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Preparation of Lyophilized Reference Standard Anti-SARS-CoV-2 Antibody

Assajun Amen[1], Sompong Sapsutthipas[2], Bhurit Songthananit[1], Kanitta Phuwanartnaranubarn[1], and Supaporn Phumiamorn[3]

[1]*Institute of Biological Products, Department of Medical Sciences, Nonthaburi 11000, Thailand*

[2]*Medical Life Sciences Institute, Department of Medical Sciences, Nonthaburi 11000, Thailand*

[3]*Medical Sciences Technical Office, Department of Medical Sciences, Nonthaburi 11000, Thailand*

ABSTRACT High-quality vaccines that meet specific standards are the primary measures during the COVID-19 pandemic. Information on vaccine quality control and testing for anti-SARS-CoV-2 neutralizing antibodies against different variants is beneficial for developing vaccination policies and models to be used. The World Health Organization has established international antibody standards, which are still limited. This study aimed to develop the reference standard anti-SARS-CoV-2 antibody for Plaque Reduction Neutralization Test (PRNT), the gold standard for detecting neutralizing antibody against each SARS-CoV-2 variant, as for quality control of SARS-CoV-2 vaccines and for testing neutralizing antibody levels after vaccination. Serum antibodies in the form of lyophilized sera were tested comparatively to the lyophilized anti-SARS-CoV-2 monoclonal antibody (mAb). The serum antibody and mAb formulations were prepared, and freeze-drying conditions were optimized. After the quality of the freeze-dried serum antibodies was evaluated, the homogeneity of the mAbs did not meet the acceptance criteria, while that of the lyophilized pooled serum antibody was found to be physically and biologically acceptable as a reference standard. The serum antibody samples were tested against the Delta, Omicron BA.2, and Omicron BA.5 variants in terms of the $PRNT_{50}$ titer (95% confidence interval) as 512.97 (473–556), 288.14 (265–313), and 161.49 (124–184), respectively. Post-production potency tests of actual utilization were conducted for stability assessment, and the results were within the acceptance criteria (geometric mean ±3 SD). Therefore, the pooled serum antibody is suitable as the reference standard antibody against SARS-CoV-2 for vaccine quality control and for evaluating post-vaccination immune response during the COVID-19 pandemic.

Keywords: Reference standard SARS-CoV-2 antibody, PRNT, Lyophilization

Corresponding author E-mail: assajun.a@dmsc.mail.go.th

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Introduction

The development and evaluation of vaccines and testing methods require traceable reference standards, generally provided by recognized international agencies such as the National Institute for Biological Standards and Controls (NIBSC), the United Kingdom. Since the standard anti-SARS-CoV-2 antibodies provided by NIBSC are still insufficient for immunization testing in vaccinated individuals and for calibration use worldwide, the World Health Organization (WHO) recommends that any country develop its reference standards. Thailand faces several problems in the supply of reference standards, such as logistical challenges, higher costs, and access delays when relying solely on international standards. Reference standards are useful and necessary for National Control Laboratories (NCLs). The key properties of a biological standard are homogeneity and stability. Anti-SARS-CoV-2 antibodies contain proteins as active ingredients, which are sensitive to thermal degradation. The lyophilization process results in high stability of biological products with a long shelf-life,[1-5] thus, drying the products at low temperatures under vacuum conditions effectively maintains the protein's physical and chemical properties.

There are two main methods for measuring neutralizing antibodies: the Plaque Reduction Neutralization Test (PRNT) and the Pseudo-typed Virus-based Neutralization Assay (PVNA). The PRNT method is a gold standard exhibiting high sensitivity and specificity. Therefore, this study prioritized PRNT as the primary method for establishing the vaccine potency, which is crucial for assigning potency values for the reference standard anti-SARS-CoV-2 antibodies. However, the PRNT is time-consuming and requires live viruses, posing an infection. The PVNA method utilizes pseudo-typed virus particles, modified to contain specific proteins or receptors that bind specific immune response components. This method is faster and safer but may not be as sensitive or specific as the live virus PRNT method. Although antibodies are currently available for each strain of the virus, the test results are different, leading to doubts regarding the accuracy of antibody level measurements in each method.[6,7] Therefore, a reference standard for anti-SARS-CoV-2 antibodies can be used to compare the values obtained by different methods, which leads to the need to establish a reference standard for anti-SARS-CoV-2 antibodies.

The Institute of Biological Products (IBP), Department of Medical Sciences, Thailand, acts as the national control laboratory for vaccines and biological products and complies with the competence requirements for reference material producers according to ISO 17034:2016. Therefore, the IBP has the potential to serve as the national reference standard for anti-SARS-CoV-2 antibodies. This study hypothesized that the preparation of the lyophilized anti-SARS-CoV-2 antibodies reference standard by IBP would be suitable and useful for the vaccine quality control and testing for anti-SARS-CoV-2 antibodies against diverse virus variants.

