Vaccine 37 (2019) 1457–1466

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Universal monoclonal antibody-based influenza hemagglutinin quantitative enzyme-linked immunosorbent assay

Wonil Chae [a,c], Paul Kim [b,c], Beom Jeung Hwang [a,c], Baik Lin Seong [a,c,*]

[a] Department of Biotechnology, College of Life Science and Biotechnology, Yonsei University, Republic of Korea
[b] Department of Integrated OMICS for Biomedical Science, College of World Class University, Yonsei University, Republic of Korea
[c] Vaccine Translational Research Center, Yonsei University, Republic of Korea

ARTICLE INFO

Article history:
Received 7 November 2018
Received in revised form 22 January 2019
Accepted 23 January 2019
Available online 11 February 2019

Keywords:
Influenza vaccine
Potency test
RNA-mediated chaperone
Consensus HA
Universal antibodies
ELISA

ABSTRACT

Seasonal and pandemic influenza infections remain a serious public health concern. Many health authorities recommend annual vaccination as the most effective way to control influenza infection. Accordingly, regulatory guidelines ask vaccine manufacturers to determine vaccine potency at the time of release and throughout shelf-life to ensure vaccine quality. The potency of inactivated influenza vaccine is related to the quantity of hemagglutinin (HA). Since 1970s, single radial immunodiffusion (SRID) assay has been standardly used for the quantitation of HA in influenza vaccine. However, SRID is labor-intensive, inaccurate, and requires standard reference reagents that should be updated annually. Therefore, there have been extensive efforts to develop alternative potency assays. In this study, we developed and tested a new HA quantitative enzyme-linked immunosorbent assay (ELISA) using a universal monoclonal antibody that can bind to HAs from various subtypes in group 1 influenza A virus (IAV). We analyzed the conserved stalk domain of HA via a library approach to design a consensus HA antigen for group 1 IAV. The antigens were expressed as a soluble form in *E. coli* and were purified by Ni-affinity chromatography. When tested with variety of HAs from IAVs or influenza B viruses (IBVs), the mAbs exhibited specific binding to group 1 HAs, with potential exception to H9 subtype. Among various conditions of pH, urea, and reducing agents, pretreatment of HA at low pH exposing the conserved stalk domain was crucially important for optimal ELISA performance. Calibration curves for various HAs were generated to determine accuracy, specificity, sensitivity, and linear dynamic range. The ELISA method shows high sensitivity and accuracy compared with the SRID assay. The HA group specific universal mAbs against the consensus stalk domain of HA are conducive to establishing an ELISA-based standard procedure for the quantitation of HA antigens for annual vaccination against influenza infection.
© 2019 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

Seasonal and pandemic influenza infections remain a serious public health concern [1]. Annual vaccination is recommended by many public health authorities. Ensuring optimal vaccine potency is important for timely release of vaccine lots in a reproducible manner. For this purpose, regulatory guidelines ask vaccine manufacturers to strictly supervise the potency at the time of release and throughout shelf-life. The quantity of hemagglutinin (HA), the major protective antigen responsible for inducing neutralization antibodies [2], is related to the potency of influenza vaccines [3,4]. Since the 1970s, single radial immunodiffusion (SRID) assay has been standardly used for HA quantitation to validate vaccine potency [5]. However, because of inherent shortcomings of this method, there is an increasing demand for an alternative potency assay. First, this method is labor-intensive, non-automated, and time-consuming. Second, because of its low sensitivity, it requires high concentrations of antigens [6]. Third, it depends on strain-specific standard reagents, purified viral HA antigen, and polyclonal anti-serum, which take two to three months to produce and are distributed only by a few WHO collaborating centers. These reagents are crucially important for timely release of vaccines to the public by annual licensure. Moreover, non-availability

Abbreviations: IAV, influenza A virus; IBV, influenza B virus; cHA, consensus hemagglutinin; mAb, monoclonal antibody; mRID, RNA interaction domain of Lysyl tRNA synthetase from mouse; D6, 6 repeated aspartic acid linker; TEV, tobacco etch virus; MCS, multi cloning site; 6xHis, hexahistidin tag.

* Corresponding author at: Department of Biotechnology, College of Life Science and Biotechnology, Yonsei University, Seoul 03722, Republic of Korea.
E-mail address: blseong@yonsei.ac.kr (B.L. Seong).

https://doi.org/10.1016/j.vaccine.2019.01.068
0264-410X/© 2019 The Authors. Published by Elsevier Ltd.
This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

or shortage of the reagents critically hinders vaccine supply, as exemplified by the 2009 pandemic [7]. Therefore, there is a need for an alternative method to determine influenza vaccine potency that can shorten the time of development and distribution.

In the current study, we developed a new HA quantitative enzyme-linked immunosorbent assay (ELISA) using a universal monoclonal antibody (mAb) that can bind to HA antigens from various subtypes and drift strains. We analyzed the conserved domain of the HA stalk to design a consensus HA (cHA) stalk for group 1 influenza A viruses (IAVs). The antigens were expressed in *Escherichia coli* based on an RNA-mediated chaperone expression system and purified as a soluble form [8,9]. Then, mAbs were generated and screened for specific binding to group 1 IAV HA. Calibration curves for various subtypes of HA antigens—recombinant HA, NIBSC standard HA reagents, and quadrivalent influenza vaccine HA antigens—were generated for optimization of ELISA-based quantitation of HA antigens with respect to accuracy, specificity, sensitivity, and linear dynamic range.

