



Neutralizing antibody responses to SARS-CoV-2 in COVID-19 patients

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Background & objectives: The global pandemic caused by SARS-CoV-2 virus has challenged public health system worldwide due to the unavailability of approved preventive and therapeutic options. Identification of neutralizing antibodies (NAb) and understanding their role is important. However, the data on kinetics of NAb response among COVID-19 patients are unclear. To understand the NAb response in COVID-19 patients, we compared the findings of microneutralization test (MNT) and plaque reduction neutralization test (PRNT) for the SARS-CoV-2. Further, the kinetics of NAb response among COVID-19 patients was assessed.

Methods: A total of 343 blood samples (89 positive, 58 negative for SARS-CoV-2 and 17 cross-reactive and 179 serum from healthy individuals) were collected and tested by MNT and PRNT. SARS-CoV-2 virus was prepared by propagating the virus in Vero CCL-81 cells. The intra-class correlation was calculated to assess the correlation between MNT and PRNT. The neutralizing endpoint as the reduction in the number of plaque count by 90 per cent (PRNT₉₀) was also calculated.

Results: The analysis of MNT and PRNT quantitative results indicated that the intra-class correlation was 0.520. Of the 89 confirmed COVID-19 patients, 64 (71.9%) showed NAb response.

Interpretation & conclusions: The results of MNT and PRNT were specific with no cross-reactivity. In the early stages of infection, the NAb response was observed with variable antibody kinetics. The neutralization assays can be used for titration of NAb in recovered/vaccinated or infected COVID-19 patients.

Key words Antibody kinetics - COVID-19 - MNT - neutralizing antibody response - PRNT - SARS-CoV-2

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The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) outbreak spread to 215 countries around the globe, has become a pandemic¹. The majority of the SARS-CoV-2-infected patients (81%) are asymptomatic or show symptoms such as fever, dry cough, difficulty in breathing and chest pain of varying severity. Only a few (14%) patients suffer from severe symptoms including acute respiratory distress syndrome. Most of the elderly and patients with comorbidities progress to severe disease leading to death². Globally, SARS-CoV-2 has infected 21,294,845 cases with over 761,779 fatalities², while India has reported 2,702,742 cases and 51,797 casualties³ till August 18, 2020. There are knowledge gaps in estimation of disease severity and numbers infected, because it is difficult to estimate mild/asymptomatic infection⁴. Such information is crucial to assess herd immunity and is essential to estimate immune response to this virus.

The kinetics of the antibodies in SARS-CoV-2 patients is not clear due to scanty data available so far. A recent study has reported ambiguity about the antibody responses against the SARS-CoV-2⁵. Validated serological assays required to study the antibody responses and sero-epidemiology, are currently lacking and are urgently needed for contact tracing, epidemiological studies and vaccine evaluation studies. Virus neutralization assays are considered as a gold standard⁶ for serological method development. In the present study, we compared the microneutralization test (MNT) and plaque reduction neutralization test (PRNT) to assess neutralizing antibody (NAb) positivity in COVID-19 patients.

Material & Methods

This study was conducted in the ICMR-National Institute of Virology (ICMR-NIV), Pune, India, during March to May 2020. The Institutional Ethics Committee approved the study. Written informed consent was obtained from all the participants.

Clinical samples: A panel of 343 serum samples was used to evaluate the performance of MNT. These included serum samples from COVID-19-confirmed patients (n=89) by real-time reverse transcription-polymerase chain reaction (RT-PCR) admitted at the designated hospitals at Pune in Maharashtra and Alappuzha in Kerala, India (samples were collected within 2 wk after PCR confirmation) and SARS-CoV-2 real-time RT-PCR-negative patients (n=58). Serum samples (n=17) from patients with other respiratory

infections included influenza A (H1N1) pdm09 (n=4), influenza A (H3N2) (n=2), human corona virus OC43 (n=3), rhinovirus (n=2), respiratory syncytial virus (n=2), influenza B (n=2), parainfluenza type 4 (n=2) were also used to test for cross-reactivity. In addition, 179 serum samples from healthy individuals, collected before the COVID-19 pandemic were used as healthy controls. Demographic data, travel history and the clinical information of all the participants were recorded.

