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Development and Optimization of an Immunoassay for the Detection of Antibodies Against SARS-CoV-2 with In-house Recombinant RBD Protein

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Abstract

COVID-19 caused by SARS-CoV-2 poses a major threat to the global community, particularly in Indonesia. Countermeasures to prevent the spread of this disease have also been implemented, including the implementation of a vaccination program. An immunoassay technique that can be used to analyze antibodies that might develop following vaccination is the indirect enzyme-linked immunosorbent assay (ELISA). We produced the recombinant spike protein used in this study. The optimization comprised adjusted concentrations of spike recombinant protein (5 and 10 ng/mL), blocking agent (2.5% and 5%), and conjugate (1:1000 and 1:5000). The optimal conditions in this study included a spiked concentration of 10 ng/mL, a blocking agent concentration of 5%, sample dilution of 1:33, and a conjugate concentration of 1:1000. The intra-assay value of this optimized indirect ELISA was 7.3, and the inter-assay value was 5.3. The commercial MyBioSource kit and immunodiagnostic were utilized as a reference in the T-test, with *P*-values of 0 and 0.313, indicating that the recombinant protein in-house ELISA kit in this study demonstrated the same ability as the commercial immunodiagnostic kit in detecting SARS-CoV-2 antibodies, allowing it to be used for post-vaccination efficacy evaluation.

Keywords: ELISA, immunoassay, RBD protein, SARS-CoV-2, Spike

Introduction

An immunoassay is a bioanalytic technique for detecting the presence of a macromolecule or a small molecule in a solution using an antibody or an antigen [1]. Given its advantages, such as test's sensitivity, durability, and low cost, immunoassay enables most laboratories to promptly and consistently diagnose illnesses. The main principle of the immunoassay technique depends on the ability of antibodies to recognize and bind to specific molecules, such as antigens [2]. The enzyme-linked immunosorbent assay (ELISA) is a popular immunoassay based on the specific interaction between antigen and enzyme. Apart from being sensitive and specific, ELISA is a relatively quick test with high affinity and low cost [3]. ELISA can be used to identify viral proteins (such as viral components), antibodies, and hormones [4,5]. Furthermore, ELISA can be used to identify the presence of food allergens [6].

A new coronavirus surfaced in December 2019 as a cause of severe respiratory sickness and quickly spread, causing a worldwide epidemic, including Indonesia. Severe Acute Respiratory Coronavirus 2 (SARS-CoV-2) is due to a novel coronavirus in the β-coronavirus family. SARS-CoV-2 has a total length of 1,273 amino acids, and it is made up of signal peptides at the N-terminus, S1 subunit, and S2 subunit. The receptor binding domain (RBD) is found in the S1 subunit, and it is an essential component during infection [7]. During viral infections, RBD proteins are useful in mediating receptor identification, cell attachment, and fusion [8, 9]. SARS-CoV-2 has been reported in Indonesia since March 1, 2020, and the number of patients with COVID-19 in Indonesia reached 1.2 million until February 20, 2021 [10]. Vaccination is a method for preventing the spread of SARS-CoV-2, and it is considered successful if the antibodies required to fight the disease have been transmitted and have neutralized the virus that infects the body. In this case, ELISA may be used to identify the presence of these antibodies, and antibody testing using

ELISA will be required as a form of review of government's vaccination program.

In this study, we developed an ELISA based on an in-house RBD SARS-CoV-2 recombinant protein as antigens. We have worked on the development of the Spike SARS-CoV-2 recombinant protein. The plates were initially coated with protein recombinant SARS-CoV-2 RBD. Then the antibodies in the serum sample, which recognize the antigen, will bind to the ELISA microtiter plate. The RBD recombinant protein of SARS-CoV-2 is an antigen used to detect COVID-19 antibodies. The SARS-CoV-2 S protein, such as other coronaviruses, is involved in receptor recognition, cell binding, and fusion during viral infection [10]. A detailed study was conducted to build a precise and accurate ELISA method and showed its ability to determine the best series to detect COVID-19 antibodies. The development of an ELISA method for detecting SARS-CoV-2 antibodies has already been carried out by utilizing recombinant full-length nucleocapsid protein [11] and recombinant full-length spike protein [12]. In this study, we only used a specific area of the RBD protein on the SARS-CoV-2 spike protein.

