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Development and validation of enzyme-linked immunosorbent assay for anti-mouse pertussis immunoglobulin G using international reference anti-*Bordetella pertussis* mouse serum NIBSC 97/642

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Purpose: In this study, an in-house enzyme-linked immunosorbent assay (ELISA) was developed and validated. The titer of ELISA was calculated using the reference line (RFL) method based on the standard curve drawn using the international reference anti-mouse serum NIBSC (National Institute for Biological Standards and Control) 97/642.

Materials and Methods: In the development step, signal to noise was depicted to select the buffers that showed the most appropriate ratio. In the validation step, standard range, precision, dilution linearity, and specificity were confirmed, and RFL and parallel line (PLL) methods were compared in precision and dilution linearity.

Results: Coating concentration for plate was achieved at 0.1 μg/mL for pertussis toxin (PT), 0.15 μg/mL for filamentous hemagglutinin antigen (FHA), and 0.25 μg/mL for pertactin (PRN). The signal to noise ratio was 22.02 for PT, 14.93 for FHA, and 8.02 for PRN with 0.25% goat serum in phosphate-buffered saline (PBS) as a dilution buffer, and 2% skim milk in PBS as a blocking buffer. Based on the precision results, we assessed the lower limit of quantification by 1, 0.2, and 1.5 EU/mL concentration for PT, FHA, and PRN which met the ICH (International Council for Harmonization) M10 criteria of a 25% accuracy and total error of 40%. In specificity, homologous serum was spiked into heterologous serum and the accuracy met the criteria. There was no difference in the results between RFL and PLL calculations (p-value=0.3207 for PT, 0.7394 for FHA, 0.2109 for PRN).

Conclusion: ELISA validated with RFL calculation method in this study is a relatively accurate assay for mouse humoral immunogenicity test.

Keywords: Enzyme-linked immunosorbent assay, Pertussis, Validation, Unit calculation, Murine

Introduction

Acellular pertussis (aP) vaccine was developed by Sato and Sato [1] in Japan, to recover the whole-cell inactivated vaccine's problems with many side effects. In the 1990s, various diphtheria-tetanus-pertussis (DTaP) vaccines were developed, and the aP vac-

cination rate appeared at a high level to date. Consequently, the large-scale whooping cough epidemic has disappeared [2]. However, despite this high vaccination rate, small-scale whooping cough epidemics have continued to occur in both developed and developing countries since the late 1990s. There are hypotheses of resurgence, the occurrence of polymorphic variants due to vaccine adoption of the *Bordetella pertussis*, the decline effect of pertussis protective immunity in adolescents and adults, which can act as a source of infection in young children, and the waning immunity of DTaP vaccine [3,4]. In order to solve this problem, the need for new pertussis vaccines is emerging [5], and the immunogenicity evaluation of the developed vaccine is very important in the development of a new vaccine.

Pertussis vaccines use different seed bacteria, purification, and inactivation methods depending on the manufacturer, and the immune mechanism of pertussis vaccines has not been accurately identified, so there are limitations in evaluating the protective effect of pertussis vaccines. In general, immunogenicity and effectiveness are primarily evaluated through the evaluation of humoral and cell-mediated immune responses and respiratory challenge, and the results of this type of animal model study show that the protective effect against pertussis is similar to that in children who actually received the vaccination. According to these studies, murine models are currently being used as the most appropriate efficacy evaluation model [6,7]. Due to this fact, the efficacy and safety evaluation of pertussis vaccines being developed or developed is preceded by murine model studies with proven correlation with human study results before clinical studies. However, in murine model studies, there are differences in the vaccination routes, components, number, dose, sample collection time, and interval, as well as an immune system different from that of humans, so there are limitations in the accurate evaluation of protective immunity.

Enzyme-linked immunosorbent assay (ELISA) is the most widely used serological assay to test the humoral immunogenicity of vaccines in animal studies. In an ELISA, converting of the optical density (OD) to a titer with the most accurate unit is critical [8-10], and various calculation methods have been suggested for titer. There are various calculation methods including the parallel line (PLL) method, single point reference line (RFL) method, mean absorbance comparison, 2 times of baseline OD value, and four-parameter curve fit model [11-18]. In a previous study comparing five different calculation methods for analyzing the clinical samples after *B. pertussis* vaccination, the RFL method exhibited the highest level of reproducibility [19].

ELISA assay is used widely not only in nonclinical tests but also in clinical tests to determine the immunoglobulin G (IgG) titer in the serum for the *B. pertussis* vaccine [11,19] but the methods and criteria have not been standardized [20,21]. The World Health Organization recommends that mouse immunogenicity test (MIT) must be validated to qualify *B. pertussis* vaccine [22], a standardized serum for *B. pertussis* (international reference pertussis antiserum) has been developed as the National Institute for Biological Standards and Control (NIBSC) supplies with the NIBSC Code 60/140 for humans [23], and NIBSC 97/642 for mice [24].

In this study, we validated the in-house ELISA assay using the RFL method for calculating the results as a unit according to previous studies [15,19,23] and some of the validation contents were compared with the PLL calculation method. NIBSC 97/642 as a standard and NIBSC JNIH-11 and JINH-12 as control sera were used in validation and settings to contribute to obtaining accurate titers as an ELISA unit (EU) in mouse models and evaluating the precise efficacy of vaccines.