2. Materials and methods

2.1. Sequence analysis for cHA stalk

HA sequences from human isolates were collected from the Influenza Virus Resource at the National Center for Biotechnology Information. The library contains sequences from H1 (11,205), H2 (159), H5 (433), and H9 (12), which are group 1 IAVs. First, highly conserved amino acid residues from each HA subtype were deduced using Seq2logo 2.0 [10] and Vector NTI Advance® version 11.5 (Thermo Fisher, Waltham, MA). Then, high frequency fragments that were consisted with highly conserved amino acid residues of each HA subtype were obtained. Finally, a consensus HA (cHA) stalk sequence for group 1 was generated by merging all high frequency fragments of subtypes H1, H2, H5, and H9, using the H1 fragment as a template.

Secondary structure prediction was conducted using Network Protein Sequence in ExPASy [11]. Secondary structures were predicted by analyzing the sequences of cHA stalk, A/Puerto Rico/8/1934 (GenBank: NC_002017.1), A/California/07/2009 (GenBank: CY266191.1), A/Canada/720/05 (GenBank: DQ009917.1), A/Indonesia/5/2005 (GenBank: EU146622.1), and A/Hong Kong/1073/99 (GenBank: AJ404626.1). Then, the predicted structure of cHA stalk was compared with the structures of the above-mentioned wild-type viral HAs.

The accessibility and polarity of the computationally designed cHA stalk were calculated and compared with those from naturally occurring infectious viruses. For the calculation, a monomer structure of the cHA stalk was built by homology modeling using SWISS-MODEL [12] based on the HA structure of A/Brevig Mission/1918 H1N1 (PDB ID: 1RUZ). The trimer structure of the cHA stalk was produced by docking the three monomer structures using ClusPro 2.0 multimer docking [13]. The accessibility and polarity of the cHA stalk and the HA stalk from A/Puerto Rico/8/34 H1N1 were calculated using Environment and Virtual Docking of Atoms (Syntekabio, Daejeon, Korea), based on the Shrake-Rupley algorithm [14,15].

2.2. Cloning, expression, and purification of the cHA stalk

The pGE-RID4 vector which has RNA interaction domain of Lysyl tRNA synthetase from mouse(mRID) as fusion protein [9] was used for soluble expression of the cHA stalk. A cHA stalk gene that was codon-optimized to *E. coli* was synthesized (BIONICS, Seoul, Korea), PCR-amplified, and inserted into the expression vector.

The *E. coli* strain BL21 star (DE3) pLysS (Invitrogen, Carlsbad, CA) was used for recombinant protein expression. *E. coli* was cultured in 15 ml of Luria-Bertani (LB) medium supplemented with 1 mM ampicillin and 1 mM chloramphenicol at 37 °C or 20 °C. After centrifugation, the pelleted cells were resuspended in phosphate-buffered saline (PBS), sonicated, and centrifuged to separate soluble and insoluble fractions. Total cell lysates and each fraction were analyzed by SDS-PAGE.

An ÄKTA prime plus chromatography system (GE Healthcare, Chicago, IL) and a HisTrap HP column (GE Healthcare, Chicago, IL) were used for purification. The supernatants in purification buffer (50 mM Tris-cl (pH 7.5), 300 mM NaCl, 10 mM imidazole, 10% glycerol, 2 mM mercaptoethanol, 0.1% Tween-20) were loaded into a Ni-nitrilotriacetic acid resin column and were eluted with a linear gradient of imidazole (10–300 mM). Each fraction was analyzed by SDS-PAGE, and the fractions enriched in mRID-cHA stalk were pooled, dialyzed against storage buffer (50 mM Tris-Cl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 0.1% Tween-20), and concentrated using Centriprep® centrifugal filter (Merck Millipore, Burlington, MA). The concentration of the antigen was determined by PAGE followed by densitometric quantitation of the coomassie-stained protein band using bovine serum albumin (Amresco, Solon, OH) with known concentration.

2.3. Generation of monoclonal antibody

Murine mAbs against the mRID-cHA stalk for group 1 IAV were generated by murine cell fusion/hybridoma [16], by ATGEN (Seongnam, Korea). In brief, six-weak-old BALB/c mice were immunized twice with the mRID-cHA stalk mixed with Freund's adjuvant once every two weeks. Positive hybridoma clones were screened using ELISA with the mRID-cHA stalk. Two selected clones, 1G5 and 2C12, were purified using Protein G resin (GE Healthcare, Chicago, IL) and dialyzed against PBS.

2.4. Reference HA antigens for ELISA

HA antigens of various origin were used; recombinant HA produced from HEK293 cells or insect cells (Sino Biological, Beijing, China), the standard HA antigens from the National Institute for Biological Standards and Control (NIBSC, Blanche Lane, UK) and quadrivalent seasonal influenza vaccine (GC flu) produced by Green Cross Pharma (Yongin, Korea). Detailed information on the HA antigens is presented in Supplemental Tables 1–3.

