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Article

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Abstract: Recently, two respiratory syncytial virus (RSV) vaccines based on the prefusion F (pre-F) antigen were approved in the United States. We developed and validated an enzyme-linked immunosorbent assay (ELISA)-based protocol for the practical and large-scale evaluation of RSV vaccines. Two modified pre-F proteins (DS-Cav1 and SC-TM) were produced by genetic recombination and replication using an adenoviral vector. The protocol was established by optimizing the concentrations of the coating antigen (pre-F proteins), secondary antibodies, and choice of blocking buffer. To validate the protocol, we examined its accuracy, precision, and specificity using serum samples from 150 participants across various age groups and the standard serum provided by the National Institute of Health. In the linear correlation analysis, coating concentrations of 5 and 2.5 μg/mL of DS-Cav1 and SC-TM, respectively, showed high coefficients of determination ($r^2 > 0.90$). Concentrations of secondary antibodies (alkaline phosphatase-conjugated anti-human immunoglobulin G, diluted 1:2000) and blocking reagents (5% skim milk/PBS-T) were optimized to minimize non-specific reactions. High accuracy was observed for DS-Cav1 ($r^2 = 0.90$) and SC-TM ($r^2 = 0.86$). Further, both antigens showed high precision (coefficient of variation < 15%). Inhibition ELISA revealed cross-reactivity of antibodies against DS-Cav1 and SC-TM but not with the attachment protein.

Keywords: respiratory syncytial virus; RSV; ELISA; vaccine; immunogenicity

1. Introduction

Respiratory syncytial virus (RSV) is a major respiratory pathogen in young children, which imposes a greater disease burden than influenza in this age group.[1] Now it is being recognized as an important pathogen in older adults and immunocompromised individuals as well, causing acute exacerbation of pre-existing cardiovascular diseases or severe pneumonia [2-4] Despite its clinical importance, the treatment and prevention tools for RSV remain limited, unlike seasonal influenza for which effective antiviral drugs and vaccines are available. Therefore, the World Health Organization is committed to develop effective RSV vaccines by actively working with pharmaceutical companies [5]. As of January 2023, 22 vaccine candidates have entered clinical trials, six of which are in phase 3 [6]. Because several vaccine candidates have recently been reported to have satisfactory safety profiles and efficacy, one or more RSV vaccines are expected to be approved in the near future.

Among surface antigens of RSV, the fusion (F) protein and attachment (G) protein comprise the major immunogens, with the F protein being the preferred target of vaccines and monoclonal antibodies.[7] The F protein exists in two forms: a prefusion (pre-F) form before fusion with the host cell membrane, and a postfusion form. Despite being more immunogenic than the postfusion form, pre-F has not been used as a vaccine target because of its structural instability, a characteristic that has led to the failure of RSV vaccine development in the past [7,8]. In the last decade, the structure of

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the pre-F form was revealed and subsequently engineered to make it more stable, DS-Cav1 and SC-TM, being the most widely used vaccine targets [9,10].

RSV vaccine candidates are based on various platforms, making it necessary to establish a standardized immunogenicity test protocol applicable to all vaccine products. Additionally, it is desirable to examine the correlation between simple and scalable enzyme-linked immunosorbent assays (ELISA) and neutralization tests, a labor-intensive gold standard. Therefore, we developed and validated an indirect ELISA-based protocol using the two most commonly modified pre-F proteins, DS-Cav1 and SC-TM, and G protein.

2. Materials and Methods

2.1. Study Population

Anonymized blood samples were collected from a total of 150 healthy children and adults, stratified by age groups: under 5 years, 5–18 years, 19–49 years, 50–64 years, and aged 65 years and above, from February to November 2021. This study was approved by the Institutional Board of Review of Korea University Guro Hospital; IRB No. 2022GR0149, and the requirement for informed consent was waived.

