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Plaque reduction neutralization test as a method for detecting functional neutralizing antibodies against live SARS-CoV-2 virus

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ABSTRACT

Introduction and objective: Since 2020, little is known about neutralizing antibody (NAb) responses to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a novel coronavirus emerging and causing COVID-19 in humans. Here, we established a plaque reduction neutralization test (PRNT) to use as a method for detecting functional NAbs to SARS-CoV-2. **Materials and Method:** Vero cells were used as target cells for infection with the new coronavirus and cytopathic effects were obviously exhibited. **Results:** The development of plaque reduction after neutralization of virus with diluted specific antiserum was assessed according to a dose-response effect and consistency of test results with pooled antibody serum, which illustrated the robustness and dynamic reduction of plaque. In addition, the PRNT was used for evaluating functional NAb responses in Thai patients after symptom onset. **Conclusion:** The PRNT can be a method for measuring antibodies against live SARS-CoV-2 and future studies will be planned to investigate functional NAb responses elicited by any COVID-19 vaccine and to evaluate long-term protection of Thai COVID-19 vaccination.

Keywords: Neutralizing antibodies, plaque reduction neutralization test, severe acute respiratory syndrome coronavirus 2

INTRODUCTION

Neutralizing antibodies (NAbs) play an important role in virus clearance and have been considered as a key immunity-booting product for prevention or treatment of viral diseases.[1-5] The level of NAbs has been used as a gold standard to evaluate the efficacy of vaccines against smallpox, polio, and influenza.[6] The perspectives on the development of NAbs against severe acute respiratory syndrome coronavirus 2 (SAR-CoV-2) are described[2] and the role and importance of NAbs in protection against SARS-CoV-2 infection has been thoroughly reviewed.[2,7-10] It has been previously shown that plaque reduction neutralization test (PRNT) is the reference method to measure functional NAbs in serum. Other methods, such as pseudotyped virus neutralization assay and microneutralization SARS-CoV-2 assay, are used for detecting the NAbs as well. Live virus is used in both PRNT and microneutralization assay. However, microneutralization uses immunostaining and need computerized counting system, whereas PRNT performs manually plaque counting. Unlike PRNT assay which requires bio-safety level 3 (BSL3) containment, pseudotyped virus neutralization assay is conducted under BSL2 facility, but limitation of SARS-CoV-2 spike protein expression on pseudovirus development may differ from native virus and be difficult to construct. In addition, the evaluation of the specific immune response to SARS-CoV-2 variants virus can be adopted and conducted by PRNT.[2,11] To date, COVID-19 is a major pandemic of international public health concern as it is a contagious acute viral respiratory infection that is spreading rapidly around the world.[12] As the COVID-19 pandemic proceeds, a safe and effective vaccine against the disease is mainly required.[2] Furthermore, to promote Thailand's vaccine security and self-reliance during the outbreak, the development of COVID-19 vaccines for the country is urgently needed. Here, the levels of SARS-CoV-2-specific NAbs in Thai COVID-19 patients have not been reported, but the immune response can be predicted after COVID-19 vaccination is launched in the country. Therefore,

the aim of this study was to establish a method for detecting functional NAbs against live SARS-CoV-2 using a PRNT. This assay can be used for measuring the functional NAb responses elicited by COVID-19 vaccination that provides long-term protection in Thai population who will be vaccinated against the disease shortly.

MATERIALS AND METHODS

Cells and Culture Medium

The African green monkey kidney (Vero, ATCC CCL81, USA) cells were grown in the commercial MEM solution supplemented with 10% heat-inactivated fetal bovine serum (Cat. No. SH30070.03, HyClone, USA) and 1% L-glutamine (Cat. No. G7513, Sigma, USA) at 37°C, 5% CO_2.

Serum Samples

SARS-CoV-2 antibody-positive human serum samples from adults were obtained from Thai patients with COVID-19 virus infection confirmed with a gold standard reverse transcription polymerase chain reaction test.[13,14] SARS-CoV-2 antibody-negative human serum samples were obtained from healthy Thai adult donors. The samples were heat-inactivated at 56°C for 30 min before performing the PRNT. All samples were kept at −20°C before use.

Positive Serum

High serum antibody titers from Thai patients were determined with the PRNT method, then pooled and distributed into small tubes for use as positive serum control using the same method.

Cell Preparation

A confluent monolayer of Vero cells was washed with phosphate-buffered saline (PBS) and then detached with 0.04% pre-warmed trypsin-EDTA solution in PBS pH 7.2–7.4. After incubation at 37°C for 2–3 min, the detached cells were adjusted to 2×10^5 cells/mL in a pre-warmed complete medium. Three milliliters of cells suspension were seeded into each well of a 6-well plate (Corning, NY). Assay plates were incubated at 37°C, 5% CO_2 overnight.

