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Research paper

# Assays for the assessment of neutralizing antibody activities against Severe Acute Respiratory Syndrome (SARS) associated coronavirus (SCV)

Shixia Wang, Pavlo Sakhatsky, Te-Hui W. Chou, Shan Lu\*

*Laboratory of Nucleic Acid Vaccines, Department of Medicine, University of Massachusetts Medical School, 364 Plantation Street, Lazare Research Building, Worcester, MA 01605-2397, United States*

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## Abstract

Accurate assessment of neutralizing antibody activities is important either for patients infected with Severe Acute Respiratory Syndrome (SARS) or for animals and volunteers immunized with the experimental vaccines against the SARS associated coronavirus (SCV). However, the current assay based on the cytopathic effect (CPE) which has been frequently cited in literature has several limitations. The CPE assay relies on the visual observation on the damage of SCV infected target cells under a microscope. It is subjected to observer variations and it is difficult to generate a quantitative determination of neutralizing activities based on the level of CPE. In the current study, we established the utility of two additional assays to measure the neutralizing activities against SCV: the plaque reduction (PR) and the neutral red staining (NRS) assays. The PR assay described in this study was modified from the traditional viral plaque reduction assay by using an improved crystal staining method to achieve better plaque formation in SCV infected Vero E6 cells. The NRS neutralization assay was adopted from a similar system used for detecting neutralizing antibody responses against human immunodeficiency virus type 1 (HIV-1). In this assay, the protective effect of neutralizing antibodies was determined by the cell viability which is measured by the uptake of neutral red dye at  $A_{540}$ . The neutralizing antibody titers can be easily determined with either of the two new assays. In this report, we described the utility of these two new neutralization assays in measuring the neutralizing activities against SCV infection from rabbit sera immunized with various forms of spike protein of SCV.

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*Keywords:* Severe Acute Respiratory Syndrome (SARS); Coronavirus; Neutralization assay; Anti-viral antibody

*Abbreviations:* SARS, Severe Acute Respiratory Syndrome; SCV, SARS associated coronavirus; CPE, cytopathic effect; PR, plaque reduction; NRS, neutral red staining; TCID<sub>50</sub>, 50% tissue culture infectious dose; HIV-1, human immunodeficiency virus type 1; MOI, multiplicity of infection; DMEM, Dulbecco modified Eagle medium.

\* Corresponding author. Tel.: +1 508 856 6791; fax: +1 508 856 6751.

*E-mail address:* shan.lu@umassmed.edu (S. Lu).

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## 1. Introduction

The severe acute respiratory syndrome (SARS) – associated coronavirus (SCV), a new member in Coronaviridae, caused highly virulent emerging infectious disease in human population spreading many parts of the world (Drosten et al., 2003; Fouchier et al., 2003; Ksiazek et al., 2003; Kuiken et al., 2003; Peiris et al., 2003; Poutanen et al., 2003). SCV can be transmitted rapidly from person to person with an approximately 11% case fatality rate. Although the first epidemic had been successfully contained and only very few new cases were reported after fall of 2003 (Enserink, 2003; Normile, 2004a,b), SARS still remains a threat due to its highly transmittable nature to human populations and the mysterious origin of SCV (Arita et al., 2003; Chan et al., 2003; Inouye, 2003; Ratzan, 2003; Sampathkumar et al., 2003; Tong and Liang, 2004). Currently, there are no proven antiviral drugs effective for this viral infection (Drosten et al., 2003; Holmes, 2003; Kuiken et al., 2003; Normile, 2004b). Developing effective vaccines against SCV is the most cost-effective approach to achieve protection in a large population susceptible to SCV infection.

It has been reported that high titers of protective antibodies were present in the convalescent sera of SCV infected patients and the passive transfer of these sera could improve the clinical outcome of SARS (Li et al., 2003a; Pearson, 2004). This implies that if a vaccine can elicit robust humoral immunity, it will be protective against SCV infection by eliminating or reducing the cell-free viral infectivity (Chantler and Davies, 1987; Burton, 1997; Maruyama et al., 1999; Roehrig et al., 2001; Burton et al., 2004). To evaluate the neutralizing antibody activities in serum samples from either SCV infected hosts or those immunized with candidate SCV vaccines, it is critical to establish highly reproducible and quantitative *in vitro* virus neutralization assays.

