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Original Article

Application and Validation of SARS-CoV-2 RBD Neutralizing ELISA Assay

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Abstract

The establishment of an approach for detecting the anti-severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2)-receptor-binding domain (RBD) neutralizing antibodies (nAbs) by a safe, easy, and rapid technique without requiring the use of live viruses is essential for facing the coronavirus disease 2019 (COVID-19) pandemic. Depending on competitive enzyme-linked immunosorbent assay (ELISA) methodology, the current study assay was designed to simulate the virus-host interaction using purified SARS-COV-2-RBD from the spike protein and the host cell receptor human angiotensin-converting enzyme 2 protein. The performance of this in-house neutralizing ELISA assay was validated using freshly prepared standards with different known concentrations of the assay. In this regard, a cohort of 50 serum samples from convalescent COVID-19 individuals with different disease severity at different time points post-recovery and a cohort of 50 serum samples from healthy individuals were processed by the in-house developed assay for detecting SARS-CoV-2 nAbs, in comparison with a commercial total anti-SARS-CoV-2 IgG antibody assay as a gold standard. The assay obtained a sensitivity of 88% (95% CI: 75.69-95.47) and a specificity of 92% (95% CI: 80.77- 97.78%). A negative strong correlation was demonstrated in the standard curve between the optical density absorbance and log concentration of the nAbs with a statistical measure of r^2 (coefficient of determination) = 0.9539. The SARS-COV-2-RBD neutralizing ELISA assay serves as a high throughput qualitative and quantitative tool that can be applied in most laboratory settings without special biosafety requirements to detect anti-RBD nAbs for seroprevalence, pre-clinical, and clinical evaluation of COVID-19 vaccines efficiency and the rapid selection of convalescent plasma donors for the treatment of COVID-19 patients.

Keywords: COVID-19, nAbs, Neutralizing antibodies, SARS-COV-2-RBD neutralizing ELISA assay

1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a member of the SARS-related coronavirus species, which is the same as SARS-CoV that caused the SARS outbreak 18 years ago (1-4). To enter into target cells, SARS-CoV-2 uses angiotensin-converting enzyme 2 (ACE2) as a receptor, and to activate the viral spike (S) protein, it uses transmembrane serine protease 2 (TMPRSS2) (5, 6). Both ACE2 and TMPRSS2 are found abundant, particularly in the upper respiratory tract (7). A growing amount of evidence suggests that antibody and T cell responses are critical for recovering from COVID-19 (8-12). As a result, antibody responses to SARS-CoV-2 have received a lot of attention as a way to accurately assess infection prevalence (13, 14). Antibodies that target the receptor-binding domain (RBD) of the S protein are particularly interesting because they can prevent virus infection and spread by

blocking virus entry into cells. Additionally, these neutralizing antibodies may be used in passive antibody therapies (13, 15). Therefore, the current study aimed to establish an approach for detecting Anti-SARS-CoV-2 neutralizing antibodies (NAbs) with a safe, easy, and rapid technique without requiring the use of live viruses, provide neutralizing antibody tests that could be used to evaluate vaccine efficiency in preclinical and clinical studies of various vaccine candidates, and monitor neutralizing titers in vaccines following mass vaccination in human populations.

2. Materials and Methods

This study was conducted at the Microbiology Department, College of Medicine, Al-Nahrain University, Baghdad, Iraq, within December 2020-June 2021.

2.1. Development of the Assay

Based on the hypothesis that serum neutralizing antibodies should also interfere with the binding of the RBD of SARS-CoV-2 (SARS-CoV-2-S RBD) to ACE2, an in-house developed *in vitro* SARS-CoV-2-RBD neutralizing enzyme-linked immunosorbent assay (ELISA) needed to be prepared for the qualitative and quantitative detection of circulating neutralizing/blocking antibodies in serum samples in an isotype-independent manner.

This assay was designed in an ELISA plate well to simulate the virus-host interaction using purified SARS-CoV-2-RBD from the S protein and the host cell receptor human ACE2 (hACE2) protein. Depending on competitive ELISA methodology, hACE2 was immobilized to the surface of the ELISA 96 microtiter plate. Afterward, a mixture of horse-reddish peroxidase (HRP)-labeled-RBD and patients' serum was added to the hACE2 adsorbed wells. After washing, if neutralizing antibodies were present in the serum, the interaction of ACE2-RBD could be neutralized (inhibited/blocked) by specific NAbs in patient serum, just like in conventional virus neutralization test (cVNT) or pseudovirus-based VNT. The direct binding was demonstrated using HRP conjugated to SARS-CoV-2-RBD protein. Depending on the amount of neutralizing antibodies present in convalescent sera, the binding of SARS-CoV-2 S RBD to ACE2 would be blocked to various degrees that should correlate with the optical density of this enzyme-linked immune sorbent-based assay. The serum samples with more neutralizing antibodies showed a lower signal intensity (16) (Figure 1).