SARS-CoV-2 Virus

The highly pathogenic SARS-CoV-2 was initially isolated from a clinical specimen from a Chinese patient infected with SAR-CoV-2 (hCoV-19/Thailand/74/2020) by the National Institute of Health, Department of Medical Sciences, Thailand, and was further propagated in Vero cells twice to get a large amount of virus. The small aliquot of the virus was kept at −70°C for single use.

SARS-CoV-2 Plaque Assay

A plaque assay was used to determine the concentration of SARS-CoV-2 used in the $PRNT_{50}$ under BSL-3 level. The monolayer of Vero cells was directly infected with 200 uL each of 1:100, 1:400, 1:1600, and 1: 6400 virus dilutions. After virus adsorption for 1 h at 37°C, 5% CO_2 with gently rocking every 15 min, 3 mL of overlay semisolid medium (containing 1.2% methyl cellulose, Sigma-Aldrich, USA) was replaced after removing excessive virus. All plates were further incubated at 37°C, 5% CO_2 for 6 days before fixation and staining. The plaques were counted and calculated as PFUs/mL. The average plaque count for virus control wells should be within the acceptable range of 40–120 plaques; no plaques were observed in cell control wells.

Fixation and Staining

To visualize the plaque formation, the plaques were directly fixed with 3 mL of 10% (v/v) formaldehyde. After 30 min, all culture media were discarded; and 1 mL of 0.5% crystal violet in PBS was added.

PRNT Method

Inactivated serum samples were fourfold serially diluted as 1:100, 1:400, 1:1600, and 1:6400 dilutions in MEM supplemented with 2% FBS. The neutralization was performed by mixing the equal volume of diluted serum and the optimal plaque numbers of SARS-CoV-2 at 37°C in water bath for 60 min. The culture medium from the assay-plates containing Vero cells was removed; and 200 uL of the virus-serum mixture was gently added. The culture plates were gently mixed every 15 min for 1 h. Three mL of overlay semisolid medium, containing 1.2% of methyl cellulose (Sigma Aldrich, USA), 1% of 10,000 units/mL penicillin-10,000 ug/mL streptomycin (Sigma, USA), and 10% FBS, was replaced after removing excessive virus. Cell control and virus control were maintained in culture medium alone and SARS-CoV-2 with culture medium, respectively. All plates were incubated at 37°C, 5% CO_2 for 6–7 days. After fixing and staining the culture plates, the number of plaques formed was counted in triplicate wells and the percentage of plaque reduction at 50% was calculated.

RESULTS

Cytopathic Effect (CPE) Induced by SARS-CoV-2 Virus in Vero Cells

To determine whether isolated SARS-CoV-2 from Thai patients infected from a Chinese visitor in Thailand could infect and show CPEs in cell line, Vero cells were selected and plaque assays were performed. Our preliminary results showed that under microscope, CPEs were obviously detected on day 3 [Figure 1b] and day 4 [Figure 1c] in infected Vero cells, where no CPEs were found in any of cell control [Figure 1a]. We, further, performed the plaque assay and the results showed that the optimal condition to detect plaques in Vero cells was 6–7 days after inoculation. As a result, SARS-CoV-2 could induce CPEs in Vero cells and the exact viral titer was also calculated based on the plaque assay condition for further use in the PRNT method.

Plaque Reduction by Functional NAbs Detected by PRNT

To determine the functional NAbs against SAR-CoV-2, an *in vitro* assay using plaque reduction was developed. The representative plaque reduction plates whose constant viral titers had been neutralized by positive serum (specifically

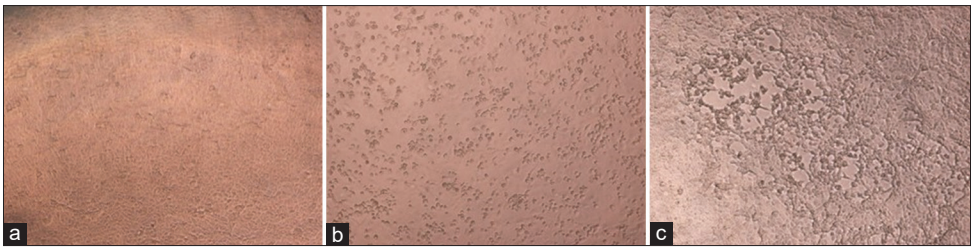

Figure 1: Cytopathic effect (CPE) of Vero cells after SARS-CoV-2 virus infection. CPE of Vero cells were observed under inverted microscope (magnification; ×10). (a) Cell control without virus, infected cells at day-3 (b), and day-4 (c)