Cells and virus: The African green monkey Vero CCL-81 cells (ATCC, USA) maintained in minimal essential medium (MEM) (HiMedia, Mumbai), supplemented with 10 per cent foetal bovine serum (FBS) (Gibco, USA) were used for virus stock preparation and virus titration.

Virus propagation and titration: SARS-CoV-2 was isolated from throat/nasal swab specimens (NIV-2020-770) of a patient in Vero CCL-81 cells at the Containment Facility of ICMR-NIV, Pune⁷. The virus stock was prepared by propagating the virus in three passages in Vero CCL-81 cells. The cytopathic effect was first observed at second post-infection day (PID) and harvested at third PID. Virus titrations were performed in Vero CCL-81 cells using 50% tissue culture infective dose (TCID₅₀) assay as defined by the Reed and Muench⁸ and was found to be 10^{6.5} TCID₅₀/ml.

Plaque assay: The virus stock was titrated by plaque assay. Briefly, 24-well plates (Corning, USA) were seeded with 4×10⁴ cells/well and incubated at 37°C in an atmosphere of five per cent CO₂ for 24 h. Ten-fold serial dilutions of the virus were inoculated in triplicates on a preformed monolayer of Vero CCL-81 cells in the 24-well plates and incubated at 37°C in five per cent CO₂ for 60 minutes. After virus adsorption, the inoculum was removed and cells were overlaid with an equal volume of 2× MEM and one per cent carboxymethylcellulose (Sigma-Aldrich, USA) supplemented with two per cent FBS. Plates were incubated at 37°C in five per cent CO₂. The plates were stained with amido black (1% amido black, SDFCL, Chennai), 1 M acetic acid (Sigma-Aldrich, USA), 0.15 M sodium acetate (Thermo Fisher Scientific, USA) after four days of incubation for visualization of plaques. Virus titres were expressed as plaque-forming units (pfu) per ml and appropriate virus dilution (containing 50-60 plaques) was used as a challenge dose for PRNT.

Microneutralization test (MNT): MNT was performed in the Biosafety Level-3 facility at ICMR-NIV, Pune, as described by Sapkal *et al*⁹. The Vero CCL-81-adapted SARS-CoV-2 Indian isolate virus was used in this assay. Two-fold serially diluted heat inactivated (56°C for 1 h) serum samples were incubated with two log TCID₅₀ of the test virus for an hour at 37°C. The test virus antibody mixture was added to a pre-formed monolayer of Vero CCL-81 cells in 96-well plates and incubated at 37°C with five per cent CO₂ for 96 h. Ten-fold dilution of the virus without antibody (virus control), SARS-CoV-2-positive serum (positive control) with 100 TCID₅₀ of SARS-CoV-2 virus and normal non-immune serum (negative control) were added in respective wells as a control in this assay. Neutralizing titre was expressed as the reciprocal of the highest serum dilution at which 50 per cent of virus added was neutralized.

Plaque reduction neutralization test (PRNT): PRNT was performed as described by Perera *et al*¹⁰, with some modifications. Briefly, four-fold serial dilutions of heat inactivated (56°C for 1 h) serum samples were mixed with an equal amount of virus suspension containing 50-60 pfu in 0.1 ml. After incubating the mixtures at 37°C for one hour, each virus-diluted serum sample (0.1 ml) was inoculated onto one well of a 24-well tissue culture plate containing a confluent monolayer of Vero CCL-81 cells. After incubating the plate at 37°C for one hour, overlay medium (2% CMC with 2% FBS in 2× MEM) was added to the cell monolayer, and the plate was further incubated at 37°C in five per cent CO₂ for 4-5 days. Plaques were observed and the plates were stained with one per cent amido black for an hour. Antibody titres were defined as the highest serum dilution that resulted in >90 per cent (PRNT₉₀) reduction in the number of plaques. To study the utility of plasma and serum samples in neutralization assays, the antibody titres in paired serum and plasma samples (n=25) of selected patients were compared¹¹.