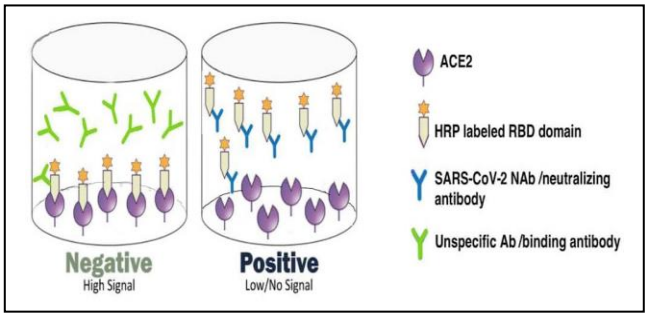

Figure 1. Schematic representation of the principle of SARS-CoV-2-RBD neutralizing ELISA assay

2.2. Assay Optimization

Initially, an experimental design was used from previous pilot studies worldwide (16, 17); however, numerous experiments were performed in this study for optimization. The ELISA plate was coated with various human ACE2 concentrations to determine the optimal concentrations of ACE2 used for coating with the labeled RBD that also added to the ACE2 coated plate at a wide range of concentrations. The optimal concentrations were determined by choosing the desired result corresponded with a well that yielded a readable signal with the least amount of ACE2 coated in combination with the least amount of labeled-RBD. Subsequent experiments were performed to obtain an acceptable standard curve and meet performance requirements using the optimal concentrations of ACE2 and labeled-RBD that were determined previously, as well as by the determination of the optimal conditions for various variables involving ELISA, including the sample/standard volumes, incubation times, incubation temperature, washing times, pH, diluent, optimal buffers, and dilution of serum samples.

2.3. Horse-Reddish Peroxidase-Receptor-Binding Domain Preparation

The conjugation procedure was performed according to the guidelines of the conjugation kit (Abcam) [ab102890]. In this study, using deionized water, 1X sodium phosphate buffer (PBS), 100 mM sodium phosphate, 150 mM NaCl, pH 7.2 (bio-world), or HEPES Buffer (CAPRICORN) diluted to 40 mM instead of PBS, were prepared as conjugation buffers for the RBD labeling reaction requiring amine-free conditions. The amount of RBD protein from a commercial supplier (Elabscience) used to be labeled is ideally corresponded to the molar ratio 1:2 RBD to HRP. Taking into account the molecular weights (22.6 KDa versus 44 KDa), for example, for 13.4 µg of required RBD protein, 52 µg of HRP are needed to be added. The molar matching calculations were conducted according to the following formula: Mass of required RBD (µg) / Protein MW (KDa) = Number of Moles of RBD, Number of Moles of RBD x 2 x HRP MW (KDa) = µg of HRP required for conjugation reaction (18).

A calculated volume of the prepared conjugation buffer was added to the required RBD stock solution that contained 13.4 µg and allowed to reconstitute for 15-30 min at room temperature with gentle agitation to achieve the RBD concentration as recommended on Abcam's HRP conjugation kit in the range of 0.5-5.6 µg/µl to give optimal results. Modifier reagent was added to the required diluted RBD to be labeled (1 µl of Modifier reagent for each 10 µl of RBD) and mixed gently. HEPES buffer (40 mM) or PBS (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2) was added as a solvent into the vial of HRP mix containing lyophilized material to generate a stock solution that was resuspended gently by withdrawing and re-dispensing the liquid once or twice using a pipette. The calculated required volume (52 µg) of HRP stock solution was pipetted into the Eppendorf tube containing the required diluted RBD (with added modifier reagent) for labeling. The reaction was shielded from light by covering the tube with aluminum foil and incubated in the dark for 3 h at room temperature (20-25°C) with constant shaking. Afterward, Quencher reagent was added (1 µl of Quencher reagent for each 10 µl of the solution) to be used as the conjugate after 30 min. Nevertheless, to improve the stability of HRP Conjugate to be stored for 6 months at 2-8°C, HRP-Conjugate Stabilizer (Elabscience) was added to the prepared HRP-RBD stock solution (over 90% of the stock solution).