Materials and Methods

Serum, antigen, and reagents

International reference anti-*B. pertussis* mouse serum NIBSC 97/642 was used as the reference standard (STD). The antiserum had 34 EU/mL for pertussis toxin (PT), 286 EU/mL for filamentous hemagglutinin antigen (FHA) and 60 EU/mL for pertactin (PRN) antibodies, respectively. The control sera were *B. pertussis* PT anti-mouse serum (NIBSC JNIH-12) and *B. pertussis* FHA anti-mouse serum (NIBSC JNIH-11) at concentrations of 200 EU/mL and 400 EU/mL, respectively. Fifteen female BALB/c mice were used to prepare negative samples; the negative serum was prepared by pooling the normal mouse serum without vaccine administration. For validation serum to verify the repeatability of precision, the serum from BALB/c mice was collected 2 weeks after inoculation with the DTaP vaccine. The antigen from NIBSC, PT (NIBSC 15/126), FHA (NIBSC JNIH-4), and PRN (NIBSC 18/154) were used for coating the ELISA plate.

ELISA settings

Normal indirect ELISA assay was established and the simple principle was shown in Fig. 1. The linearity of the STD was evaluated at five different coating concentrations of PT, FHA,

Table 1. Component of buffers used for reference line development

Type of buffers	Components
Blocking	
1% goat	Goat serum 1% in pH 7.4 PBS with 0.05% Tween 20
5% goat	Goat serum 5% in pH 7.4 PBS with 0.05% Tween 20
2% skim milk	Skim milk 2% in pH 7.4 PBS with 0.05% Tween 20
5% skim milk	Skim milk 5% in pH 7.4 PBS with 0.05% Tween 20
Dilution	
BSA 0.25%	BSA 0.25% in pH 7.4 PBS with 0.25% Tween 20
Goat 0.25%	Goat serum 0.2% in pH 7.4 PBS with 0.25% Tween 20

PBS, phosphate-buffered saline; BSA, bovine serum albumin.

and PRN antigen of each, 0.1, 0.5, 1.0, 1.5, and 2.0 μg/mL. Two-fold serial diluted STD from 1/20 to 1/81,920 was analyzed for the linearity. Unless 5 through 9 OD values between 0.1–2.5 were used. The OD values were transformed to natural logarithm for the y-axis, and dilution factors were transformed to $\log_2$ for the x-axis for the STD curve. GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used for analyzing the R^2 value. The components of buffers and coating concentrations were varied to determine the optimal conditions for improving the signal-to-noise ratio of more than 10 or close to 10, with a correlation coefficient of ≥0.95 for the STD curve (Table 1). Using different types of blocking and dilution buffers, negative mouse serum was diluted to the same dilution factor as the STD to determine the signal-to-noise ratio. Blocking and dilution buffers that gave a high signal-to-noise ratio was used to assess the optimal coating concentration, resulting in the highest recovery percentage from nominal concentrations. For the control serum, NIBSC JNIH-12 was used for PT, NIBSC JNIH-11 was used for FHA, and at least three diluted concentrations of NIBSC 97/642 were used for PRN to determine the conditions giving the most favorable recovery percentage.

Assay validation

The validation method and criteria have been described in previous studies or respective guidelines [19,22,23,25].

Standard range

Anti-mouse reference standard serum (NIBSC 97/642) was diluted two-fold, and the linearity of the STD curve with a correlation coefficient of ≥0.95 was evaluated in triplicate measurements and defined as the STD range.

Precision: intra-runs (within days) and inter-runs (between days)

NIBSC JNIH-12, JNIH-11, and 97/642 (1/2 dilution) were used as control sera with assigned nominal IgG values. Analytes for precision were prepared by diluting the control serum with dilution buffer to five (four for PRN) different concentrations; undiluted, 1/2, 1/20, 1/200, and 1/2,000. The accuracy% and coefficients of variation (CV)% of the results should meet 20% criteria and 30% of total error (sum of absolute values of CV% and accuracy%). Based on the precision results, the concentration with 25% accuracy and 40% total error was set as the lower limit of quantification (LLOQ) [22,25].

Three-time-vaccinated mouse serum was used to evaluate the CV% for the repeat test. Fifteen 4-week-old female BALB/c mice were vaccinated with the Infanrix inactivated poliovirus (IPV)/Haemophilus influenzae type b (Hib) vaccine (GSK, Rixensart, Belgium) 3 times at 2-week intervals. The serum was pooled and diluted with normal mouse serum used in the signal-to-noise part. The CV% was compared between the RFL and PLL calculation methods.

All precision assays were performed twice per day over 3 assay days, for a total of six runs. All tests were performed in duplicate on the plate. For intra-run analysis, the results of two runs on 1 day were used. For inter-run analysis, the results of six runs over 3 days were used.

Dilution linearity

Linear regression was performed using the results from six runs for precision evaluation to check whether the linearity of the results had an R^2 ≥0.95 (observed values) against the theoretical values.

Specificity

The control sera JNIH-12 and JNIH-11 were spiked in each heterologous serum. The spiked concentrations were 50% of the theoretical values and LLOQ concentrations. The accuracy of the observed values against the theoretical values was estimated.