Since the discovery of SARS, the neutralizing antibodies against SCV infection have been mainly detected by a simple microneutralization assay based on the cytopathic effect (CPE) of SCV to its target cells (Li et al., 2003b; Buchholz et al., 2004; Sui et al., 2004; Zeng et al., 2004). This method relies on the direct observation of damaged target cells from SCV infection under a microscope. However, the results

can be influenced by the subjective interpretation from the researchers, and it is not easy to quantitatively determine the neutralizing activities based on the degree of cytopathic effect. While completely protected cells can be easily distinguished from the damaged cells, partially protected cell populations are hard to evaluate. Therefore, it is difficult to come up with a titration curve to measure the strength of a neutralizing antibody with serially diluted testing antibodies.

Traditionally, various neutralization assays have been developed for many different viruses. Plaque reduction assays have been widely used to evaluate the neutralizing antibody responses against viruses that can form plaques in infected cells, such as rubella (Rhim and Schell, 1967; Sato et al., 1979), flavivirus (Russell and Nisalak, 1967; Ibrahim et al., 1968), vaccinia (Kitamura et al., 1973; Newman et al., 2003) and measles viruses (Orenstein et al., 1987; Vazquez-Rosales et al., 1996). However, unpublished data including our own have observed that the plaques formed in SCV infected target cells (such as Vero E6) were poor and could not be visualized clearly unless a microscope is used. In the current study, we have improved the plaque formation in SCV infected monolayer target cells so the plaques could be easily counted for SCV neutralizing antibody analysis.

The neutralization assay based on the target cell viability has also been widely used to study the neutralizing activities against HIV-1, in which neutral red dye is used to stain the live and healthy cells that are protected from HIV-1 infection (Montefiori et al., 1998). In HIV-1 studies, the MT-2 cells are used as the target cells (Montefiori et al., 1988, 1998). In the current report, we demonstrated that the neutral red method can be used for staining of Vero E6 cell which is a main target cell line for SCV infection.

## 2. Materials and methods

### 2.1. Antibodies

New Zealand White rabbit sera specific for different segments of SCV spike protein were produced by DNA immunization as recently reported (Table 1) (Wang et al., 2005). Rabbits were immunized with

Table 1  
Neutralizing antibody titers against SCV Urbani strain

Rabbit Sera	Spike DNA vaccines	Spike protein fragments	Neutralizing antibody titers*		
			CPE	Plaque reduction	Neutral Red staining
Pre-bleed	N/A	N/A	<30	<30	<30
RS001	N/A	N/A	<30	<30	<30
RS002	S	Full-length	2938.49 ± 1946.28	2737.5 ± 565.22	4669.16 ± 707.47
RS003	S1	Amino acids 12–798	2561.44 ± 1478.85	596.01 ± 131.71	5486.36 ± 584.52
RS004	S2	Amino acids 797–1192	492.95 ± 279.33	154.21 ± 24.44	878.63 ± 140.56
RS005	S1.1	Amino acids 12–535	4436.55 ± 2982.28	3608.08 ± 752.20	8845.09 ± 1389.23
RS006	S1.2	Amino acids 534–798	<30	<30	<30

N/A—not applicable.

\* The values are the geometric means ± standard deviation from 4 independent assays.

four bi-weekly immunizations by a gene gun and the immune sera tested in this study were collected two weeks after the 4th immunization. Sera were stored at  $-70^{\circ}\text{C}$  and heat-inactivated for 30 minutes at  $56^{\circ}\text{C}$  before use.

## 2.2. Cells and virus stocks

The African green monkey kidney Vero E6 cells (American Type Culture Collection, Manassas, VA) were used for both the propagation of SCV and the neutralization assays. The cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 100 units/ml of penicillin G, 100 mg/ml of streptomycin, 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum (FBS). All cell cultures were maintained in 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ .

The Urbani strain of SCV used in this study was obtained from the US Center for Diseases Control and Prevention (CDC, Atlanta, GA). All the procedures handling the SCV and infected cell cultures were held in a special biosafety level 3 (BSL-3) laboratory under protocol approved by Institutional Biosafety Committee (IBC). To produce the SCV stock, the virus was propagated in Vero E6 cells at a multiplicity of infection (MOI) of 0.01, culture medium was collected at day 4 and filtered through a 0.45 micron filter to remove the cell debris. The titer of the virus stock was determined in Vero E6 cell monolayer seeded at 96-well microtiter plates (Corning, NY) with 20,000 cells/well. The virus 50% tissue culture infectious dose ( $\text{TCID}_{50}$ ) was determined by a 4-day endpoint titration assay as described previously (Li et al., 2003b).

## 2.3. CPE based microneutralization assay

CPE based microneutralization assay was conducted in 96-well microtiter plates as described (Li et al., 2003b). Three fold dilutions of heat-inactivated rabbit sera were tested in triplicate wells for the presence of antibodies that neutralized the infectivity of SCV in monolayer of Vero E6 cells. 400  $\text{TCID}_{50}$  of virus in 50  $\mu\text{l}$ /well was incubated with 50  $\mu\text{l}$  of rabbit serum or tissue culture medium for 1 hour at  $37^{\circ}\text{C}$ . After the incubation, 20,000 Vero E6 cells (in 100  $\mu\text{l}$ ) were added to each well at MOI of 0.02. Results of neutralization were determined by the appearance of CPE, which was observed under a microscope on day 4 of infection. The neutralizing antibody titer was defined as the reciprocal of the highest serum dilution at which no CPE breakthrough in any of the triplicate testing wells was observed.

## 2.4. Plaque reduction based neutralization assay

The SCV plaque forming units (pfu) in the virus stock were determined by a plaque assay in 12-well tissue culture plate. Vero E6 cells ( $0.5 \times 10^6$  cells per well) were seeded onto the 12-well tissue culture plates the day before infection. On the next day, 50 pfu (16000  $\text{TCID}_{50}$  equivalents) of SCV in 100  $\mu\text{l}$  was first incubated with or without serially diluted antibodies in a total volume of 150  $\mu\text{l}$  in 96-well U-bottom tissue culture plates for 1 hour at  $37^{\circ}\text{C}$ . Then, the mixture was added into Vero E6 cells seeded 12-well plate after removing their culture medium and incubated for 1 hour at  $37^{\circ}\text{C}$ . The plates were washed with DMEM and the monolayer cells were overlaid with 1 ml of

DMEM medium containing 1% (w/v) of methylcellulose (4,000 CP; Sigma, MO). After 2 day incubation at 37 °C, Vero E6 monolayers were washed with PBS and fixed with 250 µl of pre-chilled 4% formaldehyde for 30 min at room temperature, followed by aspiration removal of the formaldehyde solution and twice PBS wash. Monolayers were then stained with 300 µl of 0.5% of crystal violet for 5 min at room temperature, and the plates were washed twice with water. After the plates were air dried, plaques were counted. The neutralization was calculated as the percentage of the number of plaques reduced with a particular testing serum as compared to the mean of the plaque counts for the three virus control wells (without sera) in the same assay. The neutralizing antibody titer was determined as the highest serum dilution which achieved 50% plaque reduction.

### 2.5. Neutral red staining (NRS) based neutralization assay

The NRS neutralization assay was performed in 96-well flat bottom plates. The set-up is similar to that of the CPE based neutralization assay. Briefly, after incubation of 400 TCID<sub>50</sub> of virus with rabbit serum in a total of 100 µl DMEM medium per well for 1 hour at 37 °C, Vero E6 cells (20,000 cells in 100 µl) was added to each well at MOI of 0.02. On day 5 of infection when more than 70% cells formed CPE in the viral control wells (without any sera), the culture medium was removed from the testing wells and 100 µl of 10% neutral red (Sigma) in DMEM medium was added to each well. After incubation for 1 hour at 37 °C, the neutral red medium was removed, and the plates were washed twice with phosphate buffered saline (PBS, pH 7.2). Then, 100 µl of acid alcohol (1% acetic acid in 50% ethanol) was added to each well. After incubation for 30 minutes at room temperature, the absorbance of neutral red stained plates was read at 540 nm ( $A_{540}$ ).