Materials and Methods

Materials

SARS-CoV-2

The SARS-CoV-2 viruses used in this study were provided by the National Institute of Health, Department of Medical Sciences, Thailand. These included the Delta (Reference no. COV2071), Omicron BA.2 (Reference no.

SEQ/22-348), and Omicron BA.5 (Reference no. SEQ/22-17241) variants.

Human serum

Twenty-one heat-inactivated convalescent serum samples from COVID-19 patients, collected within 60 days after testing negative for the virus, and vaccinated individuals having PRNT50 titers against the SARS-CoV-2 Delta strain, 513.34–1,237.00, were pooled. The sera were obtained from the study project on "Monitoring of SARS-CoV-2 Antibody Levels after Vaccination against COVID-19 (Research project code 11/2564)" that were negative for HBsAg, Anti-HIV, and HCV.[8,9]

Monoclonal antibody

Monoclonal antibody (mAb) was obtained from the Center of Excellence in Systems Biology, Chulalongkorn University, Thailand, which was specific to the SARS-CoV-2 receptor-binding domain (RBD) 3D2 at a concentration of 4.97 mg/mL, and met the following criteria: concentration ≥ 0.5 mg/mL expression host mammalian; purity ≥ 90% (SDS-PAGE) and 98% (SEC-HPLC); endotoxin ≤ 0.1 EU/mg; and the anti-SARS-CoV-2 antibody titer ≥ 1,000 units (PRNT against Delta).[8,9]

WHO international standard

WHO international standard for anti-SARS-CoV-2 immunoglobulin (human) NIBSC code: 20/136 (NIBSC, UK)[8,9].

Methods

Preparation of pooled serum

The pooled serum antibody consisted of 50% (v/v) serum and 50% (v/v) Milli-Q ultrapure water (Sigma-Aldrich, USA). The monoclonal antibody formulations were prepared as 0.0566% (w/v) mAbs, 0.0357% (w/v) L-histidine HCl monohydrate, 0.0357% (w/v) L-histidine, 0.0357% (w/v) trehalose dihydrate, and 0.01% (v/v) polysorbate 20.

Lyophilization

The collapse temperature was tested using freeze-drying microscopy (Linkam FDCS196, Linkam Scientific Instruments Ltd., UK), and the average subsidence temperature was recorded to calculate the primary freeze-drying temperature to set the lyophilization conditions. Serum antibody samples were tested in triplicate. The collapsing temperature was obtained at −15, −16.6, and −17.8°C, with a mean value of −16.5°C. Therefore, the primary drying temperature was set at −21°C (3–5°C less than the collapsing temperature). Each 0.5 mL serum antibody was added into 3-mL borosilicate type I glass clear vials (Schott, Indonesia) under sterile conditions, and a half-close rubber stopper (4405/50 Gray; West Pharmaceutical Services, Inc., USA) was placed over. The conditions obtained were used to program the lyophilizer (LyoLab LT; Lyophilization Systems Inc., USA). The serum vials were frozen at -40 °C for 180 min, followed by primary drying at -21°C and 0°C under a 200-mTorr vacuum for 1,180 min, and a 200-mTorr vacuum for 1,000 min. This was followed by secondary drying at 25°C under 100-mTorr for 360 min. Vials were then backfilled with 99.999% nitrogen gas. The formulated mAb was tested in triplicate by freeze-drying microscopy. The collapsing temperatures were −28.8, −28.9, and −28.6°C, with a mean of −28.8°C; therefore, the primary

drying temperature was set at −34°C. For lyophilization, mAb vials were frozen at -60°C for 180 min, followed by primary drying at -34°C under a 200-mTorr vacuum for 2,560 min. This was followed by secondary drying at 25°C under a 100-mTorr vacuum for 300 min. Vials were then backfilled with 99.999% nitrogen gas.

Quality control testing (visual inspection and moisture content testing)

The physical characteristics of lyophilized serum and anti-SARS-CoV-2 mAb were observed and recorded by visual inspection for 10 vials before and after dissolution. After dissolution, the lyophilized serum and mAb samples were reconstituted in 0.5 mL of distilled water for 180 seconds; the reconstitution time was determined for 10 vials. The moisture content of 3 vials was determined using volumetric Karl-Fischer titration.[10] The recommended moisture content is not more than 3%.

