A Faster, Novel Technique to Detect COVID-19 Neutralizing Antibodies

Background: The COVID-19 pandemic has spread globally in a short period of time. It is known that antibody (nAb) level can effectively predict vaccine efficacy, which leads to the exploration of vaccine trials for efficacy assessment. Thus, the current study aimed to develop a platform to quantify nAb levels faster, at lower cost, and with better efficiency.

Material/Methods: A total of 69 sera samples were collected for the research, 28 of which were from unvaccinated participants. The other 27 samples and the remaining 14 samples were from the participants who had received the first and second dose, respectively, of AZ vaccine 1 month before. With cPass assays (Genscript cPass nAb ELISA assay) used as a criterion standard and lateral flow immunoassay kit (Healgen Scientific – LFIA test kit) coupled with a spectrometer (LFIA+S) for checking each specimen, we aimed to detect the presence of neutralizing antibodies in sera and to confirm the relationship between the inhibition rate from cPass assays and the nAb index from the LFIA+S.

Results: Data analysis of the research were taken from the certified ELISA and LFIA+S, which indicated a high consistency (Pearson’s r=0.864; ICC=0.90138) between the 2 methods.

Conclusions: The dataset demonstrated that LFIA+S was affordable, had a strong correlation with results of the cPass nAbs detection kit, and has potential clinical applications, with an exclusive feature that allows non-experts to use it with ease. It is believed that the proposed platform can be promoted in the near future.

Keywords: Antibodies, Neutralizing • COVID-19 • Self Efficacy • Spectrum Analysis

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Background

Since the initial outbreak was reported, COVID-19 has been identified as a public health issue and has caused millions of infections and deaths globally [1-4]. However, there is a broad agreement that eliminating the virus is no longer feasible [5-7]. Increasing the vaccination rate has been recognized as the most effective measure against the global pandemic. Therefore, critical issues such as rapid evaluation of the efficacy of the vaccine are of urgent concern [8-10]. Recently, it has been reported that levels of neutralizing antibodies (nAbs) are highly predictive of immune protection from symptomatic SARS-CoV-2 infection. This is because nAbs can inhibit the interaction between the receptor-binding domain of the spike-1 protein (S1-RBD) and human angiotensin-converting enzyme 2 (h-ACE2) [11,12]. Currently, there are 2 standard methods available for nAbs detection. One is the 50% plaque reduction neutralization test (PRNT50) [13-15], while the other is the FDA-approved SARS-CoV-2 Neutralization Antibody Detection Kit cPassTM (Genscript cPass neutralization antibody assay) [16-18]. In addition, which is a semi-quantitative detection method and the first surrogate neutralization assay to be commercially available. However, both methods mentioned above have difficulties, such as maintaining virus viability, biosafety level 3 (BSL 3) laboratory requirement, and being time-consuming to reveal the result [19-22].

On the other hand, the paper-based SARS-CoV-2 Neutralizing Antibody Test Kit (Healgen Scientific - LFIA test kit, Healgen Scientific, LLC, Houston, USA) shows several advantages, such as low cost, short processing time, and ease of use (it can be used by a non-expert), which make LFIA an ideal commercial kit to detect the nAbs [23,24]. However, as the LFIA test provides only qualitative results, the determination of nAbs level relies on the experimental operator's subjective cognition. Therefore, a novel platform that integrates LFIA with a portable spectrometer (LFIA+S) was proposed in this research to increase sensitivity and accuracy. Furthermore, the detection method was upgraded from qualitative level to semi-quantitative level [25,26].

To determine if LFIA+S is a highly reliable method for nAbs detection, statistical analyses were performed to compare the effects of the proposed integrated platform and the normal criterion standard cPass neutralization antibody assay [27]. We found that LFIA+S results are strongly correlated with those of cPass and LFIA+S only costs around US $5 per test, which is affordable for general clinics or areas with insufficient medical care. Our aim was to provide a highly reliable platform to make it easier to detect the level I of nAbs for COVID-19 vaccine efficacy.

Material and Methods

Ethics Approval

The protocol for this research was approved by the Institutional Review Board (IRB) of MacKay Memorial Hospital (IRB No: 21MMHIS141e) and Kaohsiung Medical University Chung-Ho Memorial Hospital (IRB No: KMUHIRB-F(I)-20200141). All of the participants understood the purpose of the study and agreed to sign the participant consent form prior to their participation.

Serum Samples

A total of 69 serum samples were studied in a retrospective cohort study from June 2021 to November 2021. Results of the observational study conducted with medical personnel of MacKay Memorial Hospital and Kaohsiung Medical University Chung-Ho Memorial Hospital were included in this study. Among them, 28 serum samples were collected from the people who had not received vaccination yet; the other 27 sera and 14 sera were obtained from people who had received the first and second dose, respectively, of the AZ vaccine 1 month earlier, before the experiment.