To apply 50 µL of HRP-RBD working solution to fit 10 ng/well, a calculated volume from the prepared HRP-RBD stock solution was diluted with HRP-Conjugate Diluent (Elabscience) to the required volume at the appropriate concentration (0.2 ng/µl), which was determined in the initial experiments.

2.4. Preparation of Working Human Angiotensin-Converting Enzyme 2 Protein Solution

To immobilize the hACE2 protein at 400 ng/well in 100 µl of 1X working Coating Buffer on the microtiter plates wells, a calculated volume from Stock solution vial of Recombinant His-Tag-Human ACE2 protein in a concentration of 2.8 mg/ml (Raybiotech) was diluted immediately before usage with the prepared 1X working coating buffer from 5X stock ELISA Plate Coating Buffer (Elabscience) to a required volume at the appropriate concentration (4 ng/µl), which was determined in the initial experiments.

2.5. Plate Preparation

To coat microplate with recombinant hACE2, up to 100 µl of the prepared working hACE2 protein solution were added to each well of a new 96-well flat-bottom MaxiSorp Nunc-Immuno ELISA plate (400 ng of hACE2/well). The plate was incubated overnight at 4°C. Subsequently, after that the unbound coating hACE2 solution was discarded, washing 2 times with 200 µl of 1X Wash Buffer (Elabscience) was performed. The remaining protein-binding sites in the coated wells were blocked by adding 300 µl of blocking buffer (Elabscience) per well of the microtiter plate and incubated for at least 90 min at 37°C. Next, after washing 2 times with 200 µl of 1X Wash Buffer, the plate became ready for immediate or same-day use (19).

2.6. SARS-CoV-2-RBD Neutralizing ELISA Assay

For the quantitative assay, the preparation of standards was performed by a serial dilution of the stock purified recombinant concentrated anti-SARS-CoV-2 spike neutralizing antibody (1 mg/ml) from commercial suppliers (Elabscience) using sample diluent buffer (Elabscience) to get four points for the standard curve to calculate the unknown nAb concentration in the samples. Therefore, the standard concentrations for anti-SARS-CoV-2 spike neutralizing antibody were 10,000 ng/ml, 1,000 ng/ml, 100 ng/ml, and 0 ng/ml. Sample diluent served as the zero standard (0 ng/ml). For the qualitative assay, the prepared anti-human SARS-CoV-2 highest standard of 10,000 ng/ml was used as a positive control and the sample diluent as a negative control, and a cut-off value was calculated using the receiver operator characteristic (ROC) analysis method based on the inhibition rate of the assay (20). The serum samples were diluted with the sample diluent buffer in a 1:10 volume ratio, which was optimized in the initial experiment that included running the samples through several dilutions (1:100 v/v, 1:10 v/v), as well as non-diluted to determine empirically the appropriate required dilution of the samples and ensure accurate quantification. The assay procedure was conducted according to the method used by Tan, Chia (16) with minor modifications for optimization. For neutralization reaction, the freshly prepared standards and the pre-diluted samples were mixed with the diluted HRP-RBD solution (0.2 ng/µl) with a volume ratio of 1:1 to permit the binding of the circulating neutralizing antibodies to HRP-RBD. Afterward, each sample and the standard mixture was incubated at 37°C for 1 h for binding reaction, and 100 µL of each standard mixture and each sample mixture were transferred to the wells of the hACE2-coated microplate and incubated in dark at 37°C for 1 h. The unbound HRP-RBD, as well as any HRP-RBD bound to non-nAb, would be captured on the plate, whereas the circulating nAb-RBD-HRP complexes remained in the supernatant and were washed away 4 times with 260 µl of 1x Wash Buffer. For substrate reaction, up to 100 µL of one-component 3,3′,5,5′-Tetramethylbenzidine substrate (Elabscience) were added to each well and incubated in the dark at 37°C for 20 min to make the color blue. At the next stage, up to 50 µL of Stop Solution (Elabscience) were added to each well to quench the reaction and turn the color yellow. The absorbance was read in the microtiter plate reader (BioTek) at 450 nm immediately after adding the Stop Solution. The inhibition rate of the neutralizing antibodies for samples was determined using the following formula: inhibition % = (1-optical density [OD] values of sample/OD values of negative control) × 100 (16). Moreover, the standard curve was plotted to calculate the neutralizing antibody concentration in the samples by applying curve fitting data analysis.