ELISA assay

The assay was performed as described in a previous method for clinical samples with some modifications [23] (Fig. 1). All buffers used in the ELISA validation are shown in Table 2. The antigens from NIBSC, PT (NIBSC 15/126), FHA (NIBSC, JNIH-4), and PRN (NIBSC, 18/154), were placed in carbonate coating buffer (pH 9.6) and aliquoted into a Maxisorp ELISA

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plate (SPL Life Sciences, Pocheon, Korea) at 100 μL per well. The coating concentrations were 0.1 μg/mL for PT, 0.15 μg/mL for FHA, and 0.25 μg/mL for PRN. After overnight incubation, the plate was washed with washing buffer which is pH 7.4 PBS with 0.05% Tween 20. Blocking buffer was added and incubated for 1 hour. A two-fold serial dilution was performed using 50 μL each of NIBSC 97/642 and the sample. Then, 50 μL of dilution buffer was added to the diluted sample and NIBSC 97/642. After 90 minutes incubation 100 μL of the secondary antibody, goat anti-mouse IgG (H&L) antibody horseradish peroxidase-conjugated (Abcam, Cambridge, UK), was added to each well at a dilution of 1:100,000. After 1 hour of incubation, 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) (Thermo Fisher Scientific, Waltham, MA, USA)

Table 2. Buffers used in the ELISA assay

Buffers	Component
Blocking buffer	2% skim milk, 0.05% Tween 20 in pH 7.4 PBS
	1,000 mL PBS+20 g skim milk+0.5 mL Tween 20
	Store at 4℃ for up to 1–2 weeks
Dilution buffer	0.25% goat serum, 0.25% Tween 20 in pH 7.4 PBS
	1,000 mL PBS+2 mL goat serum+2.5 mL Tween 20
	Store at 4℃ for up to 1–2 weeks
Wash buffer 10×	Dissolve Tween 20 0.5% in pH 7.4 PBS
	1,000 mL PBS+5 mL Tween 20
	Store at RT for up to 1–2 months
	Make 1× with pH 7.4 PBS
Stop buffer	0.16M H_2SO_4

ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; RT, room temperature.

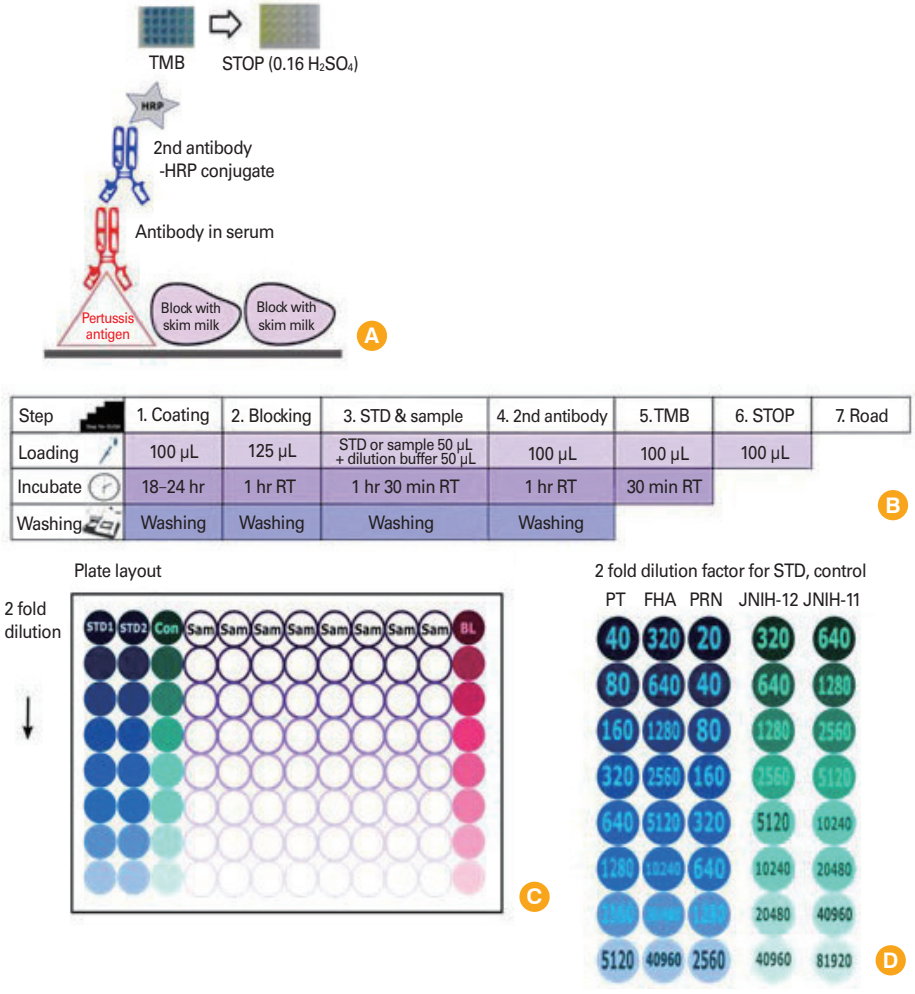

Fig. 1. In house enzyme-linked immunosorbent assay (ELISA) scheme. **(A)** Indirect ELISA scheme used in this study. The loading amount and incubation time of established ELISA step and 96 well plate layout used in this study indicated at **(B, C)**. Additionally, recommended dilution factors for each antigen of STD and control sera used in the assay was shown at **(D)**. TMB, 3,3′,5,5′-tetramethylbenzidine; HRP, horseradish peroxidase; RT, room temperature; STD, standard; Con, control serum; Sam, sample serum; PT, pertussis toxin; FHA, filamentous hemagglutinin antigen; PRN, pertactin.

and stop solution were added to the wells. The OD was measured at 450 nm using an Epoch Microplate Spectrophotometer (Biotek, Winooski, VT, USA). All OD measurements were subtracted from the blank OD value. The dilution factor of the OD was converted to the $\log_2$ and OD measurements were converted to the natural logarithm for application in the RFL method. The OD values of at least four dilution factors of STD and at least two dilution factors of the sample should be used for calculation.