Statistical analysis: Statistical analysis was carried out using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). The neutralizing endpoint as the reduction in the number of plaque count by 90 per cent (PRNT₉₀) was calculated by probit analysis. Comparative analysis for differences between two groups was performed by Mann-Whitney test. Measures of diagnostic accuracy (sensitivity, specificity, PPV and NPV) for MNT according to Standards for Reporting of Diagnostic Accuracy Studies¹² were also calculated.

The intra-class correlation was calculated to assess the correlation between MNT and PRNT. Geometric mean titres (GMT) of MNT for symptomatic and asymptomatic patients were also calculated.

Results

During the optimization of MNT and PRNT, it was found that none of the serum samples from healthy individuals (n=179) collected before the COVID-19 pandemic showed NAb positivity in both MNT and PRNT assays. Further, all serum samples (n=17) from the respiratory panel other than SARS-CoV-2 and 58 SARS-CoV-2 real-time RT-PCR-negative patients remained negative in both MNT and PRNT assay to SARS-CoV-2 at a screening dilution of 1:20. The results indicated that MNT and PRNT displayed high specificity as no cross-reactivity to human corona virus (HCoV OC-43) and other respiratory viruses was observed. Furthermore, false positivity was not observed with the serum samples from a selected panel of real-time RT-PCR-negative and healthy individuals. Among 89 serum samples from SARS-CoV-2 real-time RT-PCR confirmed patients, 64 (71.9%) were found positive and the remaining 25 serum samples (28.1%) negative by both the methods (Fig. 1A and B).

Comparison of NAb titres of MNT and PRNT: To compare the NAb titres of MNT and PRNT₉₀ (n=64), intra-class correlation analysis was performed. The quantitative results indicated that the intra-class correlation was 0.520 (Fig. 2).

Further, no significant difference in the NAb titres in paired plasma and serum samples was observed. When MNT titres were compared with PRNT, the sensitivity and specificity of MNT was 100 per cent.

NAb responses among SARS-CoV-2 patients: Of the 89 COVID-19-positive patients, 59 (66.3%) were symptomatic and 30 (33.7%) were asymptomatic. The symptomatic patients had cough 48 (81.4%), fever 36 (61.0%), sore throat 16 (7.1%), breathlessness 11 (18.6%), body ache 9 (15.3%), headache 5 (8.5%), nasal discharge 2 (3.4%) and diarrhoea 2 (3.4%). Of the 59 symptomatic patients, 42 (71.2%) showed the presence of NAb and among 30 asymptomatic patients, 22 showed NAb response (73.3%). Among the 25 (28.1%) NAb negatives, 11 patients were positive (7 symptomatic and 4 asymptomatic) in anti-SARS-CoV-2 IgG ELISA (data not shown).