2.2. Protein Construct Design, Expression, and Purification

Adeno-X 293 cells (Takara Bio Inc, Shiga, Japan) were grown in Dulbecco's modified Eagle medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with penicillin-streptomycin (Thermo Fisher Scientific) and 10% fetal bovine serum (FBS; Biowest, Nuaillé, France). Genes expressing the RSV A2 F protein (Genbank AC083301.1) were designed as previously reported and synthesized by Bionics (Seoul, Korea).[10,11] All recombinant proteins were cloned into the ectodomain of the F protein with a C-terminal fold on the trimerization domain. SC-TM was designed by replacing the p27 sequence with a seven-amino acid GS-rich linker sequence and additionally replacing three amino acids (N67I, S215P, and D486N). The DS-Cav1 protein, containing four mutations (S155C, S190F, V207L, and S290C), has been previously described[9]. Each gene was cloned into the pAdenoX vector (Takara), and the linearized vectors were transfected into Adeno-X 293 cells. Proteins were purified by Ni-NTA chromatography (Thermo Fisher Scientific), and further purified by Strep-Tactin Sepharose chromatography (Iba-Life Sciences, Göttingen, Germany).

A synthetic gene encoding the RSV A2 (Genbank AC083301.1) G protein (secreted form; sG, amino acids 48 to 298) was cloned into pET28a in-frame with a C-terminal tandem 6-histidine. Recombinant G proteins were expressed in *E. coli* BL21(DE3) and purified by Ni-NTA chromatography (Thermo Fisher Scientific).

2.3. Development and Optimization of the ELISA-Based Protocol

The activity of the three antigens was confirmed by serial dilution and inhibition ELISA. Each antigen was fixed on the hydrophilic immunoplate, Maxibinding Plate (SPL, Pocheon, South Korea), at a concentration of 0, 1, 2.5, 5, 7.5, and 10 mcg/mL and immobilized for overnight at 4 °C. Serum samples were diluted to 1:100, 1:400, and 1:1600; phosphate buffer solution (PBS; Welgene, Gyeongsan, South Korea) was used as a negative control. The secondary antibody alkaline phosphatase-conjugated anti-human immunoglobulin G (IgG; Southern Biotech, Birmingham, AL, USA), was diluted to 1:2000, 1:4000, 1:6000, and 1:8000. Alkaline phosphatase was used as a colorimetric substrate. The optical density was measured at 405 nm (OD405) and 690 nm (OD690) using a Spectramax 190 plate reader (Molecular Devices, San Jose, CA, USA), and the value obtained by subtracting OD690 from OD405 was plotted against the titers of standardized serum provided by the National Institute of Health. The standardized curve-fit four-parameter logistic method was used to calculate antibody titers of clinical samples. Concentrations at which coating antigens and dilution factors of secondary antibodies exhibited 1) high optical density at high sample concentrations, and 2) low optical density with PBS, were chosen.

In addition, the following four blocking agents were examined to select one that minimizes non-specific antigen-antibody reactions: PBS with 0.05% Tween 20 (PBS-T); 1% bovine serum albumin (BSA)/PBS-T; 10% fetal bovine serum/PBS-T; and 5% skim milk/PBS-T. All experiments were performed in duplicate and the average values were compared. The blocking agent that exhibited the

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lowest optical density and highest correlation coefficient (r^2) was chosen. All chemical reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.4. Validation of the ELISA-Based Protocol

The accuracy, precision, and specificity of the established ELISA-based protocol were evaluated as follows:

1. Accuracy: Low, medium, and high titers of National Institute of Health-provided standard serum were serially diluted 2-fold from 1:200 to 1:6,400. All experiments were performed in duplicate for each concentration. The average optical density values were plotted against the antiserum concentrations, and the r^2 values were derived by correlating the calculated antibody titers against the three RSV antigens.
2. Precision: One sample was used from each age group. A total of five samples was diluted 2-fold from 1:200 to 1:25,600. The experiment was performed six times for each sample concentration, and the coefficient of variation (CV) was calculated.
3. Specificity: All 150 samples were adsorbed with each of the three homologous/heterologous RSV antigens (10 μg/mL of DS-Cav1, SC-TM, and G protein) for an hour with gentle shaking (300 rpm) at RT following which the antibody titers were measured. Post-adsorption titers were compared with pre-adsorption titers. Cross-reactivity was determined based on whether the antibody titers were significantly lower after adsorption. Because RSV infection is mainly prevalent in young children, and also because antibody specificity might be affected by the difference in the duration of time elapsed since the last RSV infection among the age groups studied, we investigated whether there was a difference in the antibody specificity based on age.