Materials and Methods

Serum samples of vaccinated and recovered individuals (n = 6) were utilized in this study. The subjects in this trial had received two doses of the vaccination and had not received the booster shot. Six of these subjects with ages ranging from 34 to 52 years were vaccinated with the Sinovac vaccine. Three of the participants were COVID-19 survivors who were fully vaccinated and infected with SARS-CoV-2 between December 2020 and January 2021. The other three had no history of COVID-19 infection and had also been fully vaccinated. No history of comorbidity was reported among the subjects. The study was approved by IPB's Human Ethics Committee number 494/IT3.KEPMSM-IPB/SK/2021. All the participants provided informed consent to participate and their samples for the study. Serum samples were collected, heat inactivated at 56 °C for 30 min, and stored at −80 °C until being used [13].

In this study, we used the in-house RBD SARS Cov2 recombinant protein developed by other researchers in our research team. A synthetic gene (GeneBank MZ306692.1) encoding the RBD protein was expressed in the *E. coli* BL21 DE3 expression system using the pET17 vector. The purification of RBD recombinant protein expression was accomplished by affinity chromatography and dialysis. The SDS-PAGE device (Biorad) was used to analyze the quality of RBD recombinant protein expression. Commercial kits were used in this research for comparison, including MyBioSource ELISA Kit Cat No. MBS2614310 and immunodiagnostic ELISA Kit Cat No. 41A235.

In this study, SARS-CoV-2 recombinant RBD proteins were coated in different concentrations of 5 and 10 ng/mL per well to evaluate optimization. The plate was then incubated at 4 °C for 18 h. After incubation, the microplate was washed four times with a microplate washer. The blocking agent used in this optimization was skim milk (Blotto) at 2.5% and 5% concentrations. A blocking agent solution of 300 µL was added to each well and incubated at 37 °C for 1 h. The microplates were then washed four times using a microplate washer. Next, samples were inactivated. Meanwhile, standard spike antibodies were diluted to the following concentrations: 2.4, 1.2, 0.6, and 0.3 IU/mL. One hundred microliters of samples, calibrators, and controls were added to a designated well and incubated at 37 °C for 1 h. The plate was washed as previously described. Anti-human IgG peroxidase-conjugated secondary antibody was diluted in phosphate-buffered saline in ratios of 1:1000 and 1:5000, and 100 µL of the solution was added to each well. The microplates were washed four times using a microplate washer after an hour for incubation at 37 °C. Chromogen 3,3′,5,5′-Tetramethyl-benzidine was added at 100 µL per well and incubated for 20 min at room temperature in a dark room. The reaction was stopped by adding 2 N H_2SO_4, and the results were analyzed using a microplate reader at 450 nm. Furthermore, ELISA was conducted 10 times. We performed two-sample T-test for data analysis.

Table 1. The Medium Carbon Steel's Chemical Compositions

Sample	Subject Identity	Age	Gender	Date of Infection	Gene Target	
					HEX (E)	FAM (RdRp)
HU1	S + V	34	Female	January 21, 2021	33.83	34.73
HU5	S + V	52	Female	December 30, 2020	32.92	31.93
HU6	S + V	50	Female	January 12, 2021	23.06	23.87
HU8	V	47	Male	-	-	-
HU10	V	37	Female	-	-	-
HU12	V	43	Male	-	-	-

Abbreviations: S, COVID-19 survivors; V, fully vaccinated.

Results and Discussions

This study aimed to develop and optimize an ELISA kit for the detection of SARS-CoV-2 antibody with in-house recombinant RBD protein. This development was obtained systematically by considering the antigen concentration, blocking agent concentration, sample dilution, conjugate dilution, and calculating the inter-assay and intra-assay values [14, 15]. On the basis of the analysis of six serum samples, the results of in-house ELISA revealed that the optimal working conditions were as follows: 10 ng per well for coating, 5% blocking agent, 1:33 of sample dilution, and 1:1000 of conjugate dilution. An inadequate blocking concentration leads secondary antibodies to establish non-specific binding with antigens, resulting in excessive background signals and limited sensitivity [16]. This protocol was used for all subsequent experiments (Figure 1).