2.7. Assay Validation

To validate the performance of the in-house designed SARS-CoV-2-RBD neutralizing ELISA kit, it was tested using the freshly prepared standards with different known concentrations of purified recombinant anti-RBD neutralizing antibody, including the highest concentration as positive control versus sample diluent buffer as a negative control. In addition, a cohort of 50 serum samples was collected from convalescent COVID-19 individuals with a history of different severity of diseases at different time points post recovery and confirmed to be positive to total anti-SARS-COV-2 Immunoglobulin G (IgG) by commercial kits and a cohort of 50 serum samples from healthy individuals who had not been exposed to CoVID-19 infection before and whom serum samples were negative to total anti-SARS-CoV-2 IgG by commercial kits. This validation was applied to evaluate the precision, repeatability, accuracy, sensitivity, and specificity of SARS-CoV-2-RBD neutralizing ELISA assay. Notably, each of the standards and samples was employed in duplicate into the respective wells of the human ACE-2-coated microplate to ensure precision.

2.8. Statistical Analysis

The data were processed using SPSS software version 16.0.0, Microsoft Excel software 2010, and Graphpad Prism software version 7.04. The diagnostic performance

of a test or the accuracy of a test to discriminate diseased cases from normal cases was evaluated using ROC curve analysis. In addition, inter- and intra-assay coefficients of variance, sensitivity, specificity, positive predictive, and negative predictive values were measured for assessing the repeatability, performance, and precision of the in-house designed assay.

3. Results

The scatter plot between OD absorbance values (x-axis) and log-concentration values (y-axis) in figure 2 is plotted from the data in table 1 that includes the average of OD absorbance of each SARS-COV-2 neutralizing antibody standard generated by the in-house designed neutralizing ELISA kit next to the corresponding concentration and is shown in the graph as a multiple-points to fall along a straight line as a standard curve. For the in-house developed SARS-COV-2 neutralizing ELISA assay, a negative strong correlation was demonstrated between the OD absorbance and log-concentration of the nAbs with a statistical measure of r^2 (coefficient of determination) = 0.9539; furthermore, a linear regression equation was estimated for determining the unknown log-concentration of anti-SARS-COV-2 nAbs in samples, as shown as in figure 2.

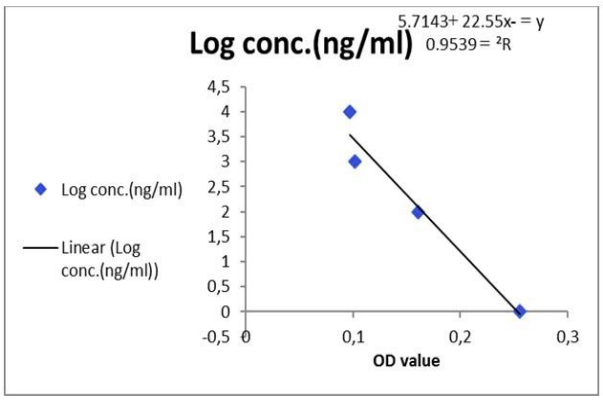

Figure 2. Standard curve of anti-SARS-COV-2-RBD nAbs for the in-house developed SARS-COV-2-RBD neutralizing ELISA assay

Table 1. Mean ± standard deviation of OD values of different anti-SARS-COV-2-RBD neutralizing antibody standards generated by the designed neutralizing ELISA kit

Standards (anti-SARS-CoV-2 nAbs)	Concentration (ng/ml)	Mean	SD
standard no. 1 (as negative control)	0	0.2555	0.003535534
standard no. 2	100	0.161	0.002828427
standard no. 3	1,000	0.1015	0.00212132
standard no. 4 (as positive control)	10,000	0.0965	0.000707107

The diagnostic ability of the in-house developed SARS-COV-2-RBD neutralizing ELISA assay was aimed to differentiate the positive and negative individuals for the presence of neutralizing antibodies. This qualitative determination of results was analyzed by the ROC curve analysis using the positive and negative control serum panels for assay validation. The optimal selection thresholds for cut-off values were determined on the basis of Youden's index (the sum of sensitivity and specificity at its maximum), as shown in table 2A. The cut-off value of 0.17 OD was accordingly estimated, which was equal to the cut-off concentration of 75.99 ng/ml or the cut-off log concentration of 1.88l; this cut-off value performed well in terms of sensitivity and specificity (Tables 2A and 2B). The sensitivity of the cut-off value was shown as a ROC plot against one minus the specificity (Figure 3). The area under the curve for ROC analysis was 1.000 (95% CI, 1.000-1.000) with a statistical significance of $P < 0.0001$. Using the established cut-off value (0.170 OD, 1.88 log, 75.99 ng/ml) in the in-house developed neutralizing ELISA, the control serum panels for the assay validation were discriminated to positive (< cut-off OD value or > cut-off concentration / log concentration) and negative (> cut-off OD value or < cut-off concentration / log concentration) results, Supplemental tables (S 1A and SB).