2.5. Correlation of Antibody Titers Determined by ELISA with Neutralizing Antibody Titers

We evaluated the correlation between antibody titers determined by ELISA and neutralizing antibody titers measured using the viral reduction neutralization test (VRNT). The VRNT was conducted on 30 samples, with six samples from each age group, and performed as follows: Hep-2 cells were seeded in a 96-well plate and cultured for 24 h at 37 °C. Sera were diluted 1/10 and inactivated at 56 °C for 30 min. RSV-RFP (RSV/A2/Kline 19 from Professor Martin ML) was diluted in PBS to a multiplicity of infection (MOI) of 2.5. 50 μL of diluted virus was mixed with 50 μL of serum sample and incubated for 1 h at 37 °C. A total of 60 μL of each virus-serum mixture was aliquoted to their designated cell-containing wells and incubated for 1.5 h at 37°C, after which the mixture was removed and incubated in 100 μL of 2% Dulbecco's modified Eagle medium overlay solution for 24 h at 37°C. Culture medium was removed from the 96-well culture plate and well were washed with 100 μL before incubating with virus-serum mixture. After 24 h, cells were fixed with 4% paraformaldehyde for 30 min and then rinsed with PBS-T (Tween-20) wash buffer. Nuclei were stained by treating with 50 μL of 6-diaminidino-2-phenylindole solution (1:50000) for 5 min at 20°C and then washed with 100 μL PBS-T. A total of 50 μL of distilled water was added to each well before imaging the sample plates using the Operetta CLS system (PerkinElmer, USA). The neutralizing dose was determined by counting the number of infected and non-infected cells. The correlation between antibody titers against the three RSV antigens measured using our ELISA protocol and the 50% neutralization dose (VRNT50) was examined.

2.6. Statistical Analyses

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 22.0 (SPSS Korea; Seoul, Republic of Korea). For the correlation between pre-F protein concentration and OD, r^2 values > 0.90 in the linear correlation analyses were considered acceptable. For precision, test conditions were considered equivalent and reproducible at a CV ≤ 20%.

3. Results

3.1. Development and Optimization of ELISA-Based Protocol

The concentration of DS-Cav1, as coating antigen, was 5 μg/mL, and the dilution factor of the secondary antibody, at which a high optical density and r^2 value greater than 0.9 was obtained, was 1:2,000 (Table S1). The SC-TM and G proteins were coated at a concentration of 2.5 μg/mL and 5

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μg/mL, respectively. The optimal dilution factor for the secondary antibody was 1:2,000 and 1:6,000 for SC-TM and G protein, respectively. For all three RSV antigens, 5% skim milk/PBS-T was used as the blocking reagent as it exhibited the lowest optical density and an r^2 value greater than 0.9, among the four blocking agents examined (Table S2).

3.2. Validation of the ELISA-Based Protocol

High accuracy was observed for all three RSV antigens. The value of r^2 was greater than 0.85 for all antisera used. Overall, the r^2 values measured with the reference antiserum were 0.9045, 0.8603, and 0.9777 for DS-Cav1, SC-TM, and G protein, respectively (Table 1). The precision of CV values was estimated to be below 15% for all RSV antigens and dilution factors. The mean CV values for DS-Cav1, SC-TM, and G protein were 2.599 %, 2.737%, and 5.129%, respectively (Table 2).

Table 1. Accuracy (dilution linearity) of the respiratory syncytial virus (RSV) enzyme-linked immunosorbent assay (ELISA) protocol for IgG antibodies against prefusion F proteins (DS-Cav-1 and SC-TM) and G protein.

	DS-Cav1 (5 μg/ml)						
	Dilution factor						r^2
	1:200	1:400	1:800	1:1600	1:3200	1:6400	
Reference sera	2.519	1.9658	1.4659	0.9846	0.7047	0.42405	0.9045
Low titer sera	2.2103	1.5678	1.0532	0.6568	0.46605	0.3265	0.9603
Medium titer sera	2.3991	1.70445	1.161	0.73195	0.4868	0.43985	0.9629
High titer sera	2.6452	2.1615	1.70685	1.21075	0.84935	0.56785	0.87
	SC-TM (2.5 μg/ml)						
	Dilution factor						R^2
	1:200	1:400	1:800	1:1600	1:3200	1:6400	
Reference sera	2.83735	2.38555	1.8442	1.2892	0.85635	0.63475	0.8603
Low titer sera	2.34215	1.73725	1.2267	0.82085	0.4996	0.37665	0.9355
Medium titer sera	2.64995	2.1299	1.50995	1.037	0.66725	0.5086	0.9051
High titer sera	2.8413	2.41785	1.90155	1.34495	0.966	0.6745	0.8514
	G protein (5 μg/ml)						
	Dilution factor						R^2
	1:200	1:400	1:800	1:1600	1:3200	1:6400	
Reference sera	1.01015	0.70585	0.4602	0.2764	0.16345	0.10335	0.9777
Low titer sera	1.14245	0.86765	0.6395	0.40675	0.2446	0.13945	0.9512
Medium titer sera	0.81455	0.5709	0.34935	0.2087	0.12385	0.0763	0.9797
High titer sera	0.97235	0.68775	0.44775	0.2775	0.1618	0.10215	0.9760

r^2, coefficient of determination.