We determined the coefficient of variability (CV) of this ELISA method after identifying the ideal parameters (Table 1). The CV was calculated to express the precision or repeatability of the test results of the immunoassay, which were described with regard to intra-assay and inter-assay values, which were 7.3 and 5.3, respectively. In addition, the acceptable intra-assay and inter-assay values were less than 10% and 15%, respectively [17], indicating that the ELISA kit can be consistent even when used in a variety of circumstances and in repeated tests.

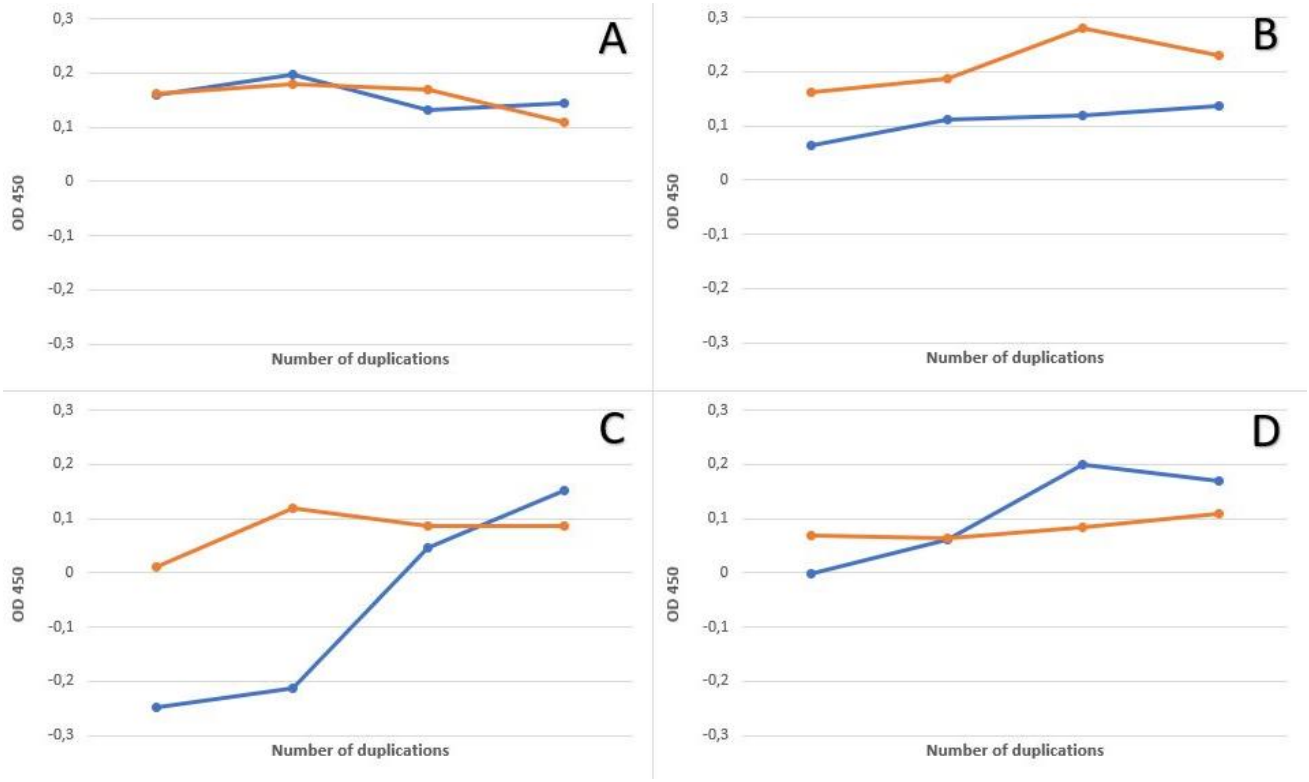

Figure 1. Optimization of an Indirect ELISA Utilizing SARS-CoV-2 RBD Protein. Graphic (A) Blocking Agent of 2.5%, Conjugate Dilution of 1:1000, with 5 and 10 ng of RBD Protein Concentrations. Graphic (B) Blocking Agent of 5%, Conjugate Dilution of 1:1000, with 5 and 10 ng of RBD Protein Concentrations. Graphic (C) Blocking Agent 2.5%, Conjugate Dilution 1:5000, with 5 and 10 ng of RBD Protein Concentrations. Graphic (D) Blocking Agent 5%, Conjugate Dilution 1:5000, with 5 and 10 ng of RBD Protein Concentrations. The Orange and Blue Lines Indicate 5 and 10 ng/mL of Recombinant Protein Concentrations, Respectively