Spectral Analysis System

The spectral analysis platform is equipped with a cassette designed for the LFIA test kit to detect the absorbance spectrum. The spectral chip provides high spectral resolution (3-5 nm), the results of which were demonstrated with a certain wavelength range (300 to 1100 nm). The real-time spectrum is used as the experimental reference for the primary absorbance wavelengths, which was to detect the use of this platform from 500 nm to 600 nm and 680 nm.

SARS-CoV-2 Neutralizing Antibody Lateral Flow Immunoassay and Detection (LFIA Test Kit)

A commercially available SARS-CoV-2 Neutralizing Antibody Test Kit (Healgen Scientific – LFIA test kit, Healgen Scientific, LLC, Houston, USA) was used to detect the presence of neutralizing antibodies in sera. First, 25 μL of serum sample was loaded and mixed with 2 drops of the diluted buffer in the well for 10 min. The loaded test solutions can be driven through a conjugated pad by capillary force. As a competitive assay, the presence of neutralizing antibodies can be detected based on the signal intensity at the test line (T-line) and the control line (C-line), as shown in (Figure 1). This is because the neutralizing antibodies can inhibit the binding between the hACE-2 proteins (coated at T-line) and S1-RBD proteins (conjugated to colloidal gold nanoparticles and stored in a conjugation pad). In other words, the increase in neutralizing antibodies within a serum sample could lead to less binding of hACE-2 and
S1-RBD/gold nanoparticles and make the T-line lighter. The testing results were considered invalid if the C-line did not appear during the 20-min waiting time.

**Novel Technique Platform of Lateral Flow Immunoassay Coupled with Spectrometer**

In this research, a patent-pending testing platform was designed to demonstrate/display the result of LFIA, as shown in **Figure 2**. A specifically designed optical system was installed on the platform to reveal the absorbance spectrum of the gold nanoparticles (**Figure 3**) displayed on the test kit (T-line). The absorbance intensity ($\Delta A$) of the gold nanoparticles can be calculated by the following formula and was later used to quantify the amounts of neutralizing antibodies in sera:

$$\Delta A = A_{\text{max}(500-600 \text{ nm})} - A_{680 \text{ nm}}$$

The signal intensity of the T and C-line can be calculated as $\Delta A_{\text{T-line}}$ and $\Delta A_{\text{C-line}}$, respectively. The value of nAb can be derived by comparing the $\Delta A$ value of both test and control line:

$$n_{\text{Ab}} = \frac{1 - (\Delta A_{\text{T-line}})}{\Delta A_{\text{C-line}}}$$

The amount of neutralizing antibody is proportional to the nAb value. Then, nAb value was transferred to the cPass ELISA inhibition percentage with a regression fitting curve. The formula of the regression curve is:

$$\text{Inhibition percentage (})\% = 56.2873 \times n_{\text{Ab}} + 73.5053$$

We compared the inhibition percentage derived from nAb with the cPass ELISA results.
A commercial FDA-approved SARS-CoV-2 neutralizing antibody ELISA kit (Genscript cPass neutralization antibody assay) as a criterion standard was compared with the LFIA kit. Serum samples and positive and negative controls were first diluted 10 times and individually mixed with a volume ratio of 1:1 HRP-RBD solution. The mixed samples were then incubated at 37°C for 30 min. We loaded 100 μL of each mixed sample to a microplate well pre-coated with hACE-2 proteins. The microplate was then incubated at 37°C for 30 min and washed with washing buffer 4 times. We added 100 μL of TMB (3, 3', 5, 5'-Tetramethylbenzidine) substrate solution to each well in the microplate and then incubated it in the dark at room temperature for 15 min to start the enzymatic reaction. We added 50 μL of stop solution to each well, and the microplate was then read by an ELISA reader (Tecan Sunrise™). Since the neutralizing antibodies inhibit the bindings between RBD and hACE-2 proteins, the lower the optical density (O.D.) at 450 nm, the more neutralizing antibody there is. The absorbance of positive control must be less than 0.3, and the absorbance of negative control must be more than 1. The inhibition percentage is obtained from the following formula:

\[
\text{Inhibition percentage (\%)} = (1 - \frac{OD_{\text{sample}}}{OD_{\text{negative control}}}) \times 100\%
\]

The serum samples with less than 30% inhibition percentage were identified as negative cases.