Table 2. Inter-assay precision of the RSV ELISA protocol for IgG antibodies against prefusion F proteins (DS-Cav-1 and SC-TM) and G protein.

Antigen	DF	Aged < 5 years		Aged 5–18 years		Aged 19–49 years		Aged 50–64 years		Aged ≥ 65 years	
		OD (mean ± SD)	CV (%)	OD (mean ± SD)	CV (%)	OD (mean ± SD)	CV (%)	OD (mean ± SD)	CV (%)	OD (mean ± SD)	CV (%)
DS-Cav1	1:200	1.105 ± 0.005	0.443	1.177 ± 0.020	1.685	1.230 ± 0.015	1.251	0.807 ± 0.012	1.505	1.187 ± 0.015	1.258
	1:400	0.704 ± 0.009	1.218	0.894 ± 0.020	2.235	0.959 ± 0.012	1.247	0.542 ± 0.011	2.1	0.897 ± 0.021	2.291
	1:800	0.454 ± 0.010	2.196	0.690 ± 0.010	1.498	0.724 ± 0.009	1.223	0.353 ± 0.008	2.206	0.671 ± 0.014	2.016
	1:1600	0.278 ± 0.002	0.864	0.478 ± 0.012	2.567	0.514 ± 0.014	2.811	0.220 ± 0.005	2.241	0.502 ± 0.016	3.137
	1:3200	0.169 ± 0.004	2.623	0.317 ± 0.010	3.106	0.347 ± 0.012	3.548	0.138 ± 0.002	1.805	0.360 ± 0.005	1.414
	1:6400	0.108 ±0.003	3.046	0.200 ± 0.006	3.129	0.228 ± 0.007	2.892	0.090 ± 0.003	2.933	0.243 ± 0.006	2.506
	1:12800	0.072 ± 0.002	2.749	0.125 ± 0.005	3.881	0.145 ± 0.008	5.202	0.063 ± 0.002	3.014	0.159 ± 0.006	3.559
	1:25600	0.058 ± 0.002	3.354	0.088 ± 0.004	4.634	0.103 ± 0.005	4.526	0.052 ± 0.003	6.283	0.109 ± 0.004	3.751
SC-TM	1:200	1.167 ± 0.011	0.982	1.343 ± 0.008	0.571	1.298 ± 0.027	2.069	0.962 ± 0.012	1.296	0.779 ± 0.012	1.567
	1:400	0.731 ± 0.021	2.877	1.088 ± 0.014	1.259	0.983 ± 0.019	1.938	0.666 ± 0.006	0.918	0.439 ± 0.011	2.484
	1:800	0.484 ± 0.006	1.304	0.870 ± 0.020	2.276	0.728 ± 0.020	2.747	0.449 ± 0.005	1.004	0.288 ± 0.005	1.670
	1:1600	0.304 ± 0.009	2.857	0.644 ± 0.010	1.604	0.530 ± 0.008	1.585	0.291 ± 0.009	3.104	0.185 ± 0.006	3.237