Table 2. Mean Intra-Assay and Inter-Assay Percentage Coefficients of Variation (CVs) for Serum Samples

	% CV Intra-assay	% CV Inter-assay
Serum Sample	7.3	5.3

Table 3. Antibody Concentration of Each Sample

Sample	MyBioSource Cat No. MBS2614310 (U/mL)	Immunodiagnostic ELISA Kit Cat No. 41A235 (IU/mL)	In-house ELISA Kit with RBD Recombinant Protein (IU/mL)
HU1	−12.305	4.199	0.947
HU5	−7.559	3.686	1.115
HU6	−9.762	2.862	0.995
HU8	−9.254	0.032	0.987
HU10	−11.457	−0.102	0.883
HU12	−7.559	0.011	0.133

We compared our in-house ELISA kit with two commercial kits, namely, MyBioSource ELISA Kit Cat No. MBS2614310 and immunodiagnostic ELISA Kit Cat No. 41A235. The results of this concentration were calculated statistically using the T-test. Based on the T-test results, the *P*-value for the in-house ELISA kit was 0 compared with the MyBioSource RBD kit, indicating a significant difference between the two kits. Meanwhile, the *P*-value for the in-house ELISA kit compared with the RBD immunodiagnostic kit was 0.313, indicating that no statistically significant difference was found between the two kits. The *P*-value between the RBD protein in-house ELISA kit and the immunodiagnostic ELISA kit was higher than 0.05, indicating that the ability of the recombinant protein in-house ELISA kit to detect SARS-CoV-2 antibodies was similar to the performance of the immunodiagnostic ELISA kit.

In this study, RBD is the domain of SARS-CoV-2, which was used as a plate coating. It could elicit a neutralization reaction from antibodies, which can be evaluated to determine the extent to which it affects the immune system [18, 19]. The concentration of the sample tested using the MyBioSource ELISA kit showed a negative result, indicating that the kit had poor performance in detecting the presence of antibodies in serum. Meanwhile, the immunodiagnostic ELISA kit and recombinant RBD protein in-house ELISA kit showed good results in detecting antibodies in serum, although the two kits had differences in the amount of antibodies present. Neutralizing antibodies will have a high affinity for RBD, and the neutralization mechanism will be the attachment of antibody's heavy chain to RBD's receptor binding motif and the suppression of the loop region, preventing the virus from infecting the host [20]. We used RBD as a research material because of its immunogenic characteristics. Apart from the RBD protein in SARS-CoV-2, other structural proteins, such as membrane, envelope, and nucleocapsid proteins, may be used for the development of future ELISA approaches.

However, the need for high-quality testing methods remains expensive. Thus, developing such methods is needed in the current conditions. Several ELISA kits for antibody detection against SARS-CoV-2 have been created worldwide [21, 22, 23, 24]. The ELISA methodology reported in this work allows for the detection of RBD human anti-SARS-CoV-2 IgG antibodies. This in-house ELISA method would be useful for a variety of diagnostic and epidemiological purposes.

Conclusions

The best conditions for the in-house ELISA kit for RBD recombinant proteins were antigen concentration of 10 ng, blocking agent concentration of 5%, and conjugate dilution of 1:1000. The inter-assay value of the recombinant RBD protein in-house ELISA was <15%, indicating that the ELISA kit can be consistent even when performed under diverse settings. The intra-assay ELISA in-house recombinant RBD and N protein value is <10%, indicating that the test kit is consistent in repeated testing. The *P*-value difference between the recombinant RBD protein in-house ELISA kit and the immunodiagnostic ELISA kit is greater than 0.05, indicating that the recombinant protein in-house ELISA kit detects SARS-CoV-2 antibodies similar to the immunodiagnostic ELISA kit, which can be used to evaluate post-vaccination success. However, further tests must be conducted to determine the ability of antibodies produced after vaccination to neutralize SARS-CoV-2 through the serum neutralization test.

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