Ethics statement

The animal studies were performed after receiving approval of the Institutional Animal Care and Use Committee in Catholic University (approval no., 2023-0193-02).

Statistics

Microsoft Excel (Microsoft Corp., Redmond, WA, USA) was used for RFL and PLL calculation methods and CV% and accuracy value. GraphPad Prism ver. 9 software (GraphPad Software) was used to estimate the R^2 of linear lines produced for the diluted samples and STD curve. RFL and PLL methods were compared with paired t-tests and the p-value was evaluated with GraphPad Prism ver. 9 software (GraphPad Software).

Results

ELISA settings

Standard curve linearity

In the coating concentration range of 0.1–2.0 μg/mL, PT, FHA, and PRN showed an R^2 value of ≥0.97, verifying that the RFL method is applicable across a coating range of 0.1–2.0 μg/mL.

Signal to noise ratio by buffers

Goat serum was added to dilution or blocking buffers to remove any nonspecific bindings as the secondary antibody origin was goat. The signal-to-noise ratio was estimated for blocking buffer containing skim milk or goat serum for the

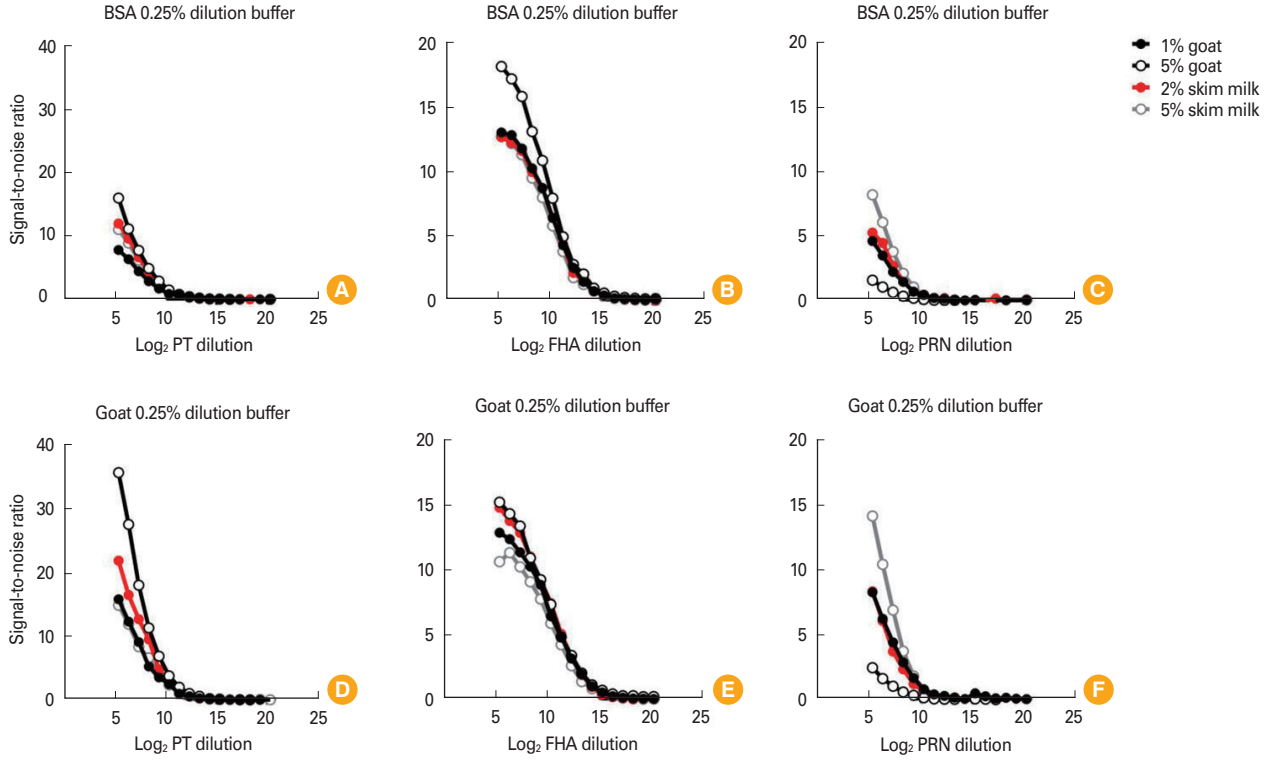

Fig. 2. Signal to noise ratio of blocking buffer. Standard (STD) was diluted two-fold from 1/40 to 1/1,310,720 and a graph was drawn using only optical density (OD) values between 0.1 and 2.5. Normal mouse serum was used by pooling the serum of five 4-week BALB/c mice and diluted 1/40. Each antigen was coated at 0.1 μg/mL for pertussis toxin (PT), 0.2 μg/mL for filamentous hemagglutinin antigen (FHA), and 0.8 μg/mL for pertactin (PRN). Four blocking buffers: goat 1%, goat 5%, skim milk 2%, and skim milk 5% were compared against two dilution buffers: **(A–C)** bovine serum albumin (BSA) 0.25% and **(D–F)** goat serum 0.25%. The signal to noise ratio was confirmed by dividing the STD OD value by the normal mouse serum OD value. The x-axis was converted to $\log_2$ dilution factor of STD.