## 3. Results

### 3.1. Neutralization of SCV as measured by CPE assay

The CPE based neutralization assay was the first and the most frequently used neutralization assay in

the SARS research (Wang et al., 2005). However, the way of determination on neutralizing antibody titers has been inconsistent and was poorly described in literature. This assay relies entirely on the observation of cell morphology to determine the effect of neutralizing antibody. As shown in Fig. 1a, after being infected with SCV for 4 days, Vero E6 cells in the microtiter plate started forming visible CPE including dissociated cell patterns. The degree of CPE can be scored as less severe (“+”, Fig. 1a, right well) or more severe (“++”, Fig. 1a, center well) as compared to the protected monolayer cells in the presence of positive anti-S rabbit sera (“–”, Fig. 1a, left well) when observed under a microscope. However, the degree of morphological changes is hard to be quantified and may be subjected to observers’ variation.

This limitation becomes even more problematic when the titers of neutralizing antibodies need to be determined. Fig. 1b depicts one sample assay in which the hyperimmune positive anti-S rabbit serum was serially diluted at 3-fold dilutions. CPE was first observed when the sera dilution reached 1:8100 (Fig. 1b, indicated by +). However, the CPE did not appear in all three testing wells at the same time and the level of CPE in each well also varied (data not shown).

In order to give a consistent scoring of the neutralizing antibody responses, the titers of positive sera can be determined either by a more complicated scoring system based on how many wells showed CPE at a given sera dilution or simply based on the last sera dilution when none of the triplicate wells has any visible CPE (1:2700 in the example of Fig. 1b). By using the latter criteria, a panel of rabbit sera elicited by DNA vaccines expressing different forms of SCV S antigen were analyzed (Table 1). Both the full length S antigen and the N-terminal S1 subunit antigen induced excellent neutralizing antibodies against SCV. The C-terminal S2 subunit also elicited positive albeit low level neutralizing activities as we recently reported (Wang et al., 2005). When the S1 subunit protein was further divided into two separate domains, S1.1 and S1.2, it was clear that the neutralizing epitope was located at the S1.1 region which has the binding domain for SCV receptor, ACE-2 (Li et al., 2003b). The S1.2 region did not show any neutralizing activities (Table 1).