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	1:3200	0.188 ± 0.005	2.417	0.430 ± 0.013	2.980	0.353 ± 0.008	2.318	0.185 ± 0.007	3.829	0.121 ± 0.001	1.030
	1:6400	0.120 ± 0.007	6.035	0.273 ± 0.006	2.282	0.236 ± 0.003	1.061	0.117 ± 0.005	4.240	0.083 ± 0.001	1.438
	1:12800	0.080 ± 0.004	4.865	0.166 ± 0.006	3.481	0.151 ± 0.004	2.600	0.081 ± 0.005	5.856	0.062 ± 0.001	2.237
	1:25600	0.061 ± 0.003	5.454	0.111 ± 0.003	2.761	0.105 ± 0.003	3.007	0.062 ± 0.007	10.717	0.052 ± 0.002	3.524
G protein	1:200	0.657 ± 0.020	2.987	0.199 ± 0.016	8.001	0.964 ± 0.032	3.355	0.746 ± 0.031	4.172	1.490 ± 0.028	1.904
	1:400	0.394 ± 0.010	2.508	0.107 ± 0.010	9.189	0.600 ± 0.023	3.852	0.434 ± 0.002	5.154	1.262 ± 0.038	2.985
	1:800	0.236 ± 0.008	3.227	0.071 ± 0.007	9.943	0.430 ± 0.015	3.424	0.285 ± 0.025	8.754	1.036 ± 0.044	4.230
	1:1600	0.136 ± 0.003	2.274	0.050 ± 0.003	6.295	0.267 ± 0.009	3.284	0.170 ± 0.014	8.251	0.821 ± 0.046	5.596
	1:3200	0.086 ± 0.005	6.356	0.039 ± 0.001	2.807	0.156 ± 0.006	4.006	0.105 ± 0.008	8.026	0.579 ± 0.038	6.544
	1:6400	0.061 ± 0.003	4.572	0.035 ± 0.001	2.674	0.098 ± 0.002	2.385	0.069 ± 0.004	5.475	0.363 ± 0.034	9.427
	1:12800	0.051 ± 0.006	11.501	0.033 ± 0.001	2.646	0.065 ± 0.002	2.849	0.049 ± 0.002	3.124	0.219 ± 0.020	8.971
	1:25600	0.041 ± 0.002	5.743	0.034 ± 0.001	2.795	0.047 ± 0.002	3.347	0.040 ± 0.001	3.521	0.134 ± 0.012	9.003

DF, dilution factor; OD, optical density; SD, standard deviation; CV, coefficient of variation.

Specificity was found to be high between the pre-F and G proteins, but low between the two pre-F proteins (DS-Cav1 and SC-TM). When adsorbed with a homologous antigen specific to each antibody, the anti-DS-Cav1, anti-SC-TM, and anti-G IgG titers decreased to 49.6%, 42.6%, and 24.5%, respectively (Table 3). The DS-Cav1-specific antibody titer decreased by an average of 44.3% when adsorbed with the SC-TM protein. Similarly, the titer of the SC-TM-specific antibody decreased by an average of 55.0% when adsorbed with the DS-Cav1 protein. Therefore, antibodies against DS-Cav1 and SC-TM showed cross-reactivity with SC-TM and DS-Cav1 antigens, respectively. In comparison, the DS-Cav1- and SC-TM-specific antibody titers were reduced by less than 10% after adsorption with G protein.

Table 3. Specificity of the RSV ELISA protocol after adsorption with homologous and heterologous proteins.

Coated protein		**DS-Cav1**				**SC-TM**				**G protein**			
Inhibitor (10 μg/mL)		**None**	**DS-Cav1**	**SC-TM**	**G protein**	**None**	**DS-Cav1**	**SC-TM**	**G protein**	**None**	**DS-Cav1**	**SC-TM**	**G protein**
Aged < 5 years	EU/ml	115.0	83.3	78.2	122.9	153.3	35.8	30.8	113.7	117.3	53.5	49.2	22.4
	%	-	72.5	68.0	106.9	-	23.3	20.1	74.2	-	45.6	42.0	19.1
Aged 5–18 years	EU/ml	193.8	73.0	67.3	121.6	148.4	69.3	65.7	122.5	78.8	44.1	41.9	17.3
	%	-	37.7	34.7	62.8	-	46.7	44.2	82.5	-	56.0	53.2	22.0
Aged 19–49 years	EU/ml	118.8	45.2	60.5	114.0	121.1	73.4	60.6	130.7	101.9	89.0	77.3	28.2
	%	-	38.1	51.0	96.0	-	60.6	50.0	108.0	-	87.4	75.9	27.6
Aged 50–64 years	EU/ml	122.7	65.7	84.0	107.7	107.0	88.0	61.1	120.4	134.3	145.8	132.1	42.3
	%	-	53.5	68.5	87.8	-	82.3	57.1	112.6	-	108.6	98.4	31.5
Aged ≥ 65 years	EU/ml	113.7	62.2	75.6	127.2	84.1	71.4	43.5	95.2	165.8	172.4	157.0	36.1
	%	-	54.7	66.5	111.9	-	84.8	51.8	113.2	-	104.0	94.7	21.8
Average	EU/ml	132.8	65.9	73.1	118.7	122.8	67.6	52.3	116.5	119.6	101.0	91.5	29.3
	%	-	49.6	55.1	89.4	-	55.0	42.6	94.9	-	84.4	76.5	24.5

EU, ELISA unit.