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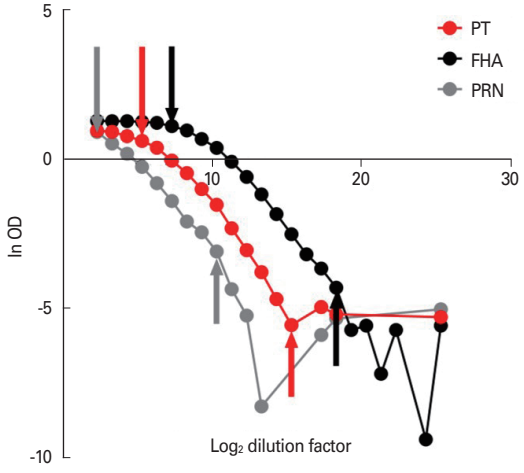
dilution buffer of 0.25% bovine serum albumin or 0.25% goat serum (Table 1, Fig. 2). The highest ratios were 35.94 for PT and 15.34 for FHA when blocking buffer containing 5% goat serum and dilution buffer containing 0.25% goat serum were used. The highest ratios were 14.28 for PRN when blocking buffer containing 5% skim milk and dilution buffer containing 0.25% goat serum were used. In this study, a blocking buffer containing 2% skim milk and a dilution buffer containing 0.25% goat serum were used (Fig. 2). With these buffers, PT showed a signal-to-noise ratio of 22.02, FHA showed 14.93, and PRN showed 8.02 (Fig. 2). Recovery was analyzed for the control sera JNIH-12 and JNIH-11 and diluted 97/642 at various coating concentrations. The most accurate level of recovery was observed at 98.69% for PT at 0.1 μg/mL coating concentration and 102.23% for FHA at 0.15 μg/mL coating concentration, respectively (Table 3). Diluted 97/642 was used as a control serum for PRN. At a coating concentration of 0.25 μg/mL, the rates of recovery were 89.02%, 92.66%, and 94.51% for the stock and 1/5 and 1/10 diluted 97/642, respectively for PRN (Table 3).

Assay validation

Range

Two-fold serial diluted 97/642 was used to identify the linearity with a ≥0.95 correlation coefficient (R^2) with a mean value



STD range (dilution factor)	
PT	40–40,960
FHA	160–327,680
PRN	5–1,280

Fig. 3. Range. More than 0.97 or 0.97 of correlation coefficient value was evaluated, when the National Institute for Biological Standards and Control (NIBSC) 97/642 was diluted 2 times starting from a dilution factor of 5 at a coating concentration of pertussis toxin (PT) 0.1 μg/mL, filamentous hemagglutinin antigen (FHA) 0.15 μg/mL, and pertactin (PRN) 0.25 μg/mL. The standard (STD) curve was drawn using 4 or more optical density (OD) values. The range of STD indicating linearity was confirmed in the section indicated by the arrow at 0.97 R^2 or high.

Table 3. Recovery of control serum with different coating concentration using skim milk 2% blocking buffer and goat 0.2% dilution buffer

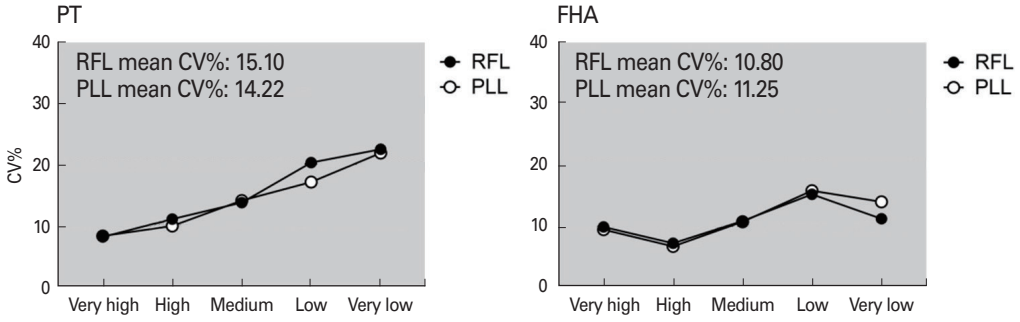
Control serum	Coating concentration (μg/mL)	Blank	R^2	Results (EU/mL)	Recovery (%)
PT: NIBSC JNIH-12 (200 EU/mL)	0.10	0.0440	0.9972	197.39	98.69
	0.20	0.0430	0.9915	169.63	84.81
	0.40	0.0450	0.9911	213.94	106.97
	0.60	0.0440	0.9948	335.67	167.83
FHA: NIBSC JNIH-11 (400 EU/mL)	0.15	0.0485	0.9994	408.90	102.23
	0.25	0.0485	0.9920	357.75	89.44
	0.45	0.0460	0.9900	345.74	86.44
	0.65	0.0595	0.5021	348.56	87.14
PRN: NIBSC 97/642					
Undiluted (60 EU/mL)	0.15	0.0668	0.9931	50.27	83.78
	0.25	0.0655	0.9949	53.41	89.02
	0.35	0.0701	0.9931	45.50	75.92
1/5 diluted (12 EU/mL)	0.15	0.0668	0.9931	8.50	70.87
	0.25	0.0655	0.9949	11.12	92.66
	0.35	0.0701	0.9931	16.54	137.87
1/10 diluted (6 EU/mL)	0.15	0.0668	0.9931	4.21	70.19
	0.25	0.0655	0.9949	5.67	94.51
	0.35	0.0707	0.9931	8.11	135.13

EU, enzyme-linked immunosorbent assay unit; PT, pertussis toxin; FHA, filamentous hemagglutinin antigen; PRN, pertactin; NIBSC, National Institute for Biological Standards and Control.