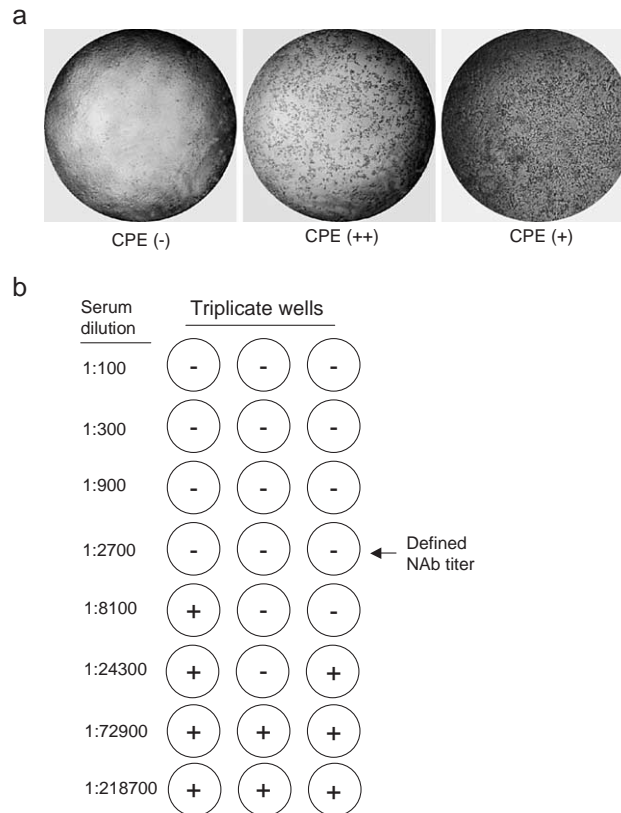


Fig. 1. (a) Sample pictures of cytopathic effect (CPE) changes in SCV infected Vero E6 cells observed under a microscope (10 $\times$ ) without (–, left) and with various levels of CPE (++, middle; and +, right). (b) A sample CPE based neutralizing assay showing the appearance of CPE in Vero E6 cells following serial dilutions of a positive anti-S rabbit serum. The arrow indicates the last serum dilution which did not show any detectable CPE in all triplicate wells.

While CPE assay is useful, the assay-to-assay variation is significant as shown by high standard deviations (Table 1). It is likely due to the variation on the observable changes of CPE when a stringent criteria (absent of CPE in any testing wells) was applied. This problem can become more severe when different researchers are involved in the scoring of CPE. CPE assay is also less effective in providing more quantitative measurements of the antibody strength, such as the concentration of sera that can inhibit 50% or 90% of infection (IC<sub>50</sub> or IC<sub>90</sub>).

### 3.2. Neutralization of SCV measured by plaque reduction assay

As noted among researchers working with SCV, Vero E6 cells can only form very tiny plaques when

conventional plaque assay technique was used (Fig. 2a). It usually requires high concentration of SCV and the resulting plaques are hard to count even under a microscope. To improve the plaque formation, we discovered that overlay of methylcellulose with proper concentration and molecular weight (4,000 CP) was very critical. With this improved method, clear plaques were formed in wells infected with SCV while the uninfected cell control well remained free of plaques (Fig. 2b).

We then performed the neutralization study in 12-well tissue culture plates with this modified plaque reduction assay. To validate the plaque reduction based neutralization assay, the positive anti-S rabbit serum RS003 and negative control rabbit serum RS001 were used. A series of rabbit sera in 3-fold dilutions were added and the numbers of plaques in



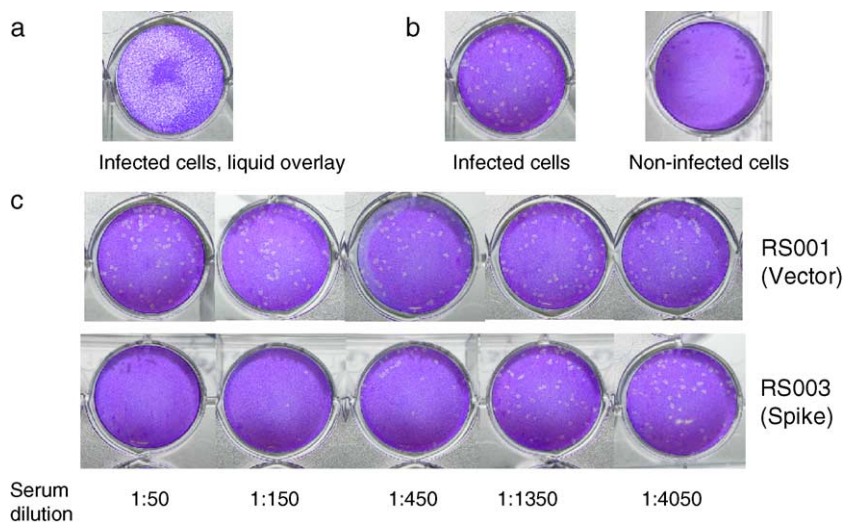


Fig. 2. Plaque assay plates fixed with formaldehyde and stained by crystal violet. (a) Poor plaques formation in SCV infected Vero E6 cells with the traditional method. (b) SCV infected Vero E6 cells formed clear plaques as transparent dots (0.5–1 mm in size) while the methylcellulose overlay method was used. (c) A representative plaque reduction based neutralization assay with the methylcellulose method. Serially diluted anti-S rabbit serum RS003 or the negative control rabbit serum RS001 were added to Vero E6 cells incubated with SCV (see Table 1 for description of these two rabbit sera).