Assessment of homogeneity and stability

The homogeneity was assessed to show the uniformity of the pooled serum in each container. A production plan using a balanced nested design, which ensures statistical consistency across replicate measurements by maintaining equal sample units per run, thereby improving variance estimation and simplifying ANOVA analysis, was implemented. A PRNT test against the Delta strain was developed to assess the homogeneity of lyophilized serum antibodies in each container. Twelve vials were tested for 3 days, with four vials tested each day and two replicates per vial. Statistical analysis was performed using two-way ANOVA with the replicates.[11,12] Homogeneity was assessed by comparing the between-unit component of variance from a homogeneity study, expressed as a between-unit standard deviation (S_{bu}) with the coefficient of variation (CV) obtained from a collaborative study on the international reference preparation (IRP) of anti-SARS-CoV-2 antibody (NIBSC Code No. 20/136) using the PRNT method. The criterion for homogeneity was set at S_{bu}< CV/3. For stability assessment, post-production monitored stability testing was conducted at −20 °C or below. The stability monitoring involved evaluating potency based on actual usage data, with predefined acceptance criteria set as within the geometric mean ±3 standard deviations. The production plan involved monitoring the use of the products and notifying customers if the criteria values were not met (geometric mean +3 standard deviations) through the institutional communication channel.

The anti-SARS-CoV-2 neutralizing antibody determination

The lyophilized pooled serum was independently determined to have the levels of anti-SARS-CoV-2 neutralizing antibody using the PRNT, PVNA, and Enzyme-linked immunosorbent assay (ELISA) methods against the Delta, Omicron BA.2, and Omicron BA.5 variants as the methods described. Twelve vials were tested over 3 days, with 4 vials tested each day against the international standard anti-SARS-CoV-2 antibody.

Plaque Reduction Neutralization Test (PRNT)

In this study, the PRNT was developed and validated by the Institute of Biological Products

(IBP), Department of Medical Sciences, Thailand.[13] Vero cells were cultured in 6-well clear tissue culture-treated multiple well plates (Corning, USA) at a density of 2×10^5 cells/well/3 mL and incubated at 37°C with 5% CO_2 for 1 day. The lyophilized serum was initially diluted to 1:10, 1:40, 1:160, and 1:640. Then, the SARS-CoV-2 virus was diluted in Minimum Essential Medium Eagle (MEM) (Sigma-Aldrich, USA) to yield 40–120 plaques/well in the virus control wells. Cell control wells, convalescent patient serum, and normal human serum were used as the assay controls. Neutralization was achieved by mixing an equal volume of diluted sera with the SARS-CoV-2 virus at 37°C in a water bath for 1 h. After removing the culture medium from the Vero cell culture plates, 200 µL of the virus-serum antibody mixture was added to the monolayer cells, and the plates were shaken every 15 min for 1 h. After removing the excess virus, 3 mL of semi-solid medium containing 1% carboxymethylcellulose (Sigma-Aldrich, USA), 1% penicillin-streptomycin (Sigma-Aldrich, USA), and 10% FBS (Gibco, USA) was overlayed. All plates were incubated at 37°C with 5% CO_2 for 7 days. Cells were then fixed with 10% formaldehyde (Sigma-Aldrich, USA) and stained with 0.5% crystal violet (Merck, USA) in PBS (Gibco, USA). The number of viral plaques was counted in triplicate wells, and the percentage of plaque reduction at 50% ($PRNT_{50}$) was calculated. The $PRNT_{50}$ titer of the test sample was defined as the reciprocal of the highest test serum dilution for which virus infectivity was reduced by 50% compared with the average plaque counts of the virus control, which was calculated using a four-point linear regression method. Plaque counts for all serial dilutions of serum were scored to ensure a dose-response.

Pseudo-typed Virus-based Neutralization Assay (PVNA).

PVNA used in this study was performed according to the method published by the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand.[14] To determine the neutralizing activity of the lyophilized serum samples, a two-fold serial dilution of the sera was prepared starting at 1:40 in the culture medium of high-glucose DMEM (Gibco, USA) without FBS. The serum samples were mixed with the pseudo-typed viruses containing the CoV-2 spike of interest at a ratio of 1:1 (v/v) in a 96-well plate (Corning, USA). The pseudo-typed virus input was normalized to 1×10^5 RLU/well. The serum-pseudo-typed virus mixture was then incubated at 37°C for 1 h. Cell suspensions of HEK293T-ACE-2 pre-transfected with pCAGGS expressing human TMPRSS2 (2×10^4 cells/mL) were then mixed with the serum-pseudo-typed virus mixture and seeded into each well of the culture plates and incubated at 37°C for 48 h. Neutralizing antibodies were determined by the luciferase activity.[13]

Enzyme-linked Immunosorbent Assay (ELISA)

The ELISA was validated by the Institute of Biological Products (IBP), Department of Medical Sciences, Thailand. An anti-SARS-CoV-2 QuantiVac ELISA (IgG) test kit (Cat No. EI 2606-9601-10G; EUROIMMUN, USA) was used. The SARS-CoV-2 IgG antibody concentration in serum was determined by its reaction with SARS-CoV-2 antigen coated in

wells, forming an Ag-Ab complex. The addition of peroxidase-labelled anti-human IgG triggered a color change upon reaction with TMB/H_2O_2 substrate, which was measured at 450 nm with a reference wavelength of 630 nm (620-650 nm). The antibody concentration was directly proportional to the absorbance, and results were calculated using point-to-point comparison with standard material by 4-parameter logistic regression. The results were reported in Binding Antibody Units (BAU). This test kit does not differentiate between viral strains because it detects the presence of antibodies against the SARS-CoV-2 antigen, which is common to all strains, rather than identifying specific viral variants.[15]

