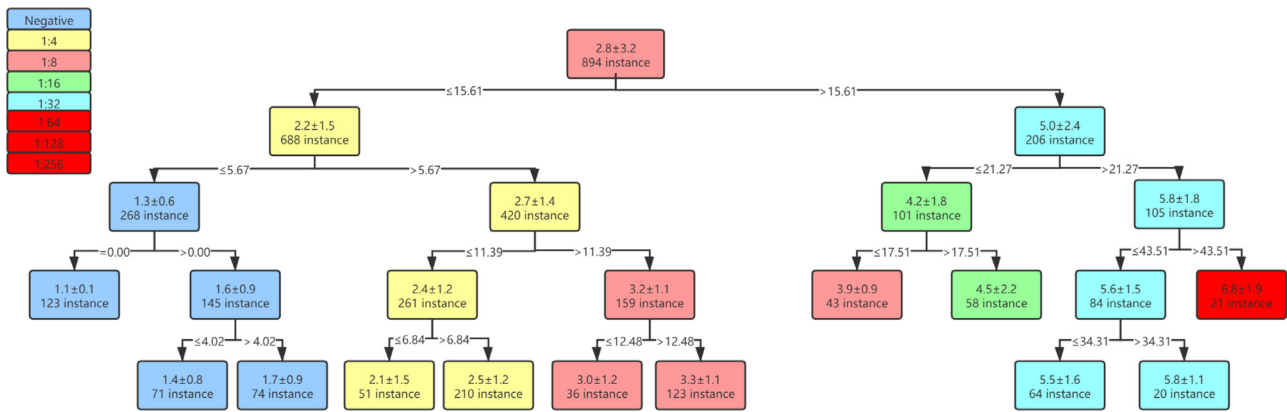
Figure 3. Decision tree model of neutralizing antibody (NAb) titers and concentrations. The values in the square lattice represent NAb concentration interval values and number of cases, respectively (e.g. the top square lattice illustrates the NAb concentrations of 894 individuals (interval value: 2.8±3.2). The values on the transverse lines represent NAb titers. They are also regarded as breakpoints to separate individuals with different NAb concentrations. The breakpoints were determined using the decision tree model. In order to differentiate titer grades, the square lattices were labelled by colours (e.g. the blue square lattice represents titers <1:4, the yellow square lattice represents titres ≥1:4<1:8 etc).
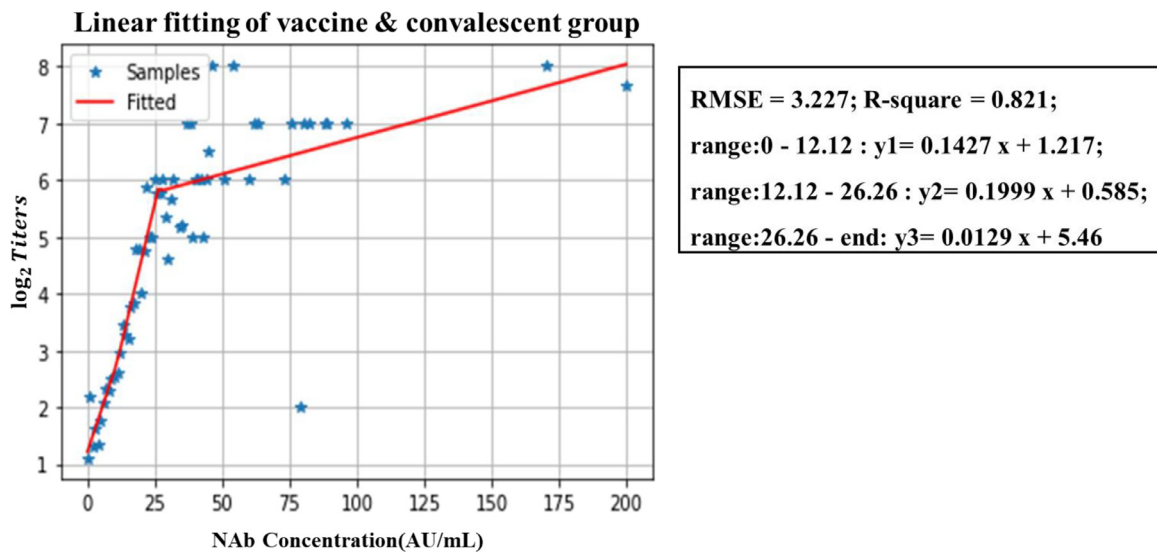

Figure 4. A combined model of the regression model of the vaccine and convalescent groups.

were 95.7% points in the 95% limits of agreement. Two models had good consistency with the gold standard (Figure 5).