each well were counted under a light source (Fig. 2c). The number of plaques in each well increased along with the dilution of anti-S rabbit serum while the number of plaques in the negative sera control wells remained unchanged. The percentage of plaque reduction in the presence of positive antibodies was calculated by using the formula below:

$$\text{plaque reduction (\%)} = \frac{\text{plaque counts in testing well} - \text{plaque counts in cell control}}{\text{plaque counts in viral control} - \text{plaque counts in cell control}} \times 100\%$$

The neutralizing antibody titer in plaque reduction assay can be reported as the reciprocal serum dilution at which 50% of plaque reduction was achieved as compared to the viral control wells. By using this assay, the neutralizing antibody activities of the same set of positive anti-S rabbit sera were analyzed (Table 1). While the plaque reduction assay demonstrated a similar pattern on the relative levels of neutralizing antibody activities as the CPE assay, the neutralizing titers determined by the plaque reduction assay was in general lower than that achieved by CPE assay. This suggested that the plaque reduction assay may be less sensitive than the CPE assay in determining the neutralizing activities against SCV infection.

### 3.3. Neutralization of SCV measured by neutral red staining

The cell viability upon viral infection can also be measured by neutral red staining. Previously, we have used this technique to evaluate the cell viability for HIV-1 infected target cell MT-2. The neutral red dye can be taken by the healthy MT-2 cells during staining and will not be washed off by PBS. The same neutral red staining method can be applied to Vero E6 cells, the target cells for SCV infection, despite they are adherent epithelial cells and form monolayer attached to the tissue culture plates. After staining and washes, the healthy Vero E6 cells will hold the neutral red dye but not the damaged cells after being infected by SCV. The proportions of healthy cells could be determined by reading the absorbance of neutral red at  $A_{540}$  of each well. It was noted that the virus/cell ratio was very important in this type of neutralization assay and the optimal ratio was to infect 20,000 cells with 400–500 TCID<sub>50</sub> of SCV viruses in 96-well plates.

Practically, a series of 3-fold dilutions of rabbit sera can be incubated with virus in triplicate wells, and viral controls (with no sera) were in octave wells as well as the cell controls (without virus and sera). When significant numbers of Vero E6 cells in SCV

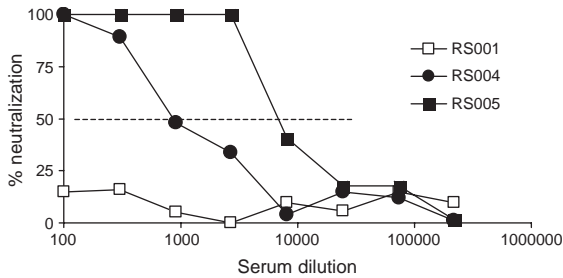


Fig. 3. SCV neutralization titers were determined by neutral red staining assays. Percent of viable cells as determined by the neutral red staining was plotted against dilutions of testing rabbit sera. Each curve represents the results of one rabbit serum: positive anti-S rabbit sera (RS004 and RS005) and the negative control rabbit serum (RS001). The levels of neutralizing activities can be shown as the percent inhibition of SCV infection to Vero E6 cells as compared to the viral control wells. For example, the neutralizing titers can be determined by using the 50% inhibition as the cut-off line as shown in the figure.

viral control wells formed CPE as compared with the cell control wells, the cells were stained with neutral red. The fraction of viable cells in the presence of neutralizing antibodies was quantified by the staining of cells, where the intensity of the staining was directly proportional to the number of viable cells. Percent neutralization was determined by the formula below:

% neutralization

$$= \frac{A_{540} \text{ of antibody testing wells} - A_{540} \text{ of viral control wells}}{A_{540} \text{ of cell control wells} - A_{540} \text{ of viral control wells}} \times 100\%$$

The neutralizing antibody titer in neutral red assay can be reported as  $IC_{50}$ , the reciprocal serum dilution at which 50% of Vero E6 cells were protected from the virus induced killing. One major advantage for neutral red staining based assay is that the levels of survived cells can be measured directly by the  $A_{540}$  reading which can be plotted against the dilution of neutralizing rabbit sera. Results from a sample experiment are shown in Fig. 3.  $IC_{50}$  can be easily determined by this method.