2.5. Antigen pretreatment

HA antigens were mixed with various pretreatment buffers with different pH, 1,4-dithiothreitol (DTT; Gold Biotechnology, St. Louis, MO) concentrations, and urea (Sigma-Aldrich, St. Louis, MO) concentrations either prior to (pre-binding) or after binding to (post-binding) the ELISA plate. Under the pre-binding condition, the antigen solution and the buffer were mixed at a 1:1 ratio and incubated at room temperature for 30 min before binding to the plate. Under the post-binding condition, the HA antigen was bound to the plate and incubated with pretreatment buffer for 30 min. The pH of the pretreatment buffer was adjusted based on the Henderson-Hasselbalch equation. DTT and urea were dissolved to desired concentration after the pH was adjusted.

2.6. Indirect ELISA

HA antigens were coated on 96-well immunoassay plates (Nunc-Immuno™ MicroWell™ 96 well solid plates; Thermo Fisher, Waltham, MA) and incubated at 4 °C overnight. Then, 5% solution of skim milk (BD Diagnostic, Franklin Lakes, NJ) was added and

incubated at room temperature for 1 h for blocking. Then, the diluted murine mAb was added and incubated at 37 °C for 2 h. Subsequently, horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich, St. Louis, MO) was added and incubated at 37 °C for 1 h. At each step, the wells were washed with PBS with 0.05% Tween-20. Lastly, 3,3′,5,5′-tetramethylbenzidine substrate solution (BD Biosciences, Franklin Lakes, NJ) was added and incubated at room temperature in the dark for 30 min. The enzymatic reaction was terminated by adding 0.2 N H_2SO_4. The optical density at 450 nm was measured using a FLUOstar Optima microplate reader (BMG Labtech, Ortenberg, Germany).

2.7. SRID assay

The SRID assay was conducted as described [5], with minor modifications. The standard HA antigen of A/Singapore/GP1908/2015 (IVR-180) (NIBSC product number: 16/292) and the corresponding standard anti-A/Michigan/45/2015-like HA serum (NIBSC product number: 16/304) were used. The diameters of precipitated circular zones were measured. The concentration of test HA antigen was calculated by the slope ratio method [17] based on duplicate SRID results.

2.8. Quantification of HA antigens by ELISA

The standard HA antigen used was from A/Singapore/GP1908/2015 (IVR-180) (NIBSC product number: 16/292). The test HA antigen was a component of quadrivalent influenza vaccine (GC flu) (kindly supplied by Green Cross Co., Korea). The standard and test antigens were treated with 10% (w/v) Zwittergent 3-14 detergent (Sigma-Aldrich, St. Louis, MO) solution at a 9:1 ratio at room temperature for 30 min. Next, both antigens were pretreated under the condition of pH 5.0 and 100 mM DTT at room temperature for 30 min and coated in duplicate on 96-well immunoassay plates. ELISA was conducted as described in Section 2.6. The test HA antigen concentration was calculated by the slope ratio method [17].

2.9. Statistical analysis

Results are reported as the mean ± standard deviation. Four-parameter linear regression was conducted to analyze ELISA results, using GraphPad Prism version 5.01 (GraphPad Software, La Jolla, CA).

3. Results

3.1. Generation and analysis of cHA stalk

The procedure for the generation and validation of consensus HA stalk for group 1 IAV is outlined in Fig. 1A and the experimental details are described in Section 2.1. Briefly, a cHA stalk sequence (Fig. 1B) was produced by merging high frequency fragments of H1, H2, H5, and H9 (Supplemental Fig. S1). The H1 fragment was used as a template considering its abundance among various subtypes. Then, the secondary structure of the cHA stalk was predicted to confirm whether the deduced cHA stalk structure was compatible with the viral HA structure. *In-silico* predictions verified that an expected secondary structure of the cHA stalk was compatible with those of naturally occurring HA of group 1 IAVs (Fig. 1C).

Surface accessibility and polarity, key scoring indicators for immunogenic epitopes [18], of the cHA stalk were calculated based on the trimer structure and were compared with those of viral HA (Supplemental Fig. S2). We found no significant difference between the deduced cHA stalk and naturally occurring HA from A/Puerto Rico/8/34 (H1N1). In summary, the cHA stalk was structurally and immunologically compatible with viral HA, and suitable for the generation of universal mAbs.

3.2. Expression and purification of mRID-cHA stalk

The cHA stalk was genetically fused with an RNA-interacting domain of lysyl-tRNA synthetase from mouse (mRID) for soluble expression (Fig. 2A) [9]. Ideally, the cHA stalk should be expressed as a soluble and properly folded form to elicit conformational epitope-directed mAbs. RIDs of eukaryotic origin can function as a transducer for RNA-mediated chaperone activity and can be used as fusion partners to expedite soluble expression of properly folded recombinant proteins [9,19]. Here, mRID ("self") was judiciously chosen because it is expected to be non-immunogenic in mice, and therefore, antibodies would be elicited predominantly to the "foreign" cHA domain, greatly facilitating the screening of clones of desired specificity.

Expression of mRID-cHA stalk was induced at various temperatures. Induction at 20 °C resulted in relatively higher solubility (~40%) than induction at 37 °C (~20%), as analyzed by SDS-PAGE (Fig. 2B), probably due to a lower translation speed [20]. In contrast, cHA stalk without fusion was expressed at a very low level, predominantly as insoluble form, at both temperatures (Fig. 2C). Thus, mRID-cHA stalk (~30 kDa) produced at 20 °C was purified by Ni^+ affinity chromatography (Fig. 2D). The protein was concentrated through a centrifugal filter (10 kDa) (1.4 mg/ml) and used for immunization of mice to generate mAbs.