Trend of NAb in different groups

In the vaccine group, NAb concentrations increased after the second vaccine injection, and peaked 10–20 days later. Approximately 75.4% of NAb vaccinees could be detected individually, and the proportion decreased to 36.8% after 60 days. The half-life of NAb in vaccinees was 10–11 weeks (Figures 6 and 7).

NAb distribution of vaccine group by age and gender

In the vaccine group, both NAb titers and concentrations had the same distribution tendencies regardless of age or gender. The NAb concentrations in subjects aged 19–50 years were higher than those in other age groups. This implies that the immune response in adults was stronger than that in elderly people. There were significant differences between males and females in the same age group ($P<0.01$). The immune response was stronger in females than in males (Table 4).

NAb protection in convalescent group

In the convalescent group, NAb increased rapidly several days after symptom onset, and declined over time. The half-life of NAb in the convalescent group was 5.7947 months. When the NAb concentration was <64 AU/mL, the convalescent patients (97% confidence interval) did not have protective capability (titer<1:160). The age (95% confidence interval) of convalescent patients whose NAb had protective capability was 30–75 years (Figure 8).

The NAb concentrations of 59 convalescent patients were tested 10 days after symptom onset and 180 days after symptom onset. The average NAb concentration decreased to 53% approximately 6 months after symptom onset (Figure 9).