Table 4. JNIH-12, JNIH-11, 97/642 accuracy%, CV%, and total error % for precision_inter-6 runs (between days) in 3 assay days for IgG compared with RFL and PLL calculation method

Nominal concentration for IgG (EU/mL)	Mean RFL	Accuracy%		CV%		Total error %	
		RFL	PLL	RFL	PLL	RFL	PLL
PT (JNIH-12)							
200	191.75	4.13	4.41	5.71	5.69	9.84	10.10
100	98.22	1.78	1.87	11.32	11.28	13.10	13.15
10	9.86	1.42	1.11	9.92	10.17	11.34	11.28
1	0.76	24.09	24.34	14.87	18.22	38.96	42.56
0.1	0.04	64.02	64.02	14.21	14.21	78.23	78.23
FHA (JNIH-11)							
400	401.29	0.32	0.74	13.80	14.22	14.12	14.96
200	238.83	18.24	18.11	11.68	11.68	29.92	29.79
20	20.10	0.52	0.56	6.82	6.80	7.34	7.36
2	1.59	20.72	17.61	11.33	14.00	32.05	31.61
0.2	0.15	24.10	24.35	9.71	9.07	33.81	33.42
PRN (97/642)							
30	25.74	14.20	13.52	9.89	9.53	24.09	23.05
15	12.71	15.29	14.84	6.16	6.82	21.45	21.66
1.5	1.16	22.34	20.99	9.22	9.75	31.56	30.74
0.15	0.10	35.43	35.43	31.91	31.91	67.34	67.34

CV, coefficients of variation; IgG, immunoglobulin G; RFL, reference line; PLL, parallel line; EU, enzyme-linked immunosorbent assay unit; PT, pertussis toxin; FHA, filamentous hemagglutinin antigen; PRN, pertactin.



Analyte	Anti-PT (EU/mL)			Anti-FHA (EU/mL)		
	Dilution factor	RFL	PLL	Dilution factor	RFL	PLL
Very high	-	1,423.18	1,421.27	-	2,811.73	2,815.62
High	1/5	246.29	247.26	1/10	329.38	331.35
Medium	1/100	10.70	10.68	1/100	43.47	43.70
Low	1/500	3.05	2.94	1/500	7.01	7.13
Very low	1/1,000	0.83	0.82	1/1,000	2.80	3.17

Fig. 4. Coefficients of variation (CV)% compared between reference line (RFL) and parallel line (PLL) calculation method with vaccinated mouse serum. Female BALB/c mice (n=15) were vaccinated with Infanrix IPV/Hib (inactivated poliovirus/Haemophilus influenzae type b) vaccine 3 times at 2-week intervals. This serum was pooled and diluted with unvaccinated normal mouse serum (n=15). The dilution factor and the mean enzyme-linked immunosorbent assay (ELISA) titer (EU/mL) corresponding to the dilution factor were displayed in a table, and the mean CV% of each five analyte was shown with graph. The assay was performed 2 times per day for 3 assay days for a total of six runs for pertussis toxin (PT) antigen. The assay was performed 2 times per day for 4 assay days for a total of eight runs for filamentous hemagglutinin antigen (FHA) antigen. EU, ELISA unit.

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of triplicate measurements. Linearity was detected at the dilution factor within the range of 40–40,960 for PT, 160–327,680 for FHA, and 5–1,280 for PRN (Fig. 3).

Precision: intra-runs (within days) and inter-runs (between days)

All results were obtained using the RFL and PLL calculation methods. Accuracy% and CV% were compared between these two calculation methods across six inter-run tests over 3 assay days (between days). Regarding precision, for intra-runs (within days) of IgG (data not shown), the CV% was below 15% for all antigens. In contrast, for PRN, the CV% was slightly greater than 15% for both the RFL and PLL methods, at the lowest concentration of 0.15 EU/mL (1/200 diluted). In addition, for inter-runs (between days), the CV% was approximately 20% for all analytes (Table 4).

The accuracy for each analyte, ranging from very high to very low IgG concentrations, was below 20% for PT, FHA, and PRN. However, at low concentrations, the level exceeded 25%. The accuracies of the RFL and PLL methods were 24.09% and 24.34%, respectively, for PT at 1 EU/mL; 24.10% and 24.35% for FHA at 0.2 EU/mL; and 22.34% and 20.99% for PRN at 1.5 EU/mL concentration of the analyte. At the lowest concentrations, the accuracy for PT and PRN was reduced to ≥35% (Table 4).