Table 2. Cut-off value results of the in-house neutralizing ELISA; **A)** Optimized ROC analysis cut-off value of positive and **B)** Concentration and log concentration limits for the optimal established cut-off in this assay

2A

Coordinates of the curve		
Test result variable (s): OD		
Positive if less than or equal To[a]	**Sensitivity (%)**	**Specificity (%)**
0.1415	83	100
0.1700	100	100

2B

Cut-off log	1.8808
Cut-off concentration ng/ml	75.99762144

OD: Optical density

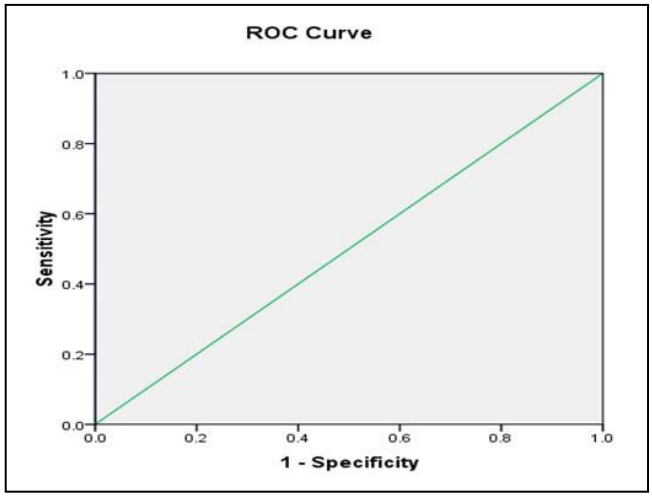

Figure 3. Receiver operating characteristic curve analysis for the optimal cut-off value for detecting the positive anti-SARS-COV-2-RBD neutralizing antibodies using the in-house developed neutralizing ELISA kit

Inhibition rate % by the in-house SARS-COV-2-RBD neutralizing ELISA assay was analyzed. The mean inhibition rate % by the in-house neutralizing ELISA in 50 serum samples used as a positive control was obtained at 52.84%, while the mean inhibition rate % in 50 negative serum control samples was estimated at 0.0 %. The cut-off inhibition rate % in the in-house developed SARS-COV-2-RBD neutralizing ELISA assay was 15%, which was equal to the cut-off value (0.170 OD, 1.88 log concentration, 75.99 ng/ml) used in this study. The results with inhibition rate value of > cut-off inhibition represented positive versus the results with inhibition rate value of < cut-off inhibition were considered negative.

As there is a positive quantitative correlation between the results of the Commercial SARS-CoV-2 IgG Assay and nAb Assays (21), the serum panels tested positive/ negative total anti-SARS-CoV-2 IgG by commercial kit was used as the gold standard to assess the sensitivity and specificity of the in-house developed SARS-COV-2 RBD neutralizing ELISA assay. The sensitivity and specificity of the in-house designed kit were measured as shown in table 3. Overall, as seen in table 4, the in-house SARS-COV-2-RBD neutralizing ELISA assay performed well, with a sensitivity of 88% (95% CI: 75.69-95.47) and a specificity of 92% (95% CI: 80.77-97.78). Furthermore, the positive and negative predictive values were demonstrated as 91.67% (95% CI 81.04-96.59) and 88.46% (95% CI 78.28-94.22), respectively. At the in-house established cut-off valve (0.170 OD, 1.88 log, 75.99 ng/ml, 15% inhibition), the assay showed a positive likelihood ratio of 11 (95% CI: 4.27-28.32) and a negative likelihood ratio of 0.13 (95% CI: 0.06-0.28).

Table 3. Agreement of the results between the in-house SARS-COV-2-RBD neutralizing ELISA assay and gold standard test at the in-house established cut-off

Experimental test	**Gold standard test**	
	+ve	**-ve**
+ve	44	4
-ve	6	46

Table 4. Validation parameters of the in-house SARS-COV-2-RBD neutralizing ELISA assay

Results		
Statistic	**Value**	**95% CI**
Sensitivity	88.00%	75.69% to 95.47%
Specificity	92.00%	80.77% to 97.78%
Positive likelihood ratio	11	4.27-28.32
Negative likelihood ratio	0.13	0.06-0.28
Positive predictive value	91.67%	81.04-96.59%
Negative predictive value	88.46%	78.28-94.22%

The analysis of the replicates of the positive and negative control samples in the same microplate yielded acceptable levels of precision (% coefficient of variation [CV]) and reliability for the in-house SARS-COV-2-RBD neutralizing ELISA assay. The levels of intra-assay precision for the positive control sample and intra-assay precision for the negative control sample were 18.70% CV and 10.62% CV, respectively, while the inter-assay precision was 10.66% CV.