Discussion

The worldwide spread of SARS-CoV-2 has deeply and rapidly affected healthcare systems. Serological tests are important to understand the antibody responses mounted upon SARS-CoV-2 infection and vaccination. People are eager to know whether or not infected individuals mount a robust antibody response to SARS-CoV-2 infection, and if the vaccine plays an immunoprotective role in avoiding re-infection. The duration of the immune response and the dynamic nature of antibody titers linked to severe, mild and
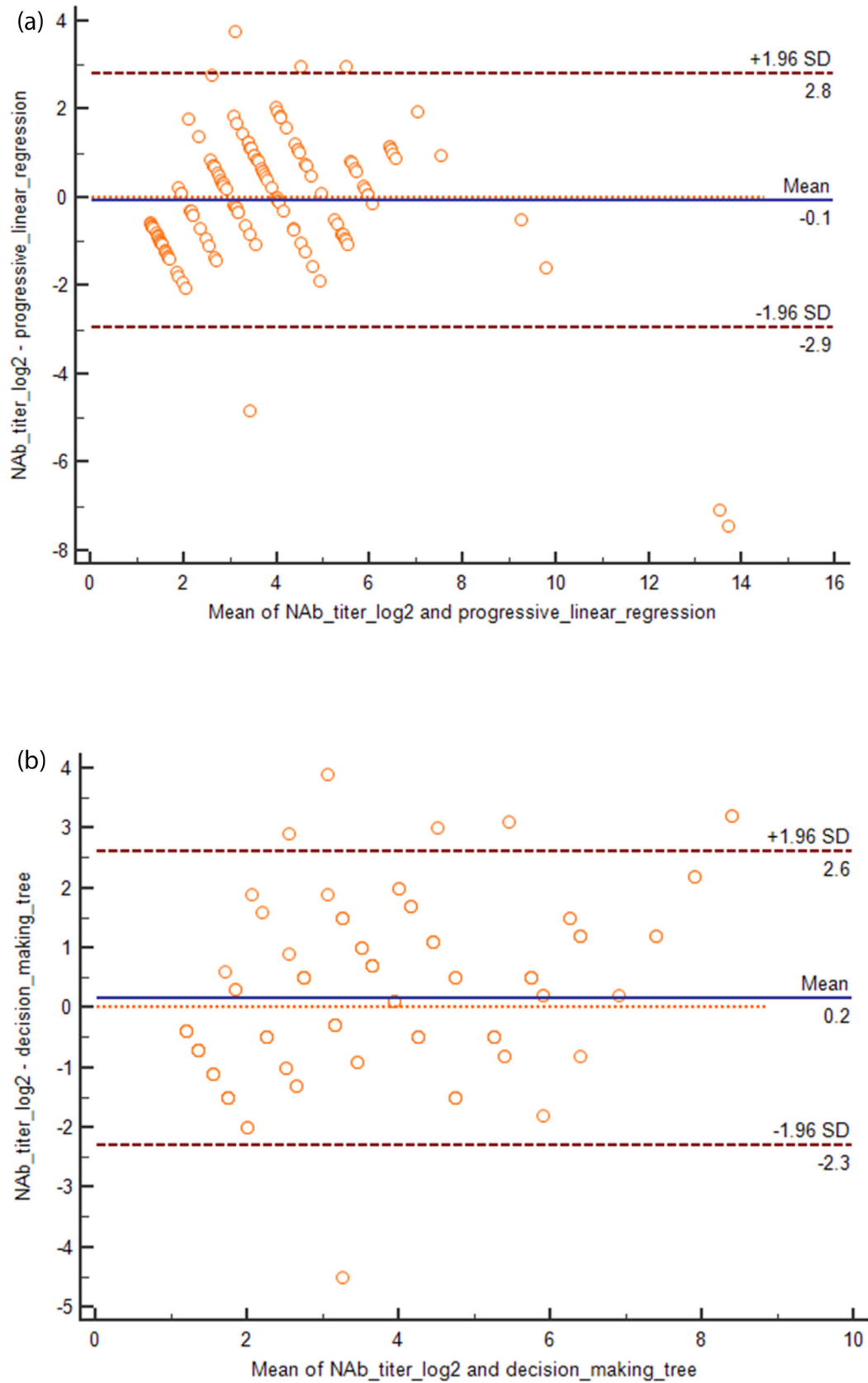
Figure 5. Bland–Altman graph of the progressive linear fitting model and the decision-making tree model. NAb, neutralizing antibody; SD, standard deviation.
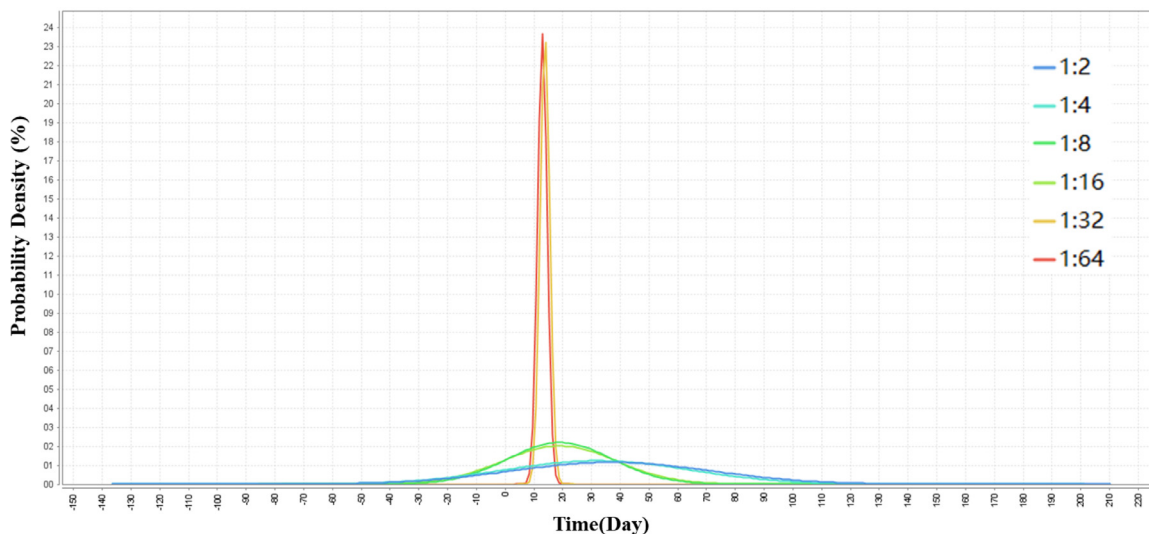
Figure 6. Probability distribution of titers and time in vaccine group.
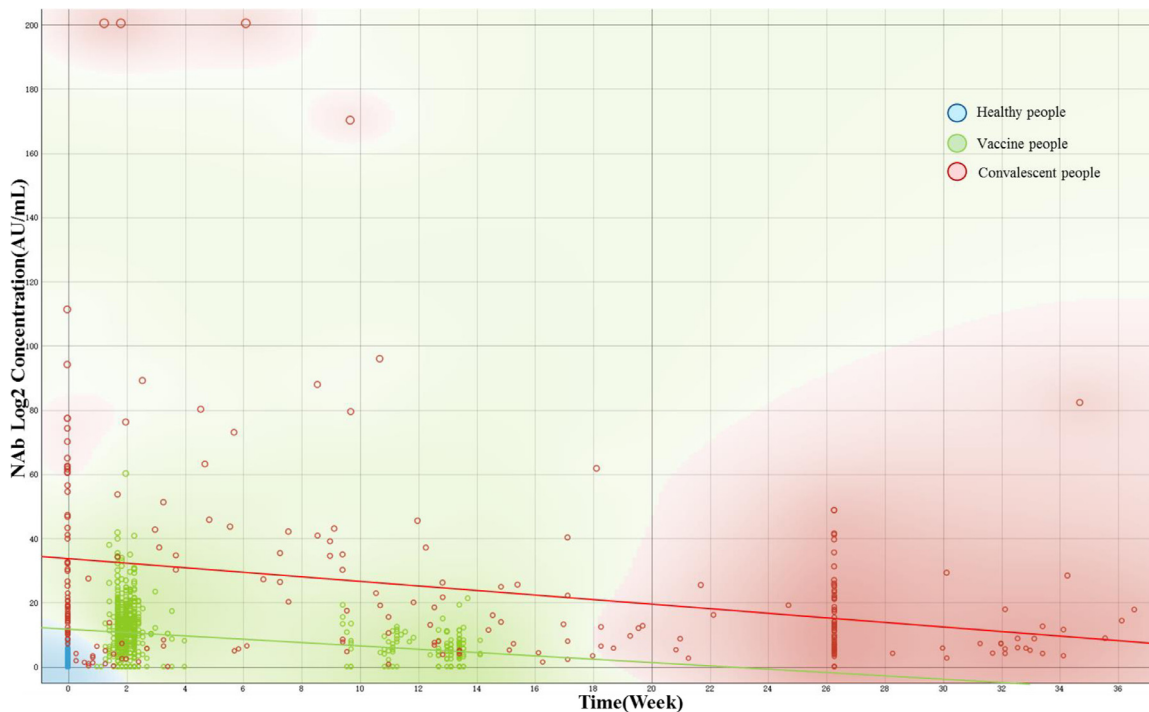