Statistical Analysis

The geometric mean was used to represent the central tendency of a skewed set of antibody-level data, and the 95% confidence interval of the geometric mean represented the range of estimates. The % Coefficient of Variation (%CV) is a measure of variation in a set relative to the mean of the antibody-level data. The *p*-value obtained from the Shapiro–Wilk normality test is a measure of the tendency of antibody-level data sets to follow a normal distribution. If the *p*-value was < 0.05, it was concluded that the data were not normally distributed. If the F-test yielded a *p*-value < 0.025, it was concluded that the variances of the two antibody-level data sets from different methods significantly differed. For normally distributed data sets, if the *t*-test yielded a *p*-value < 0.05, it was concluded that the means of the two antibody-level data sets were significantly different. For non-normally distributed data sets, if the Mann–Whitney test yielded a *p*-value < 0.05, it was concluded that the medians of the two data sets were significantly different. As the two data sets were derived from different methods, the data were transformed before different testing of the means or medians using the proportion of the geometric mean of the antibody-level data sets of the two methods. To compare different species using the same test method, one-way ANOVA and Tukey's test were used for a normal distribution, and Kruskal–Wallis and Dunn's multiple comparison tests for a non-normal distribution, with a *p*-value < 0.05 was used to determine statistical significance. Homogeneity was assessed using two-way ANOVA with replication. Statistical analyses were performed using GraphPad Prism Version 5 software (GraphPad Software, USA) and Microsoft Excel 2016.

Results

Characteristics of the lyophilized pooled serum and anti-SARS-CoV-2 monoclonal antibody

Details of the pooled serum and anti-SARS-CoV-2 mAb before and after lyophilization (Lyophilized products) were summarized in Table 1. The lyophilized pooled serum (Lot No. Lyo-110122) and mAbs (Lot No. Lyo-Ab170122) exhibited distinct post-lyophilization physicochemical profiles. Both formulations were filled at 0.5 mL per vial, with 393 and 568 vials prepared, respectively. The pooled serum presented as a coagulated dry powder and reconstituted into a yellow solution with particulate formation in 80% of tested vials,

whereas the mAbs formed a clear solution from a white freeze-dried powder. Residual moisture content was 2.43±0.15% for pooled serum and 3.65±2.23% for mAbs. Reconstitution time differed markedly, with pooled serum requiring 140.4 ± 0.41 seconds versus 10.90 ± 0.88 seconds for mAbs. Mean fill mass was comparable (0.52 g/vial vs. 0.53 g/vial), but homogeneity was sufficient only in the pooled serum, indicating superior formulation robustness relative to the mAbs. All quality control tests for the lyophilized pooled serum met the acceptable criteria, except for the appearance of the serum after dissolution, which showed a white fibrin line. This could be verified by the homogeneity assessment. While the mean of residual moisture of the lyophilized mAb was higher than 3%, which might affect the homogeneity.

Homogeneity was assessed by comparing the CV of eight values of the collaborative study WHO International Standard of anti-SARS-CoV-2 immunoglobulin (human) (NIBSC Code No. 20/136), measured by the PRNT method, using the SD of the homogeneity test. The pooled serum Lot No. Lyo-110122 demonstrated sufficient homogeneity, while the mAb Lot No. Lyo-Ab170122 formulation was insufficiently homogeneous.

Table 1 Characteristics of the pooled serum and anti-SARS-CoV-2 monoclonal antibody (mAb) before and after lyophilization (Lyophilized products).

Details (Before lyophilization)	**Pooled serum**	**mAb**
Antibody content	21 serum samples (convalescent patients and vaccinated persons)	Protein Name: 3D2
Criteria	- HbsAg Anti-HIV and HCV were negative - Potency $PRNT_{50}$ titers ≥ 500 (PRNT (Delta)	- Concentration ≥ 0.5 mg/mL, Purity ≥ 90% (SDS-PAGE) and 98% (SEC-HPLC) - Endotoxin ≤ 0.1 EU/mg Antibody titer ≥ 1000 units: PRNT (Delta)
Formulation	serum 50% and Milli-Q® ultrapure water 50%	L-histidine HCl monohydrate, 0.0566%; L-histidine, 0.0357%; Trehalose dihydrate, 0.0357%; Polysorbate 20, 0.01%.
Nominal fill volume	0.5 mL	0.5 mL
Product code (Lot No.)	Lyo-110122	Lyo-Ab170122
Number of prepared vials	393 vials	568 vials
Appearance before dissolution	Coagulated dry powder	White freeze-dried powder
Appearance after dissolution	A yellow solution with one white line per vial; found in 8 out of 10 vials	clear solution
Mean residual moisture (%)	2.43±0.15 (n = 3)	3.65±2.23 (n = 3)
Reconstitution time	140.4 s±0.41 (n = 10)	10.90 s±0.88 (n = 10)
Mean fill mass	0.52 g/vial (CV 0.73%, n = 10)	0.53 g/vial (CV 0.89%, n = 10)
Homogeneity	sufficient	insufficient