The measurement ranges of the in-house SARS-COV-2-RBD neutralizing ELISA assay, including the upper and lower limits of neutralizing antibodies concentrations, were detected in samples. The measurement range of 1.88 log/ 75.99ng/ml to 3.84 log/ 6960.6 ng/ml was indicative of the positive samples, while the measurement range of 0.009 log/1 ng/ml to 1.88 log/75.99 ng/ml was indicative of the negative results.

4. Discussion

As neutralizing antibodies can prevent viruses from infecting cells, their presence reflects a crucial component of antibody functionality. In other words, the only known indicator of SARS-COV-2 protective immunity induced by either infection or vaccination is nAbs (22, 23). Therefore, SARS-COV-2-RBD neutralizing ELISA assay was developed in this study based on the ELISA technique as a qualitative and quantitative assay to accurately determine the protective immunity by detecting the presence of anti-RBD nAbs and measuring its level in a large number of samples. Unlike the cVNT and pseudotype neutralization assay (sVNT), this SARS-COV-2-RBD neutralizing ELISA assay is simple without requiring the use of live virus or cell cultures; moreover, it is safe, cheaper, and faster than cVNT and can be performed in most laboratory settings without special biosafety requirements and it takes only 3 h to be performed.

The SARS-COV-2-RBD neutralizing ELISA assay is potentially important for the rapid selection of convalescent plasma donors with high levels of nAbs for the treatment of COVID-19 patients with severe disease and a detailed analysis of plasma donation recipients. The recipients must also be tested as part of the clinical evaluation of this therapeutic option because they may already have highly anti-RBD nAbs, in which case, additional antibody treatment may be ineffective (24). In addition, this assay can be used for the pre-clinical and clinical evaluation of SARS-CoV-2 vaccines efficiency and as a powerful tool to assess the duration and level of anti-RBD nAbs in population and cohort studies after infection or vaccination. Despite these potential advantages, new SARS-COV-2 mutations have raised concerns regarding nAb resistance in infection and vaccination responses (25-27). Therefore, more studies will be needed to determine the ability of the assay to estimate the neutralizing activity of Abs directed against variant-specific RBD domains.

In this study, the performance characteristics of SARS-COV-2-RBD neutralizing ELISA assay were validated by observing the qualitative agreement of its results for serum panels with those of the commercial total anti-SARS-COV-2 IgG antibody assay as a gold standard assay. The findings of a previous study had shown a qualitative agreement between the data of the commercial IgG serology assays and ELISA-based neutralizing assay and demonstrated that the results of positive IgG from commercial assays were linked reasonably to the presence of nAbs and they could be used as a substitute (21). Moreover, in comparison to other viral neutralization assays, recent studies found that the ELISA-based neutralizing assay was accurate in distinguishing between individuals with positive and negative anti-RBD nAbs (21, 28), with an excellent correlation in a quantitative manner (21).

The sensitivity and specificity of the in-house developed SARS-COV-2-RBD neutralizing ELISA assay were calculated by the prediction of the anti-RBD nAbs activity using commercial IgG serology assays results as a reference. Noticeably, among 50 serum samples that were collected from convalescent COVID-19 individuals at different early time points and detected with Abs by the commercial total anti-

SARS-CoV-2 IgG assays, 44 sera had a positive anti-RBD nAbs presence in the in-house neutralizing ELIZA assay (88% sensitivity). False-negative anti-RBD nAbs sera may occur when a weak Abs response develops in convalescent patients with a history of mild disease, which is below the limit of detection of the in-house neutralizing ELISA assay; therefore, it is necessary to perform other studies using other virus neutralization assays as reference. Even though commercial total anti-SARS-CoV-2 IgG assays are able to predict anti-SARS-COV-2 neutralization activity in a reasonable manner (21), these assays are indirect methods that cannot differentiate between binding and neutralizing Abs. The SARS-COV-2-RBD neutralizing ELISA assay provides the most specific and direct method for detecting neutralization function.