Figure 7. Distribution of neutralizing antibody (Ab) concentrations of different groups. Red points in the first week represent NAb concentrations of the 59 patients from symptom onset. Red points in the 26th week represent NAb concentrations of the 59 patients after recovery. The red line indicates the tendency for NAb concentrations to decline among the 59 recovered people. Green points represent NAb concentrations of the vaccinees who had received two doses of vaccine 2 weeks and 10–15 weeks, respectively, after the second dose. The green line indicates the tendency for NAb concentrations to decline among vaccinees.

Table 4
Concentration statistics for vaccinees of different ages and genders.

Parameters	Minors (<19 years)	Adults (19–50 years)	Elders (>50 years)	Male	Female
Sample	3	732	125	388	472
Mean value (AU/mL)	9.87[a]	11.37[a,b]	7.76[b]	9.69[c]	11.79[c]
Standard deviation (upper/lower) (AU/mL)	7.01	7.01/10.38	5.73/7.20	6.73/10.41	6.76/9.88

[a] P=0.7644.
[b] P (upper/lower)<0.0001/0.0003.
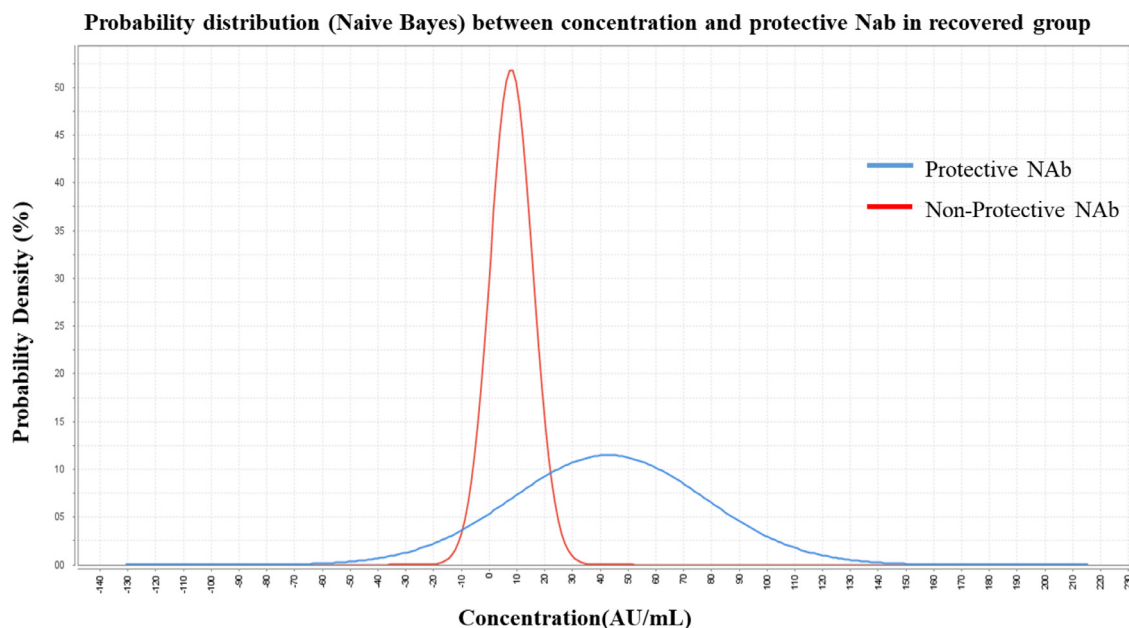[c] P (upper/lower)<0.0001/0.0044.

Figure 8. Probability distribution of concentration and protective neutralizing antibodies (NAb) in convalescent group.
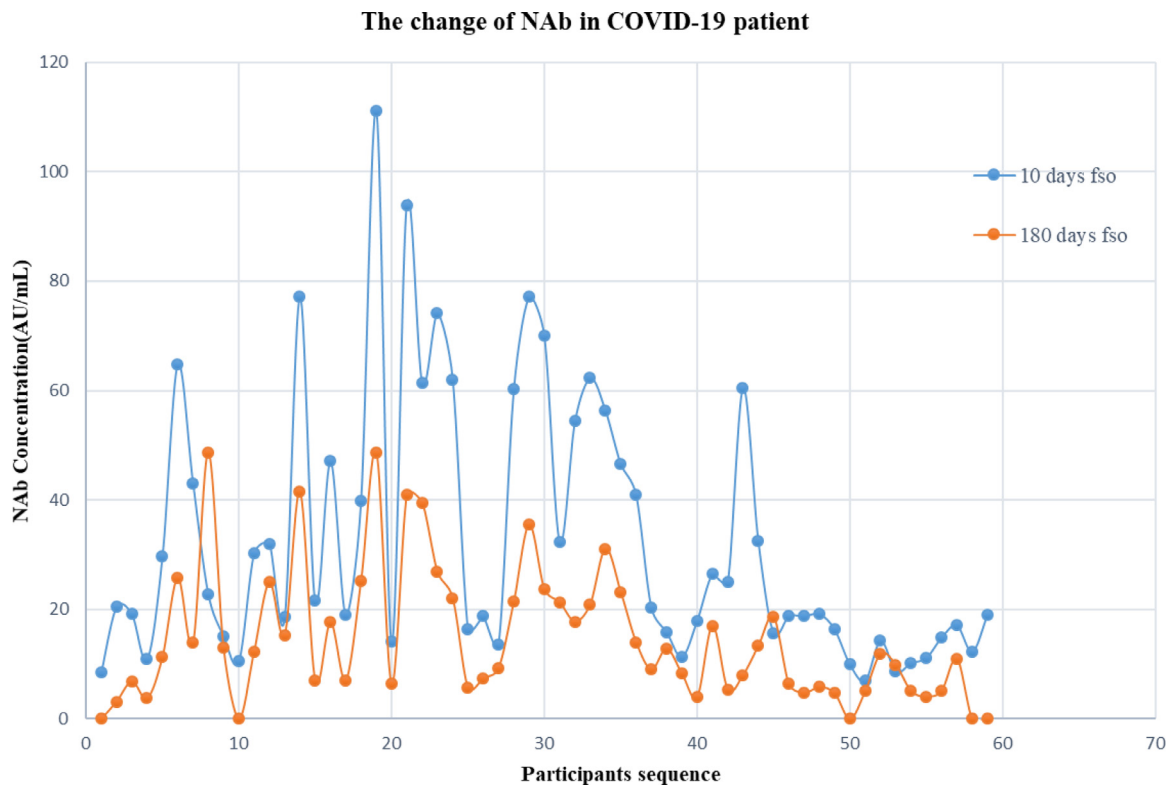