The anti-SARS-CoV-2 neutralizing antibody determination

Comparison of antibody levels against the SARS-CoV-2 Delta, Omicron BA.2, and Omicron BA.5 variants tested with the $PRNT_{50}$, PVNA, and ELISA methods of the lyophilized pooled serum Lot No. Lyo-110122 were shown in Table 2 and Figure 1. These showed neutralizing activities against the SARS-CoV-2 Delta, Omicron BA.2, and Omicron BA.5 variants. Whereas the lyophilized mAbs Lot No. Lyo-Ab170122 only specifically responded to the SARS-CoV-2 Delta variant and had a low response to the Omicron BA.2 using the PRNT method, as shown in Table 3. The WHO international standard 20/136 showed a significant response to the Delta and a low response to the Omicron BA.2 variants using the PRNT method. The PVNA method showed no response to either Omicron BA.2 or BA.5.

Table 2 Comparison of neutralizing antibody levels against the SARS-CoV-2 Delta, Omicron BA.2, and Omicron BA.5 variants tested with the $PRNT_{50}$, PVNA, and ELISA methods of the lyophilized pooled serum Lot No. Lyo-110122.

Vials	Delta		BA.2		BA.5		ELISA BAU /mL
	PRNT	PVNA	PRNT	PVNA	PRNT	PVNA	
1 (d1)	517.00	167.66	287.00	163.60	139.00	244.68	2,551.00
2 (d1)	512.00	168.30	349.00	196.29	273.00	303.30	2,881.00
3 (d1)	552.00	175.13	254.00	200.08	140.00	344.23	2,701.00
4 (d1)	654.50	173.08	312.00	201.73	146.00	324.55	2,221.00
5 (d2)	480.50	167.60	231.00	211.97	149.00	221.93	3,000.00
6 (d2)	525.00	168.85	289.00	247.24	167.00	198.62	2,931.00
7 (d2)	507.50	167.63	271.00	266.38	189.00	241.35	2,784.00
8 (d2)	574.50	210.11	289.00	347.47	72.00	295.92	2,794.00
9 (d3)	422.50	161.56	283.00	205.09	123.00	184.63	2,991.00
10 (d3)	474.50	162.57	256.00	214.78	156.00	167.51	2,822.00
11 (d3)	418.50	158.63	373.00	210.66	174.00	250.22	2,663.00
12 (d3)	562.50	162.14	292.00	233.07	160.00	191.91	3,093.00
Geometric mean	512.97	169.84	288.14	150.98	221.03	241.20	2,776.24
(95%CI)	(473-556)	(162-178)	(265-313)	(196-249)	(124-184)	(208-280)	(2,623-2,938)
%CV	12.80	7.90	13.73	21.09	30.97	24.14	8.48
Compare	$PRNT_{50}$/PVNA	3.02	$PRNT_{50}$/PVNA	1.30	$PRNT_{50}$/PVNA	0.63	-
Shapiro-Wilk (normal distribution)	0.0002	no	0.4640	yes	0.3430	yes	-
***t*-test**	nc	nc	0.0012	s	0.0004	s	-
Mann-Whitney	0.0000	s	nc	nc	nc	nc	-
WHO 20/136(d1)	51.00	125.05	<10	0	10	0	-
WHO 20/136(d2)	31.00	272.86	12	0	<10	0	-
WHO 20/136(d3)	53.00	104.21	20	0	<10	0	-

Note: s = significant, ns = not significant, nc = not calculated, d = day, WHO 20/136 diluted to 50 IU/mL, WHO 20/136 = First WHO International Standard for anti-SARS-CoV-2 immunoglobulin (human) NIBSC code: 20/136, PRNT = Plaque Reduction Neutralization Test, PVNA = Pseudo-typed Virus Neutralization Assay, ELISA = Enzyme-Linked Immunosorbent Assay, Delta = SARS-CoV-2 Delta variant, BA.2, BA.5 = Sublineages of the Omicron variant and BAU = Binding Antibody Units