3.3. mAb generation and validation

mAbs were generated by hybridoma fusion [16]. Two positive mAbs, 1G5 and 2C12, were selected and their binding specificity was screened by ELISA (Fig. 3). The two clones bound to the mRID-cHA stalk, but not to mRID. Thus, we can conclude that the two mAbs are cHA stalk specific. Also, the response curve using the mRID-cHA stalk confirmed its potential for quantitative detection of HA antigens.

3.4. Pretreatment of HA antigens

HA is composed of a HA1 globular domain and a HA2 stalk domain, which are linked by disulfide bonds [21]. HA undergoes a conformational transition at low pH, where the stalk region becomes unmasked by the HA1 domain, forming an extended alpha helix [22]. The exposure of the stalk region is expected to enhance antibody binding, and therefore, antigens were pretreated under various conditions to enhance ELISA responses.

First, low-pH solutions were used for antigen pretreatments (Fig. 4A–D). Most notably, ELISA responses were highly increased when the buffer pH was lower than 5.0. For instance, the responses with pH 3.5 buffer were approximately 3–5 times higher than those with pH 7.4 buffer. However, there was no significant change at pH > 5.0 where conformational transition is unlikely to occur. Moreover, the ELISA responses depended on the time point of pretreatment; pre-binding treatment resulted in significantly higher responses than post-binding treatment, although pH dependence remained sustained.

Considering that HA1 and HA2 domains are linked by disulfide bonds [23], treatment of HA with a reducing agent, e.g., DTT, is expected to release the HA1 from the HA2 [24] and expose the stalk region. PBS (pH 7.4) containing DTT at different concentrations was used for antigen pretreatments pre- and post-binding (Fig. 4E–H). ELISA responses were increased with increasing concentration of DTT; the responses in 100 mM DTT were approximately 1.5 times higher than those in the absence of DTT, regardless of pre- or post-binding treatment.