The neutralizing antibody titers for the same panel of testing anti-S rabbit sera as determined by the neutral red staining were compared with those measured by CPE and plaque reduction assays (Table 1). It appears that the neutral red staining was the most sensitive method in detecting positive neutralizing

activities. The overall pattern on the levels of neutralizing antibody activities among positive rabbit sera was very similar and independent of the type of assays used. Rabbit serum specific for S1.1 fragment always had the highest neutralizing activities and rabbit serum specific for S2 subunit had the least positive neutralizing activities (Table 1). These two regions on Spike protein of SCV were shown to be the two neutralizing domains (Wang et al., 2005). Rabbit sera elicited by larger S antigens (either the full length S protein or the entire S1 subunit) showed some variation of neutralizing antibody activities when different neutralization assays were used. It is possible that antibodies targeting at non-neutralizing epitopes in these sera may interfere with the antibodies targeting at the neutralizing epitope. The prebleed sera and sera from rabbits immunized with either vector control or S1.2 fragment DNA vaccine did not show any positive neutralizing activities by any of the three assays, indicating the overall high specificities of these tests. Results in Table 1 were calculated based on four independent experiments for each of the three assays (Fig. 4). Experiment-to-experiment variations or the assay reproducibility for both plague reduction and neutral red staining assays were much better than the CPE assay.

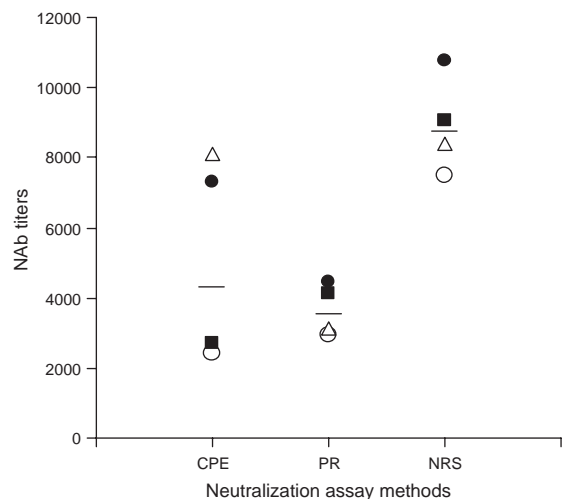


Fig. 4. The reproducibility of three types of neutralization assays: CPE, plaque reduction (PR) and neutral red staining (NRS). Four independent experiments were conducted with each of the three assays using the positive anti-S rabbit serum RS005. The short horizontal bars represent the geometric means for each assay.



#### 4. Discussions

In this report, we described the utility of two more assays that have been adapted for the measurement of neutralizing antibody responses against SCV in Vero E6 cells in addition to the previously reported CPE assay.

First, we have successfully improved the traditional plaque reduction assay to form better SCV plaques in infected Vero E6 cells. We discovered that the molecular weight of methylcellulose was very important for the plaque formation. When the 1% methylcellulose 4,000 CP in maintenance medium was used as the overlay medium, it produced easily visible plaques on the testing plates. However, other methylcellulose compounds with lower or higher molecular weights could not achieve the same results. This finding re-established the utility of plaque assay for SARS research. This assay can be used for pathogenesis studies to monitor the viral titers without using more complicated molecular or biochemical analyses to quantify the amounts of SCV. This procedure can lower the risk of handling hazardous SCV which has high potential of human transmission as shown in previous laboratory accidents (Normile, 2004a,c; Watts, 2004). The same improved plaque formation technique can also be applied to study other viruses which may also have difficulty in producing plaques with visible sizes.