On the other hand, among 50 serum samples that were collected from healthy individuals and confirmed negative to total anti-SARS-CoV-2 IgG by a commercial assay, 46 sera were found negative in the in-house developed neutralizing assay (92% specificity). The few false-positives anti-RBD nAbs sera in the in-house developed neutralizing ELISA assay might have occurred in this study due to a lower used cut-off; that is, false-positive results were recorded inhibition of over 15% cut-off (0.17 OD), which was determined using the ROC curve analysis. It is noteworthy that the current cut-off value was used to reach compromise for acceptable sensitivity and specificity of detecting anti-SARS-CoV-2-RBD nAbs by the assay. The selection of a cut-off value that gives high sensitivity over the one with low sensitivity but high specificity is determined by illness prevalence. The lower thresholds are recommended for diagnosis in high-prevalence conditions, whereas a higher threshold may be preferable for screening purposes. As a result, to optimize clinical performance and minimize false-negative results, each lab can create its cut-off values. Other possibilities for the presence of false-positive results in the assay may be the interference of the cross-reactive Abs specific to other coronaviruses with the specificity of the SARS-COV-2 serological assays (29) or due to cross-contamination.

In a recent study, a similar observation for the sensitivity and specificity of ELISA-based neutralization assay (i.e., sVNT) in a group of > 14 days after symptom onset was reported at 91.2% and 94.4%, respectively (30). Additionally, the findings of early pieces of research reporting the development of similar assays have indicated a sensitivity of 95-100% and a specificity of 100% using cohorts from Singapore and China (16). It is worth noting that the variance in values of the sensitivity reported in most studies could be partially attributed to the differences in sample collecting time. The sensitivity of serological assays is usually lower in the early stage of infection (<7 days), while it stabilizes around 21 days after the onset of the symptom (31, 32). In the current study, a strong negative quantitative correlation was demonstrated between the OD readings of the assay and the different concentrations of neutralizing Abs standard for the quantitative manner of the assay.

The general limitation of ELISA-based neutralization assay is that it is capable to detect only anti-RBD neutralizing antibodies that work by blocking the RBD-ACE2 interaction rather than neutralizing antibodies targeted against the S2 or N-terminal domains of the S protein (33, 34). Although the limitation of this study might have affected the accuracy of the assay, the current study was designed to address this limitation by including positive (n=50) and negative (n=50) control serum panels for assay validation, and the positive control serum samples for assay validation were collected at longer different time-points after the first COVID-19 diagnosis.

The SARS-COV-2-RBD neutralizing ELISA assay serves as a sensitive and specific high-throughput qualitative and quantitative tool that can be applied in most laboratory settings without special biosafety requirements to detect anti-RBD nAbs for seroprevalence, pre-clinical, and clinical evaluation of COVID-19 vaccines efficiency and the rapid selection of convalescent plasma donors for the treatment of COVID-19 patients.

Authors' Contribution

Study concept and design: Z. S. M.
Acquisition of data: A. S. A.
Analysis and interpretation of data: Z. S. M.
Drafting of the manuscript: A. S. A.
Critical revision of the manuscript for important intellectual content: Z. S. M. and A. S. A.
Statistical analysis: Z. S. M.
Administrative, technical, and material support: . S. M. and A. S. A.

Ethics

This study was approved by the Institutional Review Board, College of Medicine, Al-Nahrain University. After obtaining the consent, blood samples were drawn from participants.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Supplementary Table 1. Serum panels using the in-house designed SARS-COV-2-RBD neutralizing ELISA assay; A) Positive control samples for the assay validation and B) Negative control samples for the assay validation

A.