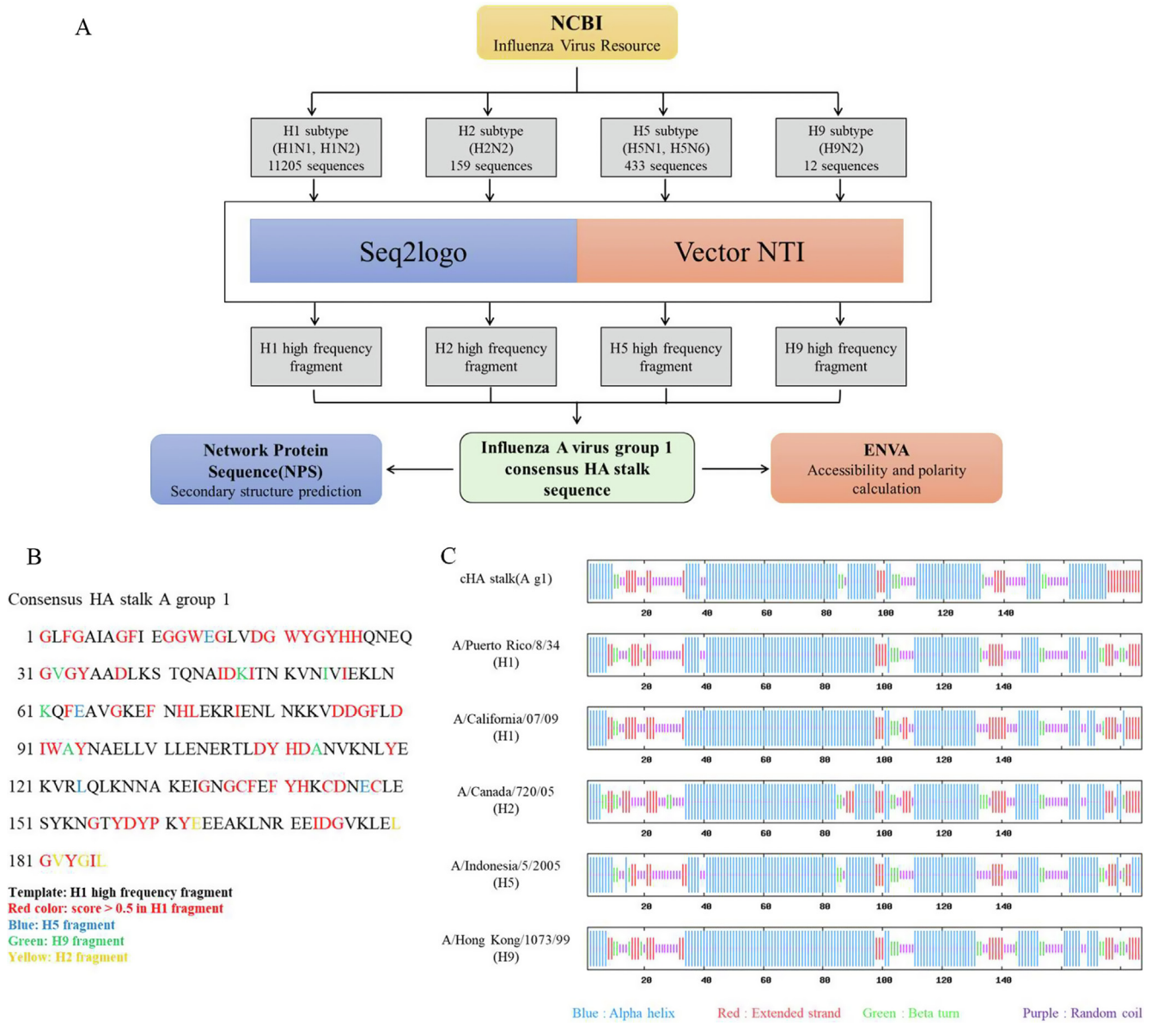

Fig. 1. Generation and validation of cHA stalk. (A) Schematic diagram of the generation and validation of the cHA stalk. (B) Amino acid sequence of the cHA stalk of group1 IAV. Detailed methods for sequence design are described in Section 2.1. (C) Comparison of secondary structures between the cHA stalk and viral HA stalks.

A potential synergistic effect between low pH and DTT was also tested. HA antigens were treated with buffers of various combinations of different pH and DTT concentrations pre- or post-binding (Fig. 5). The highest ELISA response was observed when HA was pretreated with pre-binding under the condition of pH 5.0 and 100 mM DTT.

Urea is generally used to disrupt protein structure [25], and different concentrations of urea in PBS buffer were tested (Fig. 4I–L). ELISA responses increased up to 5 M urea and were higher in pre-binding condition. Finally, antigens were pretreated with various concentrations of urea under the condition of pH 5.0 and 100 mM DTT (Fig. 4M–P). Under this condition, there was no significant difference depending on the urea concentration. Likely, low pH and reducing condition are sufficient to expose the HA stalk. In conclusion, the optimal HA antigen pretreatment was established as pre-binding treatment under the condition of pH 5.0 and 100 mM DTT.

3.5. ELISA with recombinant HA antigens

ELISAs with recombinant HA antigens produced from human cells or insect cells were conducted using the antibodies 1G5 and 2C12 (Supplemental Fig. S3). Both antibodies could bind with group 1 IAV HAs, including H1 subtype (A/California/07/2009, A/Puerto Rico/8/34, A/New Caledonia/20/99, A/Brisbane/59/2007), H2 subtype (A/Canada/720/2005, A/Japan/305/1957), H5 subtype (A/Indonesia/5/2005, A/American green-winged teal/California/HKWF609/2007), and H9 subtype (A/duck/NZL/76/1984, A/Hong Kong/1073/99), but not with group 2 IAV HAs, including H3 subtype (A/Texas/50/2012, A/Brisbane/10/2007) and H7 subtype (A/Netherlands/219/03), nor with B virus HAs including B/Phuket/3073/2013, B/Yamagata/16/1988 and B/Massachusetts/03/2010 (Supplemental Figs. S4, S5). Thus, 1G5 and 2C12 were confirmed to be specific to group 1 IAV recombinant HAs derived from eukaryotes.