As a result of estimating the LLOQ concentrations of PT, FHA, and PRN that meet the standards for accuracy within

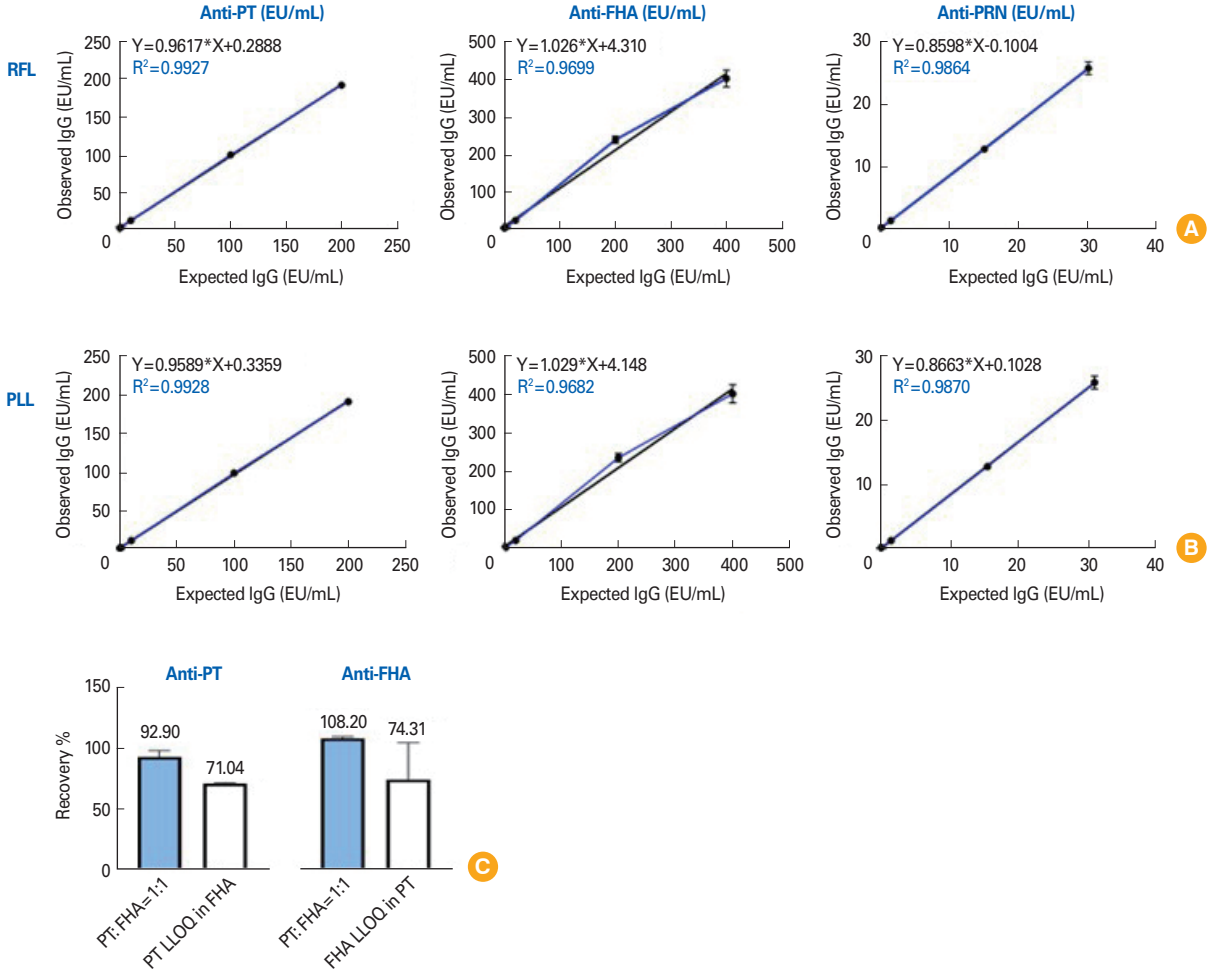

Fig. 5. Dilution linearity & specificity. National Institute for Biological Standards and Control (NIBSC) JNIH-12, JNIH-11, and stock of 1/2 diluted 97/642 (pertactin [PRN] control) were diluted of 5 analyte (4 for PRN) with dilution buffer and fitted linear line with mean observed concentration versus expected nominal titer. Graph showed as mean±standard error of mean with linear equation and R^2 value was evaluated (n=6). **(A)** Reference line (RFL) calculation method and **(B)** parallel line (PLL) calculation method. JNIH-12 and JNIH-11 control serum was used for specificity. Homologous serum was spiked in heterologous serum at 1:1 ratio or lower limit of quantification (LLOQ) of 1 EU/mL for PT and 0.2 EU/mL for filamentous hemagglutinin antigen (FHA) to estimate recovery %. A total of two tests were performed and the mean recovery % value and standard (SD) were shown at graph **(C)**. EU, enzyme-linked immunosorbent assay unit; IgG, immunoglobulin G; PT, pertussis toxin.

25% and total error within 40%, they were confirmed to be 1, 0.2, and 1.5 EU/mL, respectively. At concentrations above the LLOQ, CV% and accuracy% were all within 20%, and total error% was all within 30%, satisfying the U.S. Food and Drug Administration (FDA) and the International Council for Harmonization (ICH) M10 standards (Table 4).

Precision with control serum, there was no difference in CV% and accuracy% between the RFL and PLL calculation methods, and both calculation methods showed the same LLOQ concentration. p-value between RFL and PLL calculation showed 0.3207 for PT, 0.7394 for FHA, and 0.2109 for PRN. Repeatability results with vaccinated mouse serum—CV% for PT and FHA were also compared between RFL and PLL methods. The average CV% of the total five concentrations for PT antigen was 15.10% calculated with RFL and 14.22% with PLL, and for FHA antigen was 10.80% calculated with RFL and 11.25% with PLL. Both methods showed a CV% within 20%, and in the case of PT, at a very low concentration, which is below the LLOQ, RFL showed a CV% of 22.36%, and PLL showed a CV% of 21.75% (Fig. 4).