The differences in antibody specificity based on age group were examined. The post-adsorption anti-DS-Cav1 IgG antibody titer was found to decrease more significantly in individuals aged 65 years and above than in children aged < 5 years; anti-DS-Cav1 titers after/before adsorption with DS-Cav1, SC-TM, and G protein were 72.5%/22.6%, 68.0%/27.4%, and 106.9%/35.2%, respectively. Conversely, anti-SC-TM and anti-G protein antibody titers showed a greater decrease after adsorption in children aged < 5 years than in those aged 65 years and above. Anti-DS-Cav1 and anti-SC-TM IgG antibody titers were positively correlated (Figure 1). Although the r^2 value derived from the entire study population was low (0.1742), the linear relationship became clearer when divided by age group and remained statistically significant. The correlation was strongest in individuals aged 19–49 years ($r^2 = 0.6552$) and lowest in children aged < 5 years ($r^2 = 0.3879$).

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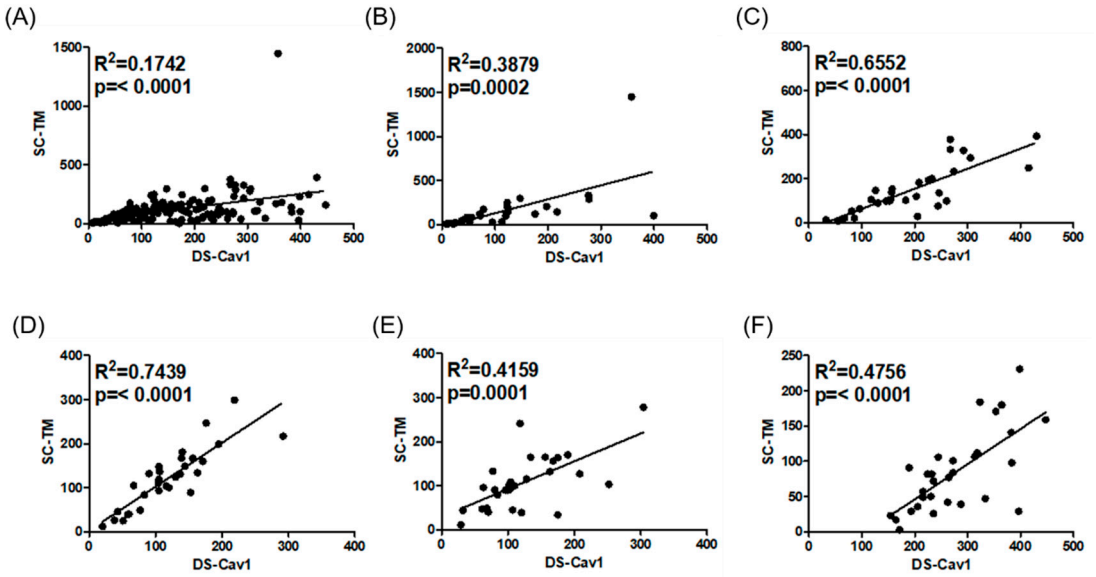

Figure 1. Correlation between anti-DS-Cav1 IgG titers and anti-SC-TM IgG titers. (A) total population, (B) aged < 5 years, (C) aged 5–18 years, (D) aged 19–49 years, (E) aged 50–64 years, and (F) aged 65 years and above.

3.3. Correlation with Neutralizing Antibody Titers

The correlation between IgG titers and neutralizing antibody titers against the three RSV antigens was weak. The value of r^2 between IgG titers and VRNT50 was 0.1449 (p = 0.526), 0.0496 (p = 0.237), and 0.2655 (p = 0.004) for DS-Cav1, SC-TM, and G protein, respectively (Figure 2). When examined based on age group, the r^2 values were estimated to be in the range of 0.1158–0.4795, 0.0309–0.1437, and 0.0042–0.6080 for DS-Cav1, SC-TM, and G protein, respectively.