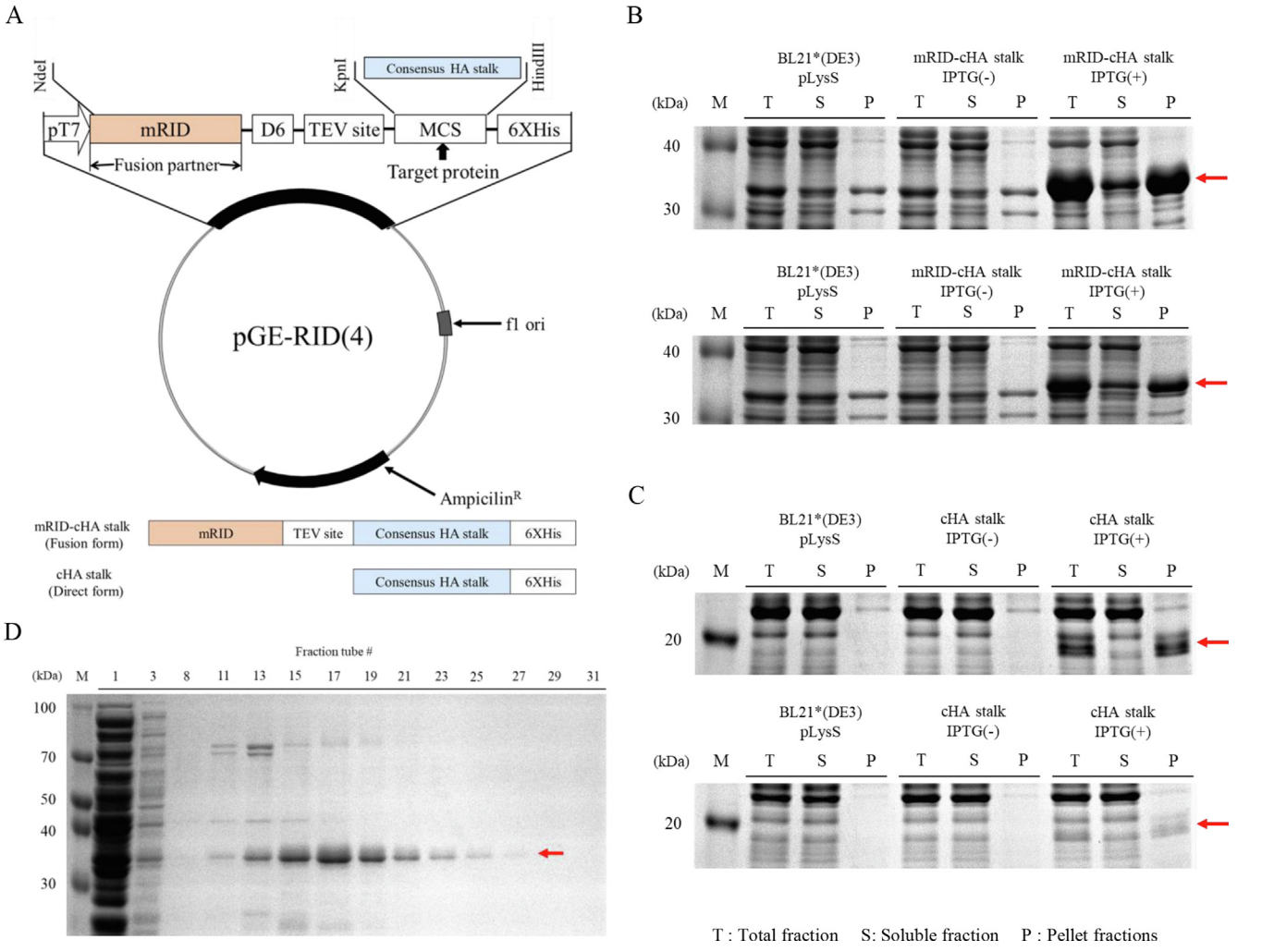

Fig. 2. Expression and purification of cHA stalk antigens. (A) Schematic diagram of the pGE-RID4 vector and structure of the mRID-cHA stalk (fusion form) and cHA stalk (non-fused form). The vector has an mRID, a D6 linker, a TEV protease recognition site, a multi-cloning site (MCS) and a 6xHis tag sequence under the control of the T7 promoter. (B) Expression profile of the mRID-cHA stalk at 37 °C (upper panel) and 20 °C (lower panel). The red arrow indicates the mRID-cHA stalk (32.2 kDa). BL21*(DE3) pLysS indicates expression profile of endogenous proteins without transformation, and mRID-cHA stalk IPTG (+) and mRID-cHA stalk IPTG (−) with or without induction of transformed cells, respectively. (C) Expression profile of the cHA stalk at 37 °C (upper panel) and 20 °C (lower panel). The red arrow indicates the cHA stalk (22.6 kDa). BL21* (DE3) pLysS indicates expression profile of endogenous proteins without transformation, and cHA stalk IPTG (+) and cHA stalk IPTG (−) with or without induction of transformed cells, respectively. (D) Purification profile of the mRID-cHA stalk using Ni^{+} affinity chromatography. The red arrow indicates the mRID-cHA stalk. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

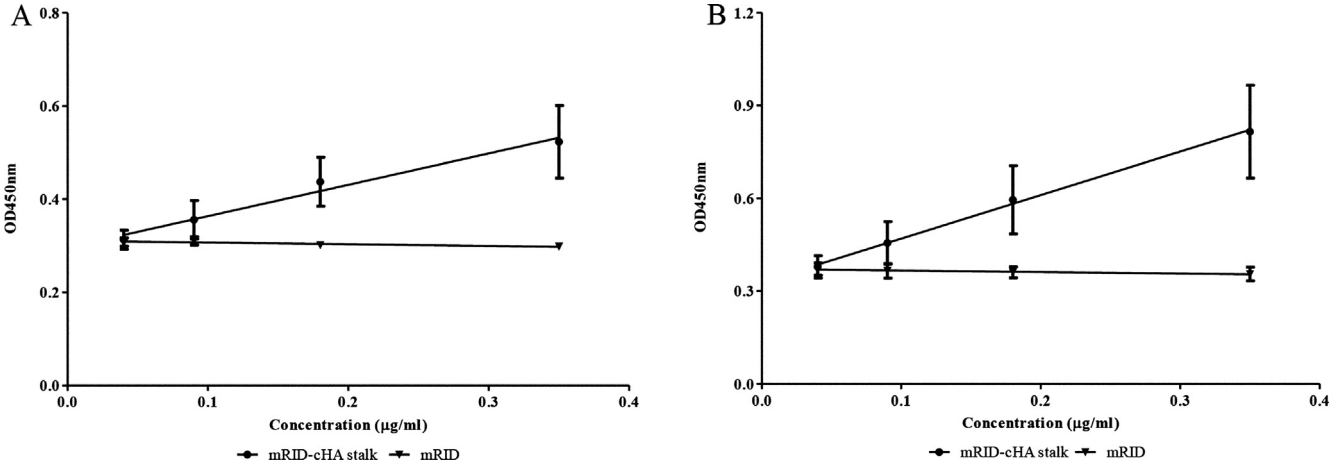

Fig. 3. ELISA with mRID and mRID-cHA stalk using the universal antibodies. Calibration curves were determined by four-parameter regression. Error bars indicate the standard deviation in five replicates. (A) 1G5 (B) 2C12.