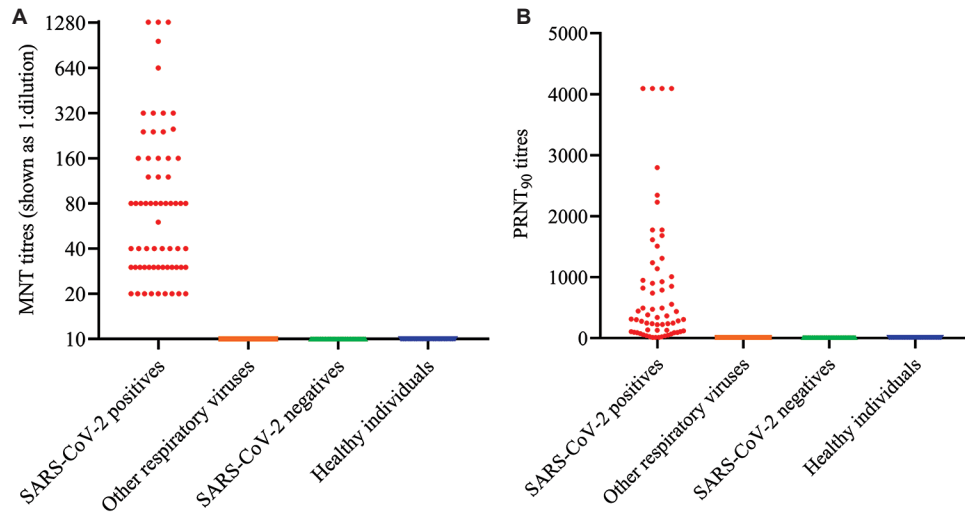


Fig.1. SARS-CoV-2 neutralizing antibody assay performance: The SARS-CoV-2 neutralizing antibodies titres of 89 serum samples of real-time RT-PCR confirmed COVID-19 patients were compared with 58 samples from real-time RT-PCR-negative patients, 17 samples from respiratory panel viruses including HCoV OC-43 (to test for cross-reactivity), 179 samples from healthy donors. (A) Microneutralization test. (B) Plaque reduction neutralization test (PRNT₉₀).

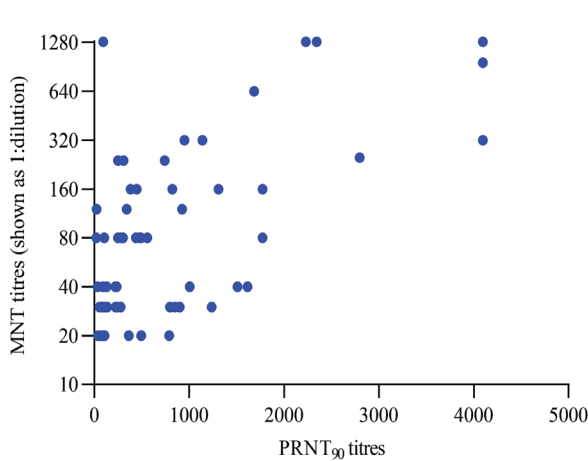


Fig. 2. Correlation of neutralizing antibody titres measured by microneutralization test and plaque reduction neutralization test. The neutralizing antibody titres of MNT were compared with that of PRNT₉₀ (n=64) by intra-class correlation.

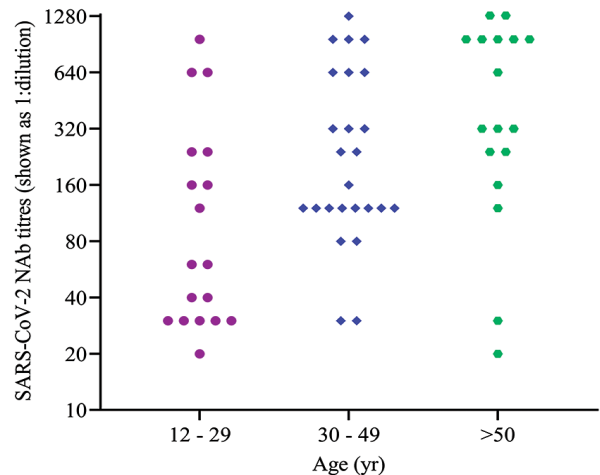


Fig. 3. Age-wise neutralizing antibody (NAb) response to SARS-CoV-2 infection: NAb titres among the patients (post onset of disease day 10 onwards) of age group 12-29 yr (n=18), 30-49 yr (n=25), and >50 yr (n=17) were compared.

Further, the NAb titres were found to be variable among the 64 COVID-19-positive patients [post onset of disease (POD) day 10th onwards]. The median age of the patients (n=60) was 36.5 yr (ranging from 3 to 70 yr); 62.5 per cent were males.

As represented in Figure 3, among patients of age group 12-29 yr (n=18), 15 showed low-to-medium NAb titre (20-320) and three patients showed higher titre (640-960). In 30-49 yr (n=25) age group, 18 patients showed low-to-medium NAb titre (20-320) and only seven patients showed higher titre (640-

1280). Among the 17 patients of above 50 yr of age, low-to-medium NAb titre (20-320) was observed in nine patients and high titre was noted in eight patients. The GMTs of NAb were 173.8 for symptomatic patients and 234.5 for asymptomatic patients.