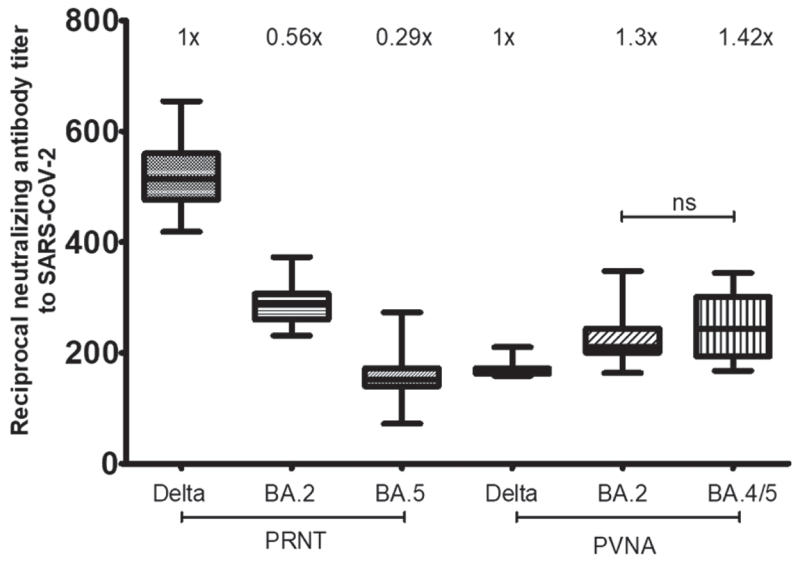

Figure 1 Comparison of neutralizing antibody levels of the lyophilized pooled serum Lot No. Lyo-110122 against the Delta, BA.2, and BA.5 SARS-CoV-2 variants using the PRNT and PVNA test methods. The Kruskal–Wallis and Dunn's multiple comparison tests were used, and $p < 0.05$ indicated significance (ns = not significant)

Table 3 Comparison of neutralizing antibody levels against the SARS-CoV-2 Delta and Omicron BA.2 variants tested with the $PRNT_{50}$ method of the lyophilized mAbs Lot No. Lyo-Ab170122.

Vials	Delta	BA.2
1 (d1)	3,580.00	< 10
2 (d1)	2,404.50	< 10
3 (d1)	3,625.00	10.00
4 (d1)	3,649.00	15.00
5 (d2)	2,912.50	< 10
6 (d2)	2,881.00	12.00
7 (d2)	3,297.00	10.00
8 (d2)	2,563.00	12.00
9 (d3)	1,561.50	< 10
10 (d3)	2,188.50	< 10
11 (d3)	1,967.00	< 10
12 (d3)	1,937.50	< 10
Geometric mean (95%CI)	2,621.10 (2,236-3,072)	nc
%CV	27.57	nc
WHO 20/136 (d1)	55.00	< 10
WHO 20/136 (d2)	46	12
WHO 20/136 (d3)	45	20

Note: nc = not calculated, d = day, WHO 20/136 diluted to 50 IU/mL, WHO 20/136 = First WHO International Standard for anti-SARS-CoV-2 immunoglobulin (human) NIBSC code: 20/136, Delta = SARS-CoV-2 Delta variant and BA.2 = Sub-lineages of the Omicron variant

Discussion

The preparation of pooled serum and monoclonal antibodies (mAbs) showed clear differences in stability during freeze-drying. Pooled human serum needed only water in its formulation with no added stabilizers because it stayed naturally stable during lyophilization.[16] By contrast, mAbs usually need complex stabilizer mixtures. This high stability of human serum comes from its natural composition, which allows it to withstand freeze-drying well. Human serum also serves as a protective agent (lyoprotectant) for other biological samples during freeze-drying, giving it two valuable roles in biological preservation.[17]

The appearance of lyophilized serum antibodies after reconstitution, which appeared as a white fibrin line, could be affected by incomplete clotting during serum collection. This might occur because of the conversion of fibrinogen into fibrin.[18] Fibrin deposition can interfere with the formation and counting of viral plaques, leading to inaccurate results. Conversely, fibrin deposition can create a background signal that interferes with plaque counting and leads to overestimating the antibody titer[19]. The results of the homogeneity assessment showed that fibrin did not sufficiently affect the antibodies to cause inhomogeneity. To prevent fibrin formation, it is important to allow complete blood clotting before separating serum. After separation, the serum was stirred, filtered, and gently stirred again before being filled into the vial. On the contrary, the Lot. No. Lyo-Ab170122 mAb formulation showed insufficient homogeneity. This issue might be attributed to the mean residual moisture of the mAbs over 3%. High residual moisture levels can lead to protein denaturation, consequently affecting the homogeneity of the formulation.[20]

The neutralizing antibody of the WHO international standard 20/136 to the SARS-CoV-2 Delta variant obtained using the PVNA method was significantly higher than that of the $PRNT_{50}$ method, but the antibody levels against Omicron BA.2 and BA.5 were < 20 by both methods. Previous data were consistent with those of our study, determining the antibody level of the WHO international standard 20/136 prepared from convalescent patient sera during the Delta strain outbreak and previous outbreak strains.[21] Following WHO guidelines, the relative potency of the secondary standard against the international standard in IU/mL was determined using parallel-line analysis. This approach was the preferred option for data analysis.[22] However, it should be noted that this approach should only be performed when the WHO standard is appropriately responsive to the SARS-CoV-2 variant/s being studied. Previous research on anti-SARS-CoV-2 antibody titers has been limited in terms of reporting values in international units per milliliter (IU/mL). The $PRNT_{50}$ method is widely accepted and frequently used for quantifying SARS-CoV-2 antibodies[6,7] and was chosen as the appropriate method for our study, which was sufficient for the intended purpose.