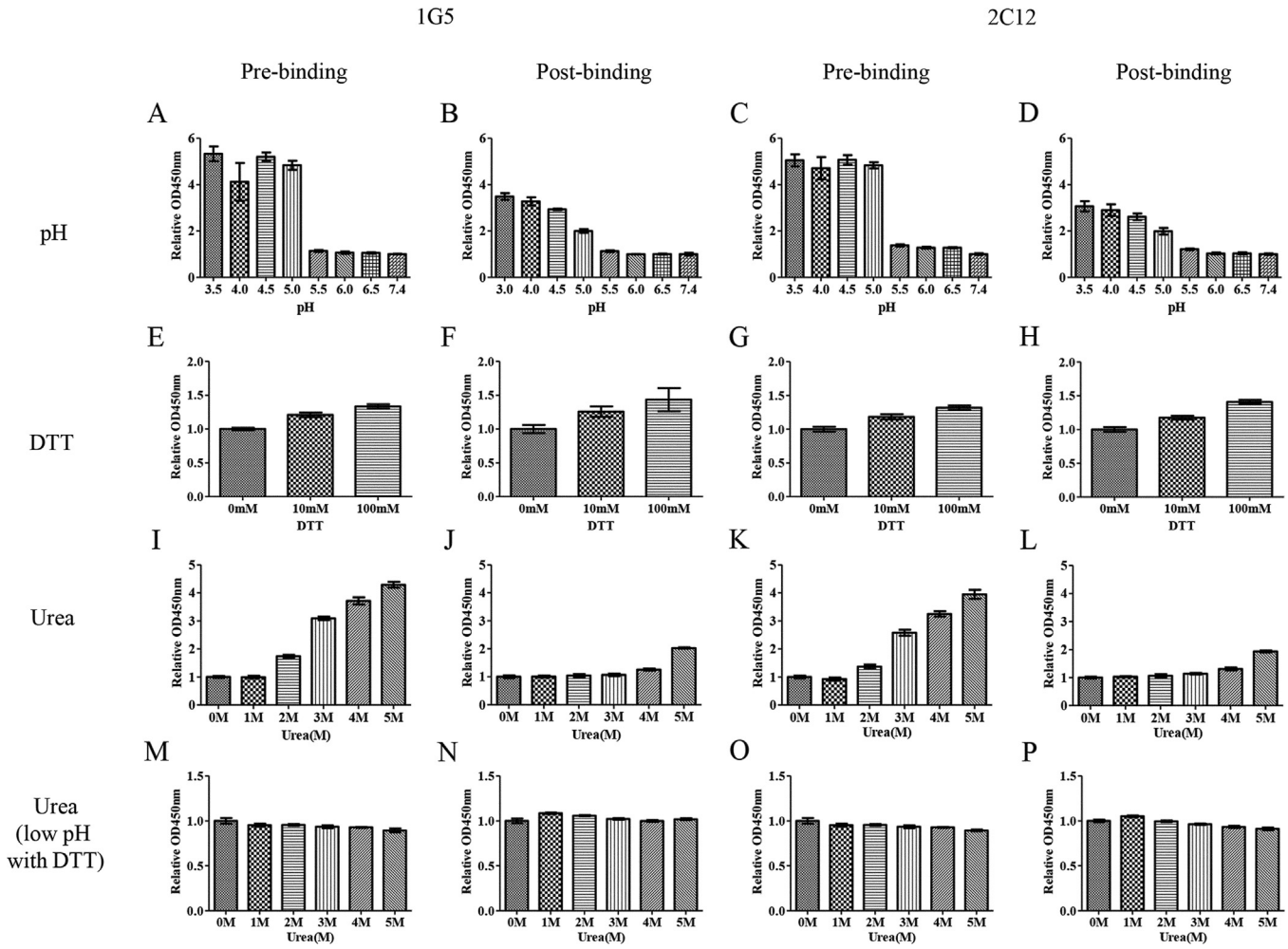

Fig. 4. Comparison of ELISA responses under different conditions of antigen pretreatment. Treatments were carried out pre- or post-binding, as indicated in the figure. Plates were coated with HA antigen, GC flu A/Singapore/GP1908/2015 IBR-180 (H1N1) (GC Pharma, Korea), at 5 μg/ml (100 μl) per well. The relative OD450nm values were calculated by dividing the value of the experimental group by that of the control group. Error bars indicate standard deviation in five replicates. (A–D) ELISA responses under NaOAc buffer at different pH. (E–H); at different concentration of DTT in PBS buffer (pH 7.4); (I–L) at different concentrations of urea in PBS buffer; (M–P) at different concentrations of urea in condition of pH 5.0 with 100 mM DTT).

Statistical analysis based on linear regression were implemented to validate the ELISA results with respect to linearity, sensitivity, and reproducibility (Supplemental Table 4). In terms of linearity, both antibodies showed high coefficient of determination (R^2) values: for 1G5, R^2 was 0.9955 ± 0.0035 and for 2C12, R^2 was 0.9949 ± 0.0059. Detailed results are presented in Table S4. Thus, there was a strong positive correlation between HA quantity and ELISA response.

Regarding sensitivity, the limit of detection (LOD), except for the H9 subtype, were lower than ∼0.04 μg/ml. However, the LOD values varied depending on the strain, probably reflecting differences in the affinity of the antibodies to HAs from different strains. Specially, the H9 subtype had lower affinity than the other HAs tested. The variations in ELISA response among different strains may be due to sequence variations or slight structural differences in the target epitope(s). Moreover, the lack of H9 reference sequences at the time of consensus sequence deduction may explain, in part, the low affinity to the H9 subtype.