We also applied neutral red staining method to SCV infected Vero E6 cells. Previously the same technique was used for HIV-1 neutralization assay in which HIV-1 infected MT-2 cells were transferred to 96-well microtiter plates pre-coated with poly-L-lysine to capture the suspended MT-2 cells for neutral red staining (Montefiori et al., 1998). The captured healthy cells could uptake and hold neutral red dye without being washed off by PBS but not the infected cells which formed syncytia or CPE. The cell viability could be easily determined by measuring the absorbance of neutral red dye released from cells after extracting with acid alcohol. Although the Vero E6 cells are adherent epithelial cells attached to the bottom of tissue culture plates, we demonstrated that direct staining of the Vero E6 cells infected with SCV in the plate worked very well. In order to achieve the best reading, special attention should be placed on the number of cells seeded on the plate, virus/cell ratio,

and the concentration of neutral red. Too many cells seeded would generate high background of the staining due to over-grown cells. On the other hand, if the cell numbers were too low, the difference between the  $A_{540}$  reading of cell controls and the  $A_{540}$  reading of viral controls would also be small as the denominator to calculate the percentage of the neutralization. The preferred range of  $A_{540}$  differences between viral infected well and cell controls well should be around  $\sim 1$ .

Similar to several other reports, we initially used the CPE assays to evaluate the neutralizing antibody responses raised by SCV S DNA vaccines (Wang et al., 2005). The CPE assay is simple and does not require any special reagents or devices other than a regular light microscope. However, the scoring of SCV induced CPE under microscope could be highly subjective and sometimes the results reported by different observers may vary. Since the CPE score is mainly based on the morphology of cell conditions, which is an evolving process, it is difficult to standardize the scoring based on the degrees of CPE. In our previous study, a more stringent standard was used for the CPE based neutralization assay: it was scored positive once any CPE-like morphology of the cells was observed. Although CPE assay is useful, it would be ideal to develop more quantitative and objective assays.

Our study demonstrated that an improved plaque reduction assay has been established to measure the neutralizing antibody activities against SCV infection. Previously plaque assays have been used successfully for a wide range of viral infection and neutralization studies including vaccinia (Kitamura et al., 1973; Newman et al., 2003), measles (Orenstein et al., 1987; Vazquez-Rosales et al., 1996) and rabies (Yoshino and Morishima, 1971; Wiktor and Clark, 1973; Cardoso and Pilz, 2004). However, the neutralizing antibody titers observed by the plaque reduction assay in our study were lower than those determined by the CPE assay or the neutral red staining assay. One important factor for such difference may be due to the different amounts of virus used in these assays. The MOI was 0.032 in the plaque reduction assay and 0.02 in both the neutral red staining and the CPE assays. Different neutralizing titers were also observed for neutralizing antibodies against vaccinia between a plaque reduction assay and a biomarker assay (Earl et al., 2003).

Therefore the plaque reduction assay may be more useful in differentiating the high titer and low titer anti-SCV antibodies. A unique advantage of plaque reduction assay is that the plates can be stored for a longer time than the other assays.

On the other hand, the neutral red staining based assay gave higher neutralizing antibody titers than CPE and plaque reduction assays for the same set of positive rabbit sera. Besides the fact that less virus was used in neutral red staining assay than plaque reduction assay, the measured  $A_{540}$  range optimized in our neutral red staining assay provided sufficient reading range to fully demonstrate the neutralizing potential of positive rabbit sera. The main advantage of neutral red staining method is that the quantitative measurement of neutralizing activities can be accomplished by a plate reader which improves the efficiency and reproducibility.

Although the neutralizing antibody titers were different depending on the type of assays used in our study, the general pattern of neutralizing activities among this set of testing rabbit sera was very consistent. None of the negative control sera showed any detectable neutralizing activities indicating the high specificity of these assays. Therefore, these two additional neutralizing assays against SCV can serve as useful tools for SARS research including several clinical trials recently started in human volunteers to test the immunogenicity of candidate SARS vaccines.

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