To understand the kinetics of SARS-CoV-2-specific NAb response during the disease progression, 38 sequential serum samples were collected at different time points from 12 patients. The generation of NAb plotted against POD indicated that NAb were first detectable from day 4 to day 10 and increased rapidly after POD

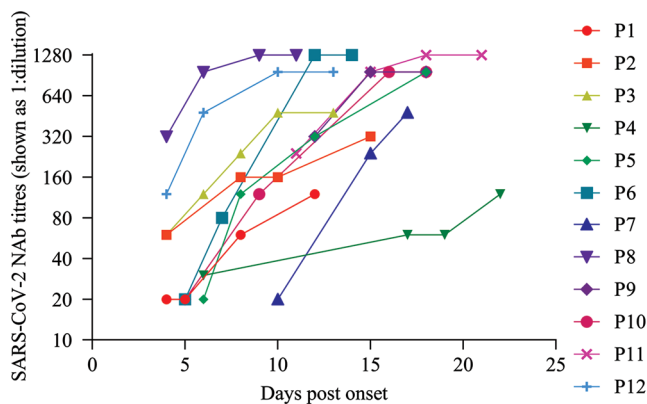


Fig. 4. Kinetics of neutralizing antibody (NAb) response among COVID-19 patients: Kinetics of SARS-CoV-2 NAb titres in sequential blood samples collected at different time points from 12 representative COVID-19 patients. SARS-CoV-2-specific NAb appeared on days 4-10 during the COVID-19 disease progression.

11-22. However, in patient Number 8, NAb response was initiated at POD 4 with a titre of 320 and reached highest titre (1280) within in a week time (on day 9 and day 11), while in patient Number 4, NAb response was marginal on POD 6, gradually increased on POD 17 and had maximum titre of 120 on day 22 (Fig. 4).

Discussion

In the present study, our primary objective was to optimize MNT and PRNT by using an Indian isolate of SARS-CoV-2. The MNT was relatively less time consuming, less labour intensive with higher throughput and required less volume of serum as compared to PRNT. During optimization, it was found that the qualitative results of the optimization panel for MNT and PRNT were comparable^{10,13}. The results indicated that the MNT and PRNT displayed high specificity as no cross-reactivity to human corona virus OC-43 and other respiratory viruses was observed. Furthermore, false positivity was not observed with the serum samples from a selected panel of real-time RT-PCR-negative and healthy individuals.

Analysis of MNT and PRNT₉₀ NAb titres indicated a positive correlation between the MNT and PRNT assays when tested with different spectrums of samples having variable antibody titres. Furthermore, low, medium and high NAb titres of serum samples were comparable. Hence, our strategy of using MNT as a screening assay for large-scale seroepidemiological studies and confirming all positive results by PRNT₉₀ assays can be considered. Understanding NAb response among COVID-19 patients is a crucial requirement, because the presence of neutralizing antibodies would provide greater assurance

of protection in the absence of approved and validated preventive and therapeutic options.

In our study, the majority of patients (71.9%) developed NABs to SARS-CoV-2 while 28.1 per cent (n=25) of patients failed to develop NABs; of these, 11 patients were positive by anti-SARS-CoV-2 IgG ELISA and many of them had similar POD. The role of cellular immune responses (T cells or cytokines) in the recovery of such patients needs to be explored^{14,15}. NAb titres were reported to be high among elderly and middle age group COVID-19 patients which was consistent to the earlier reports^{14,16}.

Among sequential samples of 12 patients, 10 displayed a similar trend of NAB kinetics. In these patients, the titres reached their peak at 10 to 15 days after disease onset and remained stable thereafter until day 22. The differences were observed in the titres of the NAB, possibly due to individual immune responses to SARS-CoV-2 infection and viral shedding. However, further studies are needed to understand the differences in NAB kinetics among COVID-19 patients.

The present study with preliminary observations had several limitations. The systematic follow up, sequential sampling and clinical profiling was limited. The sample size of COVID-19-positive patients was small to correlate the disease severity with NAb titres. More systematic studies with controlled follow up of patients and titration of NAb among the recovered patients will provide useful information for passive antibody therapy against SARS-CoV-2 virus infection. Further, it is pertinent to study the protective role of NAB during reinfection in recovered COVID-19 patients.

In conclusion, our findings indicated that during early stages of infection, the NAb response was observed with variable antibody kinetics. The neutralization assays can be used for titration of NABs in recovered/vaccinated or infected COVID-19 patients.

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Conflicts of Interest: None.

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