**Statistical Analysis**

**t Test**

A 2-tailed t test was used to evaluate whether there was any statistically significant difference between the pre-vaccinated and vaccinated groups with the LFIA+S method. In this study, results yielding a P value of 0.05 were identified as borderline of statistical significance, P values under 0.01 were statistically significant, and P values below 0.001 were highly statistically significant.

**Receiver Operating Characteristic Curve**

The receiver operating characteristic (ROC) is an effective way to evaluate the overall accuracy of the proposed LFIA+S method by calculating the area under the curve (AUC). In general, the value of AUC ranges from 0 to 1, where a values of 0.9 to 1 reflect an outstanding result, values of 0.8 to 0.9 is considered excellent, and 0.8 to 0.7 is acceptable.

**Pearson’s r**

Pearson’s r was used to evaluate the correlation between cPass ELISA and LFIA+S. Pearson’s r between 0 to 0.25 was the absence of correlation, Pearson’s r between 0.25 and 0.5 was poor correlation, Pearson’s r between 0.5 and 0.75 was moderate to good correlation, and Pearson’s r between 0.75 and 1 was very good to excellent correlation.

**Bland-Altman Analysis**

Bland-Altman analysis was used to evaluate the agreement between cPass ELISA and LFIA+S. The upper limit of the 95% confidence interval was defined by adding 1.96 times standard deviations (SD) to the mean difference between these 2 methods; the lower limit of the 95% confidence interval was defined by subtracting the 1.96 times SDs from the mean difference. Data with 95% confidence interval were agreements between these 2 methods.

**Interclass Correlation Analysis**

We used intraclass correlation coefficient (ICC) analysis to estimate the reliability between cPass ELISA and the proposed LFIA+S. Coefficient of ICC below 0.5 was poor reliability, coefficient between 0.5 and 0.75 was moderate reliability, coefficient between 0.75 and 0.9 was good reliability, and coefficient above 0.9 was excellent reliability.

**Results**

The results are shown in Figure 4. The increasing concentration of neutralizing antibodies could replace the S-RBD and bind with h-ACE2, which leads to a lighter color of the T-line. However, no obvious relationship was found between the antibody concentration and C-line color. Therefore, 4 categories were defined based on the visual observation: T-line deeper than C-line (negative), T-line as deep as C-line (weak positive), T-line lighter than C-line (medium positive), and T-line fade away (high positive).
In addition, absorbance was utilized to reveal the spectrum result of the test kits. The spectral peak intensity ranges from 520 nm to 540 nm and depended on the colloidal gold, which is a determining marker for S-RBD. Figure 5 illustrates the results of the spectral observation, which once again confirms that the deeper the color of the T-line (HPOS), the higher the absorbance of the colloidal gold, and the C-line (NEG) does not consistently change with concentration variation.

After scanning the test kits with a spectrometer, an nAb index was calculated by a spectral result formula. To evaluate the diagnostic ability of nAb, we first considered those specimens
with an inhibition rate greater than 30% in the cPass kit as positive samples, and we viewed the others as negative samples. We used the t-test to compare the nAb value between positive and negative samples. As shown in Figure 6, the P value was less than 0.001, which shows that these 2 independent groups were significantly different. Then, we calculated the area under the receiver operating characteristic curve (ROC curve). This value was greater than 0.9, which means that nAb is a good predictor for distinguishing negative results from positive ones (Figure 7).

We performed both cPass assays and lateral flow test for each specimen. To confirm the relationship between the inhibition rate from cPass assays and the nAb index from lateral flow test, we performed linear regression analysis, showing a correlation coefficient of 0.864 (Figure 8).

We converted the inhibition percentage of cPass ELISA and LFIA+S. The Bland-Altman analysis showed a 94.2% (65 out of 69) confidence interval between cPass ELISA and LFIA+S (Figure 9). ICC analysis showed excellent reliability between the cPass and LFIA+S, and the coefficient was 0.90138, greater than 0.9 (Figure 10). With the information above, we are more confident that LFIA+S is a reliable solution to predict nAbs.

**Discussion**

With the outbreak of the COVID-19 pandemic and the threat of SARS-CoV-2 virus variants, there have been more reinfection cases [1,5,28,29]. Even fully vaccinated people still have a high risk of getting infections. It is a necessity that the vaccine effectiveness (VE) be tested, which can be analyzed by neutralizing antibody level (nAb level). As a result, it is important to
detect the nAb level in sera after being vaccinated [7,30,31]. In currently used detection methods, PRNT<sub>50</sub> is a criterion standard for verifying the nAb, which was first used to detect neutralizing antibodies in 2014. Although it has higher accuracy and sensitivity than other tests, such as hemagglutination and many commercial enzyme immunoassays, the main disadvantage is that it takes days to get results [10,15,18,22,32]. In addition, because live viruses are used while conducting PRNT<sub>50</sub>, it requires extremely strict operating procedure and high requirements for biosafety level and personnel operations. Therefore, it cannot completely meet a large number of testing requirements. To address the shortcomings of PRNT<sub>50</sub>, the first USFDA-EUA-approved commercial neutralizing antibody ELISA kit (Genscript cPass neutralization antibody assay) has turned out to be a better alternative way to quantify nAb.