The level of neutralizing antibodies in the lyophilized serum Lot No. Lyo-110122 was determined using different methods and compared using means or medians (according to the data

distribution). The antibody levelsof the $PRNT_{50}$ method showed significant differences of neutralizing antibodies to the Delta, Omicron BA.2, and BA.5, which were 3.02-, 1.3-, and 0.63-fold that of the PVNA, respectively ($PRNT_{50}$ divided by PVNA). While the ELISA effectively detected antibodies against SARS-CoV-2, it could not differentiate between responses to specific variants or lineages. This is primarily because conventional ELISAs utilize conserved or ancestral viral antigens. Antibodies generated following infection with different variants commonly display considerable cross-reactivity with these antigens, preventing discrimination based on overall binding signal. Distinguishing variant-level responses requires assays specifically designed to capture differential antibody binding to variant-specific epitopes or assess neutralizing activity.[15]

Determination of neutralizing antibodies against different live SARS-CoV-2 variants using the $PRNT_{50}$ method revealed significant differences in antibody titers against the Delta and Omicron variants. The neutralizing antibodies against the Omicron variants BA.2 and BA.5 also showed a substantial reduction, 0.56- and 0.29-fold, respectively, relative to the Delta variant. Our data also showed that the antibody titers of the Delta and Omicron BA.5 variants were 1.78- and 0.52-fold relative to Omicron BA.2, respectively, corresponding to the study which has reported the neutralizing antibody levels of the 1st generation vaccinated and recovered patients in 2020 during the outbreak of the Delta and earlier variants.[14] There was also another report describing the antibody levels against Omicron BA.5 0.22- and 0.67-fold relative to Omicron BA.2.[6] Moreover, the level of neutralizing antibodies to different variants by the PVNA method showed that the median level to the Delta strain was significantly different from that of Omicron BA.2 and BA.5. In contrast, the median antibody level of Omicron BA.2 was comparable to that of BA.5 with non-significant differences, and compared with each other, the level of neutralizing antibodies was 1-, 1.3-, and 1.42-fold relative to the Delta variant, respectively, differing from the PRNT method. The advantage of the PVNA method is that it can be performed in biosafety level 2 (BSL2) and takes only 2 days to finish testing, whereas the PRNT requires biosafety level 3 (BSL3) and takes 3–7 days after the incubation period with the virus.[6,7]

In the aspect of homogeneity, sufficient homogeneity was observed in the evaluation of the lyophilized pooled serum prepared Lot No. Lyo-110122. The geometric mean was within ±3 standard deviations, indicating that it had properties as a reference standard antibody. As a consequence, the data generated from this study provided a valuable foundation for developing reference standards for other SARS-CoV-2 variants. For the establishment of this reference standard, it is not used as a secondary reference standard since its value was not defined in IU, but in $PRNT_{50}$ titers, the value of which was sufficient and widely accepted as a reference standard for quality control of COVID-19 vaccines and the measurement of the immune responses.

However, improving the sensitivity of the PVNA method to match that of the PRNT is

critical to enable more rapid testing, be safer without using the live virus, and be practical in laboratories lacking BSL-3 facilities, particularly in rural areas. Strengthening inter-agency collaboration among research institutes, hospitals, and vaccine manufacturers to share data and updated standards is essential for maintaining the relevance and effectiveness of these materials. Given Thailand's experience with multiple COVID-19 waves and the rapid spread of variants like Delta and Omicron, these measures are vital for ensuring timely and accurate responses to future public health challenges.

Post-production stability of the reference standard was assessed at -20°C or below through potency evaluation using actual usage data against ±3 standard deviation criteria. Control charting of these data enabled trend analysis to predict when stability would fall outside these limits. This predictive capacity facilitates timely decisions regarding the preparation of subsequent lots to ensure continuous availability, and conversely, informs discontinuation if the strain or variant is no longer relevant.

Conclusion

Evaluation results of the standard antibody Lot No. Lyo-110122 exhibited suitable physical and biological characteristics to use as a reference standard antibody against SARS-CoV-2 in lyophilized form for vaccine quality control and neutralizing antibody testing in vaccinated populations. Standard antibody levels against the SARS-CoV-2 Delta variant were determined by PRNT at 512.97 (473-556) (Geometric mean, 95% CI), Omicron variants BA.2 at 288.14 (265-313), and BA.5 at 161.49 (124-184). For further research, one should also consider testing for SARS-CoV-2 variants that are endemic at particular times.