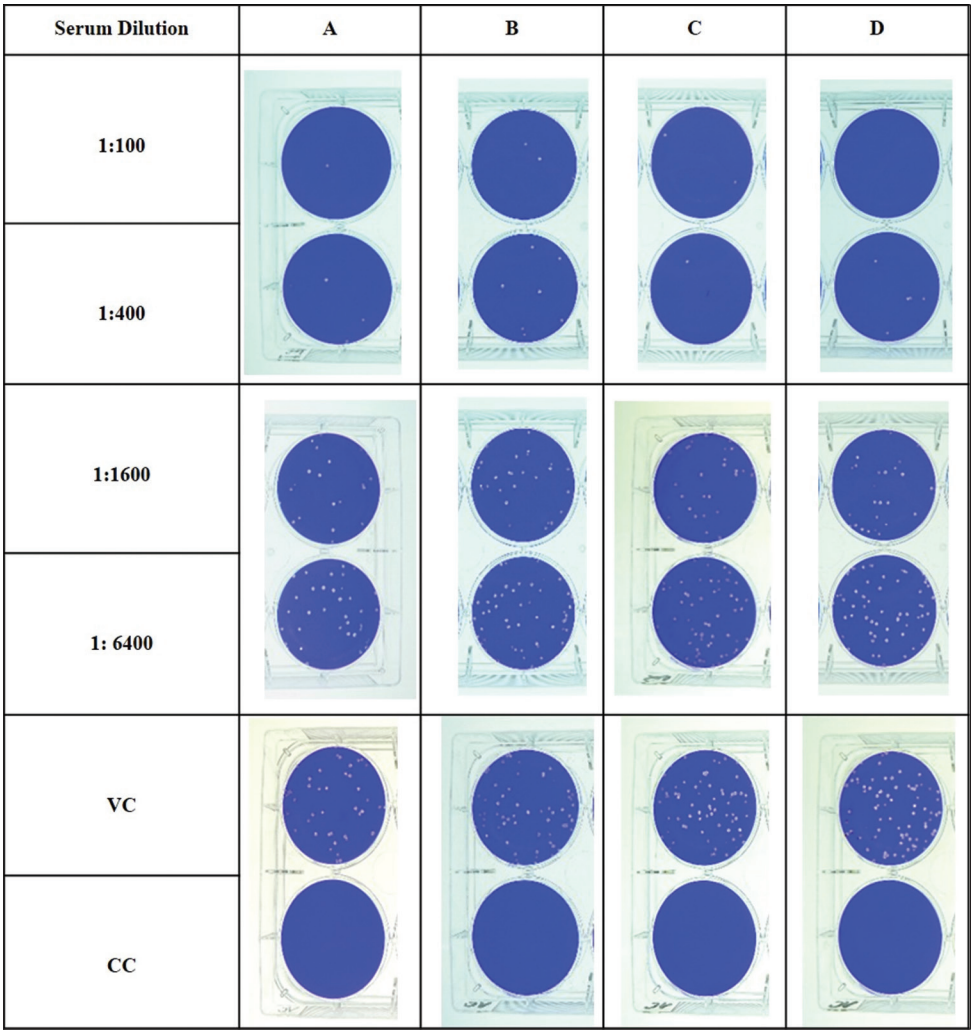

Figure 2: Morphology of SARS-CoV-2 virus plaques. Viral reduction plaques were observed in four independently experiments, Column A-D were the representative wells of the tests after incubation with SARS-CoV-2-specific serum antibodies. The first four rows in were infected with the challenge SARS-CoV-2 virus dose along with serum dilution at 1:100, 1:400, 1:1600, and 1:6400, respectively. The fifth and sixth rows were virus control and uninfected cell, respectively

pooled antiserum obtained from Thai patients with 1:100, 1:400, 1:1600, and 1:6400 dilutions) are shown in Figure 2. Serially, plaque reductions were observed when the positive sera were diluted. The number of plaques clearly reduced at 1:100 and 1:400 dilutions. The highest number of plaques was observed at 1:6400 dilution, which was similar to the number of plaques in the virus control. The data indicated that the specifically pooled antisera could neutralize SAR-CoV-2 and plaque reductions were detected in PRNT.

Robustness in the Detection of the NAbs against SAR-CoV-2 Virus

To examine the robustness of the serum at 1:100, 1:400, 1:1600, and 1:6400 dilutions used in the PRNT, we independently performed the assays using nine positive serum samples. The percentage of plaque reduction was then calculated. The data showed the consistency of plaque reduction in each dilution point [Figure 3], illustrating the plaque reduction kinetics.