Table 1 provides a simplified comparison and analysis. The results of the statistical analyses show that LFIA+S has an accuracy highly similar to that of cPass in the detection of nAb, and also has high specificity and sensitivity. Our results show that these 2 methods are highly correlated. The 69 clinical sera data were transferred from nAb value to percentage inhibition with the regression fitting formula. When comparing these serum data with Bland-Altman analysis, the result showed that 65 sera were in the critical interval, and only 4 entries were out of the interval. The agreement between these 2 methods was 94.2% (65 out of 69). ICC analysis showed the coefficient of ICC of the 2 methods was 0.90138, which demonstrated excellent reliability. In terms of the cost, LFIA+S does not require expensive reagents, and it only needs colloidal gold test paper for detecting neutralizing antibodies, at US $5 per test paper.

This current study has several limitations. The first limitation was the design of the optical platform; all the databases we established for comparing the concentration of gold nanoparticles to absorption spectrum information can only be conducted using cPass requires professional specialists to operate and a rigorous experimental environment, which needs a huge budget for large-scale testing. In the light of the defects of existing methods, the purpose of the current study was to develop a method that has the same high accuracy as cPass but is easier to use, quickly provides results, and is more affordable [33-35].

The LFIA+S method proposed in the current study is believed to be a more convenient way to get the nAb result. It takes only 3 min to perform, and it is easy to carry out because of the small size of the kit. In the current research, the LFIA+S method could significantly differentiate between the positive and negative sera at P<0.001. The area of the ROC curve was 0.9901, which indicated excellent accuracy. Comparing the proposed method with the cPass ELISA kit, Pearson’s r was 0.864, which meant that these 2 methods are highly correlated. The 69 clinical sera data were transferred from nAb value to percentage inhibition with the regression fitting formula. When comparing these serum data with Bland-Altman analysis, the result showed that 65 sera were in the critical interval, and only 4 entries were out of the interval. The agreement between these 2 methods was 94.2% (65 out of 69). ICC analysis showed the coefficient of ICC of the 2 methods was 0.90138, which demonstrated excellent reliability. In terms of the cost, LFIA+S does not require expensive reagents, and it only needs colloidal gold test paper for detecting neutralizing antibodies, at US $5 per test paper.

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This current study has several limitations. The first limitation was the design of the optical platform; all the databases we established for comparing the concentration of gold nanoparticles to absorption spectrum information can only be conducted using
the LFIA test kit produced by Healgen Scientific. In other words, using testing kits of other brands requires rebuilding the database. To assess all brands will require a significant storage space that makes real-time data and cloud computing impractical. Furthermore, it the number of collected samples should be larger; the insufficient sample size turned out to be the primary obstacle to fine-tuning the platform sensitivity. In the future, it will be essential that more vaccinated people are recruited so as to provide medical institutions with more valid data on vaccine efficacy.

Conclusions

As COVID-19 vaccine coverage increases, it is essential to investigate whether the vaccine efficacy is sufficient to prevent the spread of the pandemic. In reality, the existing methods for detecting nAbs level, PRNT$_{50}$ and cPass, rely heavily on professional personnel, strict experimental laboratory settings, and expensive detection costs (more than US $100/per test), yet the testing efficiency is low and it takes 3–5 days to get results. However, the results of the current study demonstrated that LFIA+S has a high correlation with cPass; furthermore, LFIA+S has several other advantages that the existing methods lack. LFIA+S features low testing cost (less than US $5/per test), higher effectiveness and efficiency (results can be shown in 3 min), and is more portable and easier to use. Ultimately, we hope that this study can serve as a valuable source that provides a new method for nAbs detection. Our proposed method shows great promise as a testing tool for evaluating the level of nAbs. We expect that the clinical application of LFIA+S can provide adequate data support for CDC to control the pandemic and implement reasonable vaccination standards.

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Statement

The staff of SpectroChip provided experimental technical support in this research. In addition, SpectroChip provided a spectrometer (the ONE InstantCare chromogenic rapid screening analyzer) for experimental use. SpectroChip had no other direct or indirect financial involvement in the study.

Declaration of Figures’ Authenticity

All figures submitted have been created by the authors, who confirm that the images are original with no duplication and have not been previously published in whole or in part.

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