Figure 9. Variation tendency of neutralizing antibody (NAb) concentrations in 59 convalescent patients. fso, from symptom onset.

asymptomatic COVID-19 manifestations will need to be factored into calculating prevalence based on serosurveys (Krammer and Simon, 2020). NAb is a protective antibody which blocks the S protein to conjugate ACE2 on alveolar epithelial cells (Datta et al., 2020). There is a need to investigate the dynamics of the vaccine immune responses and the duration of protection based on antibody titers. This study developed an automated CLIA to replace the MNT to detect NAb levels.

Many serological assays have been used to measure antibodies (Ainsworth et al., 2020). Qualitative analysis includes enzyme-

linked immunosorbent assays, lateral flow assays, western-blot-based assays, etc. In terms of quantitative analysis, the Abbott SARS-CoV-2 immunoglobulin (Ig) G assay detects anti-N IgG using a two-step chemiluminescent microparticle immunoassay method with acridinium-labelled anti-human IgG (Bryan et al., 2020). The DiaSorin SARS-CoV-2 IgG assay, another two-step chemilumines-cent microparticle immunoassay, targets undisclosed epitopes in the SARS-CoV-2 S protein and uses an isoluminol-conjugated anti-human IgG. The Roche Anti-SARS-CoV total antibody assay is a two-step bridging electrochemiluminesent immunoassay using

ruthenium-labelled and biotin-conjugated N protein. The Siemens SARS-CoV-2 total antibody assay is a one-step bridging CLIA method that detects antibodies against the RBD using acridinium and biotinylated S1 RBD (Muecksch et al., 2021). The present study used a CLIA, in accordance with Siemens' method, to test the concentration of NAb against the RBD. As a result, strong positive correlation between NAb titers and concentrations was seen in the convalescent and vaccine groups.

In this study, data originated from healthy people (*N*=675), convalescent patients (*N*=232 individuals) and vaccinees (*N*=860). The CLIA was able to distinguish between these three groups using the geometric mean concentration of NAb ($P<0.05$). Through comprehensive analysis of the experimental data and literature, two methods were used to determine the cut-off value and borderline range of NAb concentration. In the study by Peterhoff et al. (2021), the cut-off value and borderline range for NAb concentration were determined from the healthy group data. In the present study, the cut-off value and borderline range were validated using the ROC curve based on data from vaccinees and convalescent patients. The ROC curve outperformed the method reported by Peterhoff et al. (2021). The ROC curve analysis indicated AUC of 0.901 ($P<0.001$), sensitivity of 87.4% and specificity of 78.1%. This was much better than the results reported by Matusali et al (2021).

As NAb titers and concentrations had a non-linear relationship, several estimating methods were applied to build the mathematical model. The progressive linear fitting model and decision-making tree model had low absolute error, strong correlation and high R^2 values. Therefore, both the progressive linear fitting model and the decision-making tree model were used to establish a mathematical model and explore the relationship between NAb titers and concentrations. The transform values between titers and concentrations can be mutually validated by two mathematical models.

According to this mathematical model analysis, it was predicted that the NAb concentration in convalescent patients would decrease by half in 5.79 months. To verify the accuracy of this forecast, 59 convalescent patients were tested 10 and 180 days after symptom onset, respectively. The concentration of NAb was found to decrease by an average of 53% after approximately 6 months. This mathematical model analysis can also predict the half-life of NAb concentration in vaccinees. After the second vaccine dose, the NAb concentration increased and reached a peak after 10–20 days. For three-quarters (75.4%) of vaccinees, the NAb concentration was higher than the cut-off value. This suggests that the effective rate of the vaccine was 75.4%. This result is similar to a published report of a clinical trial of Chinese vaccine (Palacios et al., 2020). The mathematical model predicted that the NAb concentration would decrease to 36.8% after 60 days. The half-life of NAb concentration in vaccinees was 10–11 weeks. This suggests that people should receive a third dose of vaccine approximately 3 months after the second dose. However, as none of the patients had received a third dose of vaccine, it was not possible to evaluate NAb concentration after the third dose. Vaccine duration was not fully evaluated, but these results suggest that the vaccine should be injected every 6 months, unless it has been improved.