Dilution linearity

This analysis was based on the six runs performed for the inter-assay evaluation of precision. Linear regression was confirmed from slope of the theoretical values versus observed values. The results indicated that the linearity was ≥0.95 for all samples, specifically 0.9927 for PT, 0.9699 for FHA, and 0.9864 for PRN (Fig. 5). Results from PLL calculation method showed equivalent results from RFL method, 0.9928 for PT, 0.9682 for FHA, and 0.9870 for PRN.

Specificity

In the case of serum mixed 1:1 with PT-specific NIBSC JNIH-12 (200 EU/mL) and FHA-specific NIBSC JNIH-11 (400 EU/mL), the average value of two tests was 92.90 EU/mL and 216.40 EU/mL. Recovery was 92.9% for PT and 108.2% for FHA, and when spiking with homologous serum at the concentration of LLOQ in the heterologous serum, recovery was 71.04% for PT and 74.31% for FHA (Fig. 5).

Discussion

Humoral immunogenicity of mice (non-clinical) is a good indicator for useful *B. pertussis* vaccine development. The World Health Organization and European Pharmacopeia recommend performing the MIT to verify lot-to-lot consistency across the final products of the *B. pertussis* vaccine [22]. Although there is no standardized method, ELISA is recommended and the assay should be validated by measuring the LLOQ, and reproducibility to reveal consistent IgG antibody response. The assay should include international reference serum (NIBSC 97/642) to unify the units, and controls (JNIH-12, JNIH-11) should be used in each test. Thus, a standardized ELISA must be validated to examine humoral immunogenicity and enable quality control of commercial vaccines. In this study, we optimized and validated in-house ELISA for MIT to satisfy the recommendation of the World Health Organization.

In the validation step, the STD range was evaluated for PT as a 40–40,960 dilution factor, FHA as a 160–327,680 dilution factor, and PRN as a 5–1,280 dilution factor with a ≥0.98 correlation coefficient (Fig. 3). A hook effect was observed at dilution factors ≥40 for PT and ≥160 for FHA slightly. FHA showed good linearity at the lowest dilution factors of 327,680 among PT and PRN that this result could influenced the lowest LLOQ of FHA (Fig. 3).

We found the LLOQ at precision assay that meets the criteria of 25% CV% and accuracy and 40% of total error (sum of absolute values of CV% and accuracy%) suggested by recently revised ICH M10 [22] and the FDA Bioanalytical Method Validation Guidance for Industry [25]. Based on the precision results across five different concentrations, criteria were satisfied at 1, 0.2, and 1.5 EU/mL concentrations for PT, FHA, and PRN, respectively (Table 4). Moreover, the dilution linearity had an R squared value of 0.95 or more (Fig. 5), specificity had PT and FHA recovery within 10%, and LLOQ was within 30% which the assay validated with reasonable results (Fig. 5).

Recently, many multiplex ELISAs have been developed to reduce the volume of samples to identify antibodies for a large number of antigens at the same assay, multiplex flow-cytometric immunoassays were compared with conventional ELISA in terms of the sample volume, test time, and labor [16,26]. However, the multiplex ELISA system is considerably more expensive, as it requires a device like a flow cytometer, an expert to manage the device, and software to plot graphs. The ELISA proposed in this study involves a conventional approach and no need for an expensive device and the actual amount of sample used in the assay is less than the required amount of 50 μL, depending on which dilution factor is applied. In this study, the signal-to-noise ratio was increased by using goat serum, the secondary antibody origin, as a dilution buffer, this made a cost reduction by lowering the coating concentration setting to PT 0.1 μg/mL, FHA 0.15 μg/mL,

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and PRN 0.25 μg/mL (Table 3, Fig. 2).

There were some studies using parallel-line analysis (PLL) in ELISA using international reference serum (97/642) [11-13]. This calculation method is a widely used method for unit calculation along with RFL. This method draws a sample graph in addition to the standard graph and resets the slope with an average value of the two slopes. RFL is a calculation method that uses a calculation formula based on the assumption that the slope of the standard graph is the same as the slope of the sample graph, and does not calculate the slope of the sample graph separately. PLL or RFL calculations can be done using Excel, but using professional software is faster and more accurate. In a previous study, our laboratory established the RFL calculation method for analyzing the pertussis-vaccinated human serum with ELISA using Excel without separate software [23]. In this study, an established Excel form was applied to the mouse serum and ELISA was set up. Precision and dilution linearity were compared and verified with the PLL method. There were no meaningful differences between the results of the RFL and PLL methods in the CV% and accuracy% and dilution linearity (Table 4, Figs. 4, 5). These results slightly contrast to those of previous reports showing that the RFL gives more reproducible results [19], or that the new version of the modified PLL showed different results and was preferred over RFL [27]. With the focus on the results in this study, both methods seem to conclude with equivocal results. Either of these methods can be selected based on the laboratory conditions.

In conclusion, in this study, the in-house ELISA satisfied validation parameters including precision (Table 4, Fig. 4), dilution linearity, and specificity (Fig. 5) and met the criteria of FDA and ICH M10, suggesting that the ELISA is suitable for producing accurate results in non-clinical mouse samples.

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