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การจัดเตรียมแอนติบอดีมาตรฐานอ้างอิงต่อเชื้อ SARS-CoV-2 ชนิดทำแห้งแบบแช่เยือกแข็ง

อัศจรรย์ อาเมน[1] สมปอง ทรัพย์สุทธิภาสน์[2] ภูริต ทรงธนนิตย์[1] กนิษฐา ภูวนาถนรานุบาล[1] และ สุภาพร ภูมิอมร[3]

[1]สถาบันชีววัตถุ กรมวิทยาศาสตร์การแพทย์ นนทบุรี 11000

[2]สถาบันชีววิทยาศาสตร์ทางการแพทย์ กรมวิทยาศาสตร์การแพทย์ นนทบุรี 11000

[3]สำนักวิชาการวิทยาศาสตร์การแพทย์ กรมวิทยาศาสตร์การแพทย์ นนทบุรี 11000

บทคัดย่อ วัคซีนที่มีคุณภาพได้มาตรฐานเป็นเครื่องมือหลักในการแก้ปัญหาการระบาดใหญ่ของโรคโควิด 19 ข้อมูลการควบคุมคุณภาพวัคซีนและการทดสอบแอนติบอดีลบล้างไวรัส (neutralizing antibody) ต่อไวรัส SARS-CoV-2 สายพันธุ์ต่าง ๆ เป็นประโยชน์ในการกำหนดรูปแบบการให้วัคซีน เพื่อการควบคุมคุณภาพของวัคซีนและการตรวจระดับแอนติบอดีภายหลังได้รับวัคซีน แม้องค์การอนามัยโลกจะมีการผลิตสารมาตรฐานระดับสากลแต่มีปริมาณจำกัดไม่เพียงพอต่อความต้องการ ดังนั้นการศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อจัดเตรียมและกำหนดค่าแอนติบอดีมาตรฐานต่อเชื้อ SARS-CoV-2 สำหรับใช้ในการทดสอบโดยวิธี Plaque Reduction Neutralization Test (PRNT) ซึ่งเป็นวิธีมาตรฐานสำหรับการตรวจหาระดับแอนติบอดีต่อเชื้อ SARS-CoV-2 แต่ละสายพันธุ์ คณะผู้วิจัยได้จัดเตรียมแอนติบอดีจากซีรัมผู้ป่วยและผู้ได้รับวัคซีนในรูปผงแห้งแช่เยือกแข็ง เปรียบเทียบกับโมโนโคลนอลแอนติบอดี (mAbs) โดยได้จัดเตรียมสูตรและศึกษาสภาวะการทำแห้งแบบแช่เยือกแข็งที่เหมาะสม ในการประเมินคุณภาพของแอนติบอดีที่ผ่านการทำแห้งแบบแช่เยือกแข็ง พบว่าความเป็นเนื้อเดียวกันของแอนติบอดีชนิด mAbs ไม่เป็นไปตามเกณฑ์ยอมรับ ในขณะที่แอนติบอดีจากซีรัมมีคุณสมบัติทางกายภาพและชีววิทยาอยู่ในเกณฑ์ยอมรับ สามารถนำมาใช้เป็นแอนติบอดีมาตรฐานอ้างอิงต่อเชื้อ SARS-CoV-2 ในรูปผงแห้ง โดยพบว่ามีค่า Neutralizing antibody ต่อสายพันธุ์เดลตา โอไมครอน BA.2 และโอไมครอน BA.5 ที่ทดสอบโดยวิธี $PRNT_{50}$ titer (ช่วงความเชื่อมั่น 95%) เท่ากับ 512.97 (473–556), 288.14 (265–313) และ 161.49 (124–184) ตามลำดับ สำหรับการประเมินความคงตัว โดยการประเมินความแรงของผลิตภัณฑ์ที่ใช้งานจริงภายหลังการผลิต พบว่าอยู่ในเกณฑ์การยอมรับ (ค่าเฉลี่ยเรขาคณิต ±3 SD) ดังนั้นแอนติบอดีจากซีรัมจึงเหมาะสมสำหรับใช้เป็นแอนติบอดีมาตรฐานอ้างอิงต่อไวรัส SARS-CoV-2 เพื่อควบคุมคุณภาพวัคซีน และประเมินระดับแอนติบอดีลบล้างไวรัสหลังการฉีดวัคซีนในช่วงการระบาดของโรคโควิด 19

คำสำคัญ: แอนติบอดีมาตรฐานอ้างอิงต่อ SARS-CoV-2, PRNT, การทำแห้งแบบแช่เยือกแข็ง