The protective capability of the SARS-CoV-2 vaccine is another important subject. Matusali et al. reported that, according to Italian guidelines, NAb titer ≥1:160 was a selection criterion for convalescent plasma for treating patients with COVID-19 (Agenzia Italiana del Farmaco et al., 2021; Matusali et al., 2021; Peterhoff et al., 2021). This implies that people with NAb titer≥1:160 can be protected from SARS-CoV-2 infection. According to the protective capability of NAb reported by Matusali et al. (2021), only 5.35% of vaccinees had protective capability of NAb after receiving the second dose of vaccine. It is recommended that people should receive a third dose of vaccine in order to increase the protective capability of NAb.

In the convalescent group, the NAb concentration increased rapidly for several days after symptom onset (generally 10–15 days), and then declined over time. The half-life of NAb in convalescent patients, predicted by the mathematical model, was approximately 5.8 months. Recent reports have indicated that SARS-CoV-2 NAb titers decline over time, while another study indicated that NAb titers remained stable for ≥3 months after infection (Ibarrondo et al., 2020; Seow et al., 2020; Wajnberg et al., 2020). As reported by Matusali et al. (2021), a neutralizing response was detected as early as 5 days after symptom onset, and very high levels could be reached in the first weeks of symptoms. NAb could be detected up to 10–11 months after symptom onset, but NAb titers declined in one-quarter of individuals (Matusali et al., 2021). Muecksch et al. (2021) reported that the NAb titer decreased by approximately 25% per 2-week sampling interval, and decreased by approximately 45% over 4 weeks (Muecksch et al., 2021). This finding was similar to the present study in both the convalescent group and the vaccine group.

Convalescent patients had higher NAb titers than vaccinees, which conforms to the immunological mechanism. The half-life of NAb was twice as long in the convalescent group compared with the vaccine group. The protectiveness and effectiveness of NAb in the convalescent group were much higher than in the vaccine group. These findings may help to guide clinical treatment and diagnosis. The chemiluminescent method may be used to measure vaccine efficacy, to identify suitable therapeutic plasma, and to identify people who have been infected by SARS-CoV-2.

Combining all the information above, it is practicable to use CLIA to replace MNT. As CLIA is a fast, convenient and common method, it is possible to provide a common test for evaluating vaccine efficacy. The use of machine learning may also provide a new method for medical staff to study clinical data and find the internal mechanism among various clinical indexes.

This study has some limitations. First, no other medical information was obtained, so irrespective factors that may have influenced the models could not be excluded. Second, because the COVID-19 pandemic was reaching an end in China, it was difficult to find enough high NAb titer samples, and this influenced the precision and accuracy of the mathematical model. Third, the sample size of convalescent patients was not sufficient, so the results may have a deviation. Fourth, plasma was not collected after the first vaccination or 6 months after the second vaccination. Thus, it was not possible to analyse dynamic changes in NAb titers in vaccinees. Meanwhile, it was not possible to record the NAb titers of convalescent patients at different times (1 month, 3 months, 6 months, 1 year, etc.). The authors believe that dynamic changes in SARS-CoV-2 NAb will be identified with further study.

Conclusion

SARS-CoV-2 NAb is an essential biomarker to diagnose COVID-19 seroconversion and prognosis, and vaccine effectiveness. In this study, an automatic CLIA was used to detect NAb, and to estimate equivalence of NAb concentrations tested by CLIA and NAb titers tested by MNT. Some mathematical models were established to evaluate the effectiveness, protectiveness and durability of the SARS-CoV-2 vaccine. As the mathematical models predicted, vaccine-induced NAb durations do not exceed 6 months. NAb protective capabilities in vaccinees are much lower than those in convalescent patients. In order to enhance the immunity of vaccinees, there is a need for three doses of vaccine. Individuals who wish to retain immune capability should receive a dose of vaccine every 6 months. NAb should be tested periodically for vaccine immunity.

Conflict of interest statement

None declared.

Funding

Ethical approval

This work was approved by the Ethics Committees of Guangdong Provincial Centre for Disease Control and Prevention and Shenzhen Third People's Hospital. MNTs were performed in a BSL-3 facility at China Guangdong Provincial Centre for Disease Control and Prevention.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2021.12.316.

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