Determination of the NAbs in COVID-19 Thai Patients

The characteristic of immune response to SARS-CoV-2 infection in patients played a crucial role in the vaccine and therapeutic development. To investigate the pattern of humoral immune response induced by SARS-CoV-2 infection in Thai patients, the PRNT was applied. The serum samples were randomly collected from the patients after onset of COVID-19 symptoms. In Figure 4, high levels of $PRNT_{50}$ titer were observed at >15 days ranging from 570 to >1280. Taken together, NAbs collected from patients were dynamically observed according to the day after onset and PRNT is the potential method to investigate functional NAbs in SARS-CoV-2-infected patients.

DISCUSSION AND CONCLUSION

For the evaluation of the specific immune response to SARS-CoV-2, a useful serological test involving NAbs has been developed.[15] It has been shown previously that PRNT is the most virus-specific serological test and is validated and demonstrated to be suitable to detect and measure NAbs in serum.[13,16-18] In this study, we established a PRNT assay as a method to detect the functional NAbs from Thai patients against live SAR-CoV-2. Pooled positive anti-serum samples were collected from Thai patients who showed high antibody titers ($PRNT_{50}$) more than 500 against SARS-CoV-2; and the PRNT assay was extensively performed to observe the consistency of those titers [Figures 2 and 3] after obviously exhibiting CPEs in the target Vero cells [Figure 1]. We also showed that the $PRNT_{50}$ titer obtained from negative sera could be easily distinguished from those generated from a pooled positive serum [Figure 2]. In addition, the robustness of antibody titers by the $PRNT_{50}$ was shown since it had illustrated dynamic reduction range of the plaques [Figure 3]. During the COVID-19 outbreak in Thailand, we got some serum specimens from COVID-19 patients and tried to evaluate the functional antibodies to SARS-CoV-2 in Thai patients after symptom onset with the PRNT to use the method as a standard method for supporting the country's COVID-19 vaccination efforts. It was found that Thai COVID-19 patients showed different levels of immunity; and functional NAbs in human sera by PRNT could be detected, but the results could not be interpreted confidently, because more information regarding age groups and severity levels was required. In summary, our data have demonstrated that the PRNT can be used as a method for measuring functional NAbs against live SARS-CoV-2; and future studies will be planned to investigate functional NAb responses elicited by a COVID-19 vaccine and to evaluate long-term protection in vaccinated Thai population.

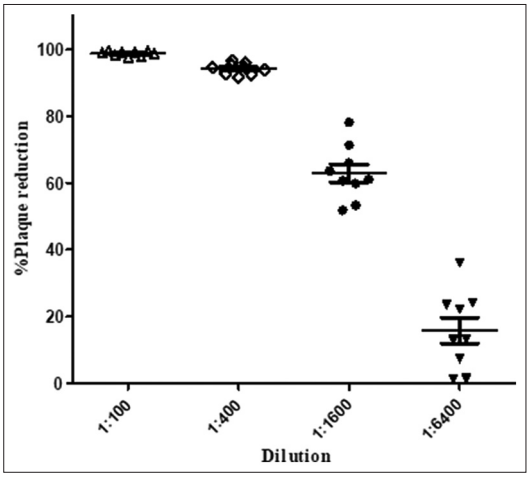

Figure 3: Robustness of antibody titers by plaque reduction neutralization test$_{50}$. The number of plaques was plotted against serial dilutions (1:100, 1:400, 1:1600, and 1:6400, respectively) of positive serum samples, illustrating the dynamic range of plaque reduction

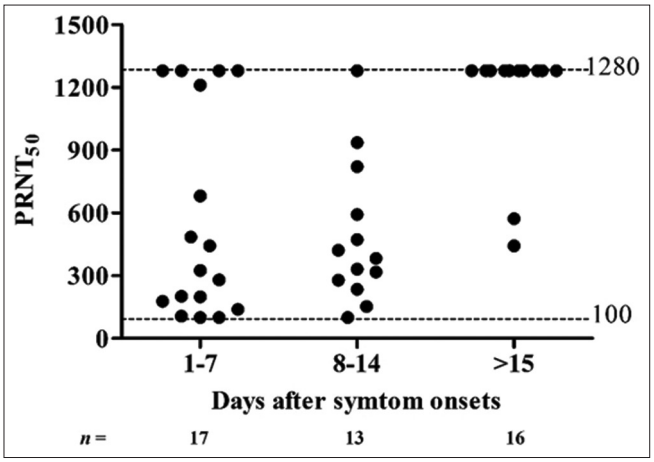

Figure 4: Evaluation of neutralizing antibody titers from Thai patients after symptom onsets by plaque reduction neutralization test. The serum samples were randomly collected from the patients and grouped by the days after onset of COVID symptoms, n = number of patients of each group

ETHICS STATEMENT

The study protocol was reviewed and approved by the Human Research Ethics Committee of the Department of Medical Sciences (approval letter no. EC192/63). A written informed consent was obtained from each of all participants in this study.

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