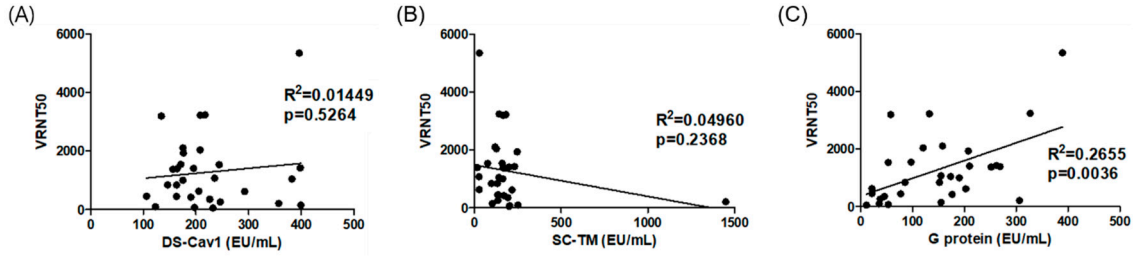

Figure 2. Correlation between anti-DS-Cav1 IgG titers and anti-SC-TM IgG titers. (A) total population, (B) aged < 5 years, (C) aged 5–18 years, (D) aged 19–49 years, (E) aged 50–64 years, and (F) aged 65 years and above.

4. Discussion

We established an ELISA protocol using two modified pre-F proteins, DS-Cav1 and SC-TM, and G protein, which showed good accuracy and precision. Although the two types of pre-F proteins showed remarkable cross-reactivity, high specificity was observed between pre-F and G proteins in adults. There was poor correlation between IgG antibody titers and neutralizing antibody titers measured using the VRNT.

When adsorbed with G protein, the SC-TM antibody titer was barely reduced (94.9% of the pre-adsorption titer) across all age groups; however, the DS-Cav1 antibody titer was considerably reduced by 37.2% in young children aged 5-18 years. Similarly, when adsorbed with pre-F proteins, the anti-G protein antibody titer decreased by approximately 50% on average in children. Young children are repeatedly exposed to diverse respiratory viral antigens, which results in non-specific antibody responses; therefore, the proportion of antibodies with lower specificity may be higher in children than in adults. As reported previously, antibody responses to RSV infection are influenced by independent factors such as level of development of the child's immune system and the presence or absence of maternal antibodies.[12] In addition, instability of the prefusion form of the soluble DS-Cav1 pre-F protein might influence the immune response to the protein; the G protein is highly

glycosylated and shows variable antigenicity among RSV strains, except for the centrally conserved domain.[13,14]

The low correlation between IgG pre-F antibody titers and neutralizing antibody titers might be due to the small amounts of functional antibodies. For this study, blood samples were collected from healthy individuals when RSV was in low circulation in the community because of the COVID-19 pandemic. A higher correlation can be expected when neutralizing antibody titers and post-vaccination RSV antibody titers are measured. Further studies are warranted to validate this assay in RSV vaccine recipients.

This study had some limitations. First, neither paired acute/convalescent sera nor postvaccination sera were available. Thus, specificity and correlation analyses were limited. Second, we did not test the neutralizing activity against the two RSV strains (A and B). However, the F protein genetic sequence is highly conserved between RSV strains, in contrast to the G protein, which shows approximately 50% genetic difference between RSV A and B strains.[15]

Since 2022, pre-F protein-based RSV vaccines with high efficacy in older adults and pregnant women, have been reported[16-19] A single dose of a protein-subunit vaccine or mRNA vaccine showed an efficacy of over 80% against RSV-related lower respiratory tract diseases in adults aged 60 years and above with an acceptable safety profile during one RSV season[16-18] The efficacy of the vaccine against medically-attended severe RSV-related lower respiratory tract disease in infants younger than 90 and 180 days of age has been reported to be 81.8% and 69.5%, respectively[19] Pre-F is mainly used as the target antigen for RSV vaccines, but some companies are also developing vaccines containing the G protein, nucleocapsid, and matrix proteins to enhance cell-mediated immunity. The advent of RSV vaccines is welcome news at a time when RSV is rampant again after its transient disappearance at the onset of the COVID-19 pandemic [20–22]. Therefore, there is an urgent need to establish a standardized vaccine immunogenicity evaluation method. The ELISA protocol (based on two pre-F antigens and G proteins) developed and validated in this study will be helpful in evaluating vaccines that are to be introduced in the near future.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: Determination of coating antigen concentration and dilution factor of secondary antibody; Table S2: Determination of optimal blocking buffer.

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