Positive serum samples to total anti-SARS CoV-2 IgG by commercial kit	Absorbance OD readings (1:10) sample dilution factor	Positive/negative	log	Concentration ng/ml
sample 1	0.148	1	2.3769	238.1770983
sample 2	0.25	0	0.0768	1.193438379
sample 3	0.085	1	3.79755	6274.079248
sample 4	0.109	1	3.25635	1804.471387
sample 5	0.088	1	3.7299	5369.081545
sample 6	0.093	1	3.61715	4141.426899
sample 7	0.098	1	3.5044	3194.478724
sample 8	0.096	1	3.5495	3544.051303
sample 9	0.101	1	3.43675	2733.694632
sample 10	0.096	1	3.5495	3544.051303
sample 11	0.09	1	3.6848	4839.494493
sample 12	0.113	1	3.16615	1466.054111
sample 13	0.097	1	3.52695	3364.728293
sample 14	0.099	1	3.48185	3032.843496
sample 15	0.096	1	3.5495	3544.051303
sample 16	0.101	1	3.43675	2733.694632
sample 17	0.094	1	3.5946	3931.877693
sample 18	0.085	1	3.79755	6274.079248
sample 19	0.093	1	3.61715	4141.426899
sample 20	0.103	1	3.39165	2464.052745
sample 21	0.09	1	3.6848	4839.494493
sample 22	0.102	1	3.4142	2595.374301
sample 23	0.107	1	3.30145	2001.935127
sample 24	0.139	1	2.57985	380.0581062
sample 25	0.092	1	3.6397	4362.144019
sample 26	0.083	1	3.84265	6960.653256
sample 27	0.102	1	3.4142	2595.374301
sample 28	0.098	1	3.5044	3194.478724
sample 29	0.096	1	3.5495	3544.051303
sample 30	0.104	1	3.3691	2339.375838
sample 31	0.09	1	3.6848	4839.494493
sample 32	0.281	0	-0.62225	0.238643714
sample 33	0.095	1	3.57205	3732.931323
sample 34	0.1	1	3.4593	2879.386738
sample 35	0.091	1	3.66225	4594.624246
sample 36	0.089	1	3.70735	5097.415087
sample 37	0.089	1	3.70735	5097.415087
sample 38	0.099	1	3.48185	3032.843496
sample 39	0.258	0	-0.1036	0.78777102
sample 40	0.321	0	-1.52425	0.029905426
sample 41	0.252	0	0.0317	1.075721875
sample 42	0.248	0	0.1219	1.324036629
sample 43	0.084	1	3.8201	6608.455958
sample 44	0.092	1	3.6397	4362.144019
sample 45	0.097	1	3.52695	3364.728293
sample 46	0.104	1	3.3691	2339.375838
sample 47	0.091	1	3.66225	4594.624246
sample 48	0.092	1	3.6397	4362.144019
sample 49	0.106	1	3.324	2108.62815
sample 50	0.099	1	3.48185	3032.843496

B.

Sample series	OD	Positive/negative	Log	concentrationng/ml
sample 1	0.207	0	1.04645	11.1288426
sample 2	0.177	0	1.72295	52.83844158
sample 3	0.253	0	0.00915	1.021292165
sample 4	0.185	0	1.54255	34.87787365
sample 5	0.199	0	1.22685	16.85970611
sample 6	0.164	1	2.0161	103.7767343
sample 7	0.224	0	0.6631	4.603625638
sample 8	0.215	0	0.86605	7.345984371
sample 9	0.2	0	1.2043	16.00663346
sample 10	0.231	0	0.50525	3.200737072
sample 11	0.241	0	0.27975	1.904364162
sample 12	0.186	0	1.52	33.11311215
sample 13	0.193	0	1.36215	23.02236845
sample 14	0.201	0	1.18175	15.19672484
sample 15	0.168	1	1.9259	84.31405951
sample 16	0.185	0	1.54255	34.87787365
sample 17	0.189	0	1.45235	28.33674748
sample 18	0.211	0	0.95625	9.041698059
sample 19	0.201	0	1.18175	15.19672484
sample 20	0.198	0	1.2494	17.75824323
sample 21	0.243	0	0.23465	1.716524474
sample 22	0.206	0	1.069	11.72195366
sample 23	0.154	1	2.2416	174.4214932
sample 24	0.202	0	1.1592	14.42779623
sample 25	0.198	0	1.2494	17.75824323
sample 26	0.188	0	1.4749	29.84695288
sample 27	0.219	0	0.77585	5.968291135
sample 28	0.196	0	1.2945	19.70153207
sample 29	0.215	0	0.86605	7.345984371
sample 30	0.194	0	1.3396	21.85747549
sample 31	0.224	0	0.6631	4.603625638
sample 32	0.253	0	0.00915	1.021292165
sample 33	0.196	0	1.2945	19.70153207
sample 34	0.186	0	1.52	33.11311215
sample 35	0.217	0	0.82095	6.621402676
sample 36	0.21	0	0.9788	9.523574862
sample 37	0.199	0	1.22685	16.85970611
sample 38	0.184	0	1.5651	36.73668801
sample 39	0.231	0	0.50525	3.200737072
sample 40	0.214	0	0.8886	7.737488205
sample 41	0.225	0	0.64055	4.370689956
sample 42	0.193	0	1.36215	23.02236845
sample 43	0.191	0	1.40725	25.54171179
sample 44	0.218	0	0.7984	6.286370884
sample 45	0.221	0	0.73075	5.379600187
sample 46	0.184	0	1.5651	36.73668801
sample 47	0.169	1	1.90335	80.04791051
sample 48	0.198	0	1.2494	17.75824323
sample 49	0.214	0	0.8886	7.737488205
sample 50	0.247	0	0.14445	1.394601089