Reproducibility is represented by the coefficient of variation (CV). The CV was 4.018 ± 1.106% for 1G5 and 4.377 ± 1.223 for 2C12. Detailed CV values for all ELISAs are presented in Table S4, and indicate that the results of repeated ELISAs were reliable. In summary, the universal mAbs, 1G5 and 2C12, are specific to multiple subtypes of HAs within group 1, without cross-reactivity to other subtypes of HA, with a high degree of linearity, sensitivity, and reproducibility.

3.6. ELISA with standard HA antigens

ELISA with the standard HA antigens were also conducted using 1G5 and 2C12 with statistical indicators in terms of linearity, sensitivity, and reproducibility (Supplemental Fig. S3). All the standard antigens were egg-derived and supplied by the NIBSC.

Both antibodies bound specifically with the group 1 IAV HAs of H1 subtype (A/Michigan/45/2015 (NYMC X-275), A/Singapore/GP1908/2015 (IVR-180), A/California/7/09 (NYMC-X181), A/Puerto Rico/8/34, A/Brisbane/59/2007 (IVR-148)), H2 subtype (A/Singapore/1/57), H5 subtype (A/Anhui/1/05 IBCDC-RG-6), and H9 subtype (A/chick/Hong Kong/G9/1997 NIBRG-91), but not with group 2 IAV HAs of H3 subtype (A/Hong Kong/4801/2014 (NYMC X-263B), A/Switzerland/9715293/2013 (NIB88), A/Texas/50/2012 (NYMC X-223A)), nor with B virus HAs of B/Brisbane/60/2008 (NYMC BX-35), B/Phuket/3073/2013 and B/Massachusetts/02/2012 (Supplemental Figs. S6 and S7). The binding specificities were consistent with those of recombinant HA antigens (Supplemental Figs. S4 and S5). These results confirmed the specificity of 1G5 and 2C12 for multiple subtypes of group 1 IAV HAs.

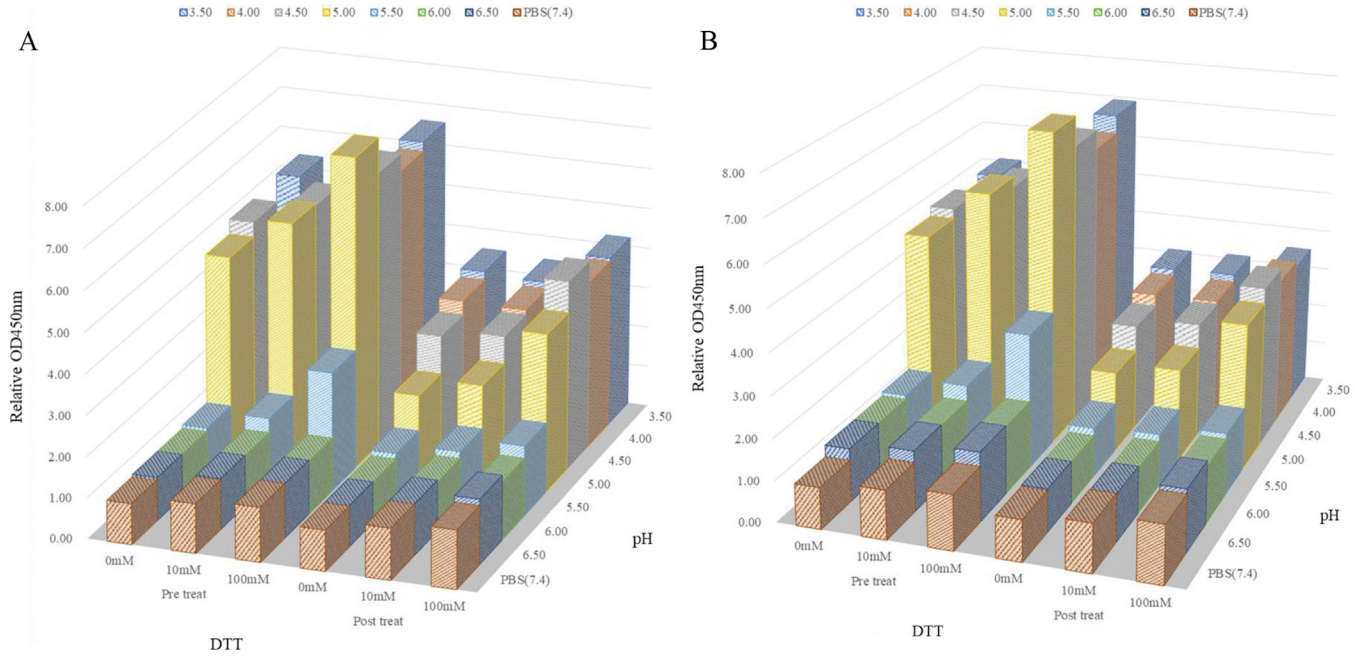

Fig. 5. ELISA responses under pretreatment with NaOAc buffer at different pH and with various concentrations of DTT. Plates were coated with HA antigen, GC flu A/Singapore/GP1908/2015 IBR-180 (H1N1) vaccine antigen, at 5 μg/ml (100 μl) per well. (A) 1G5, (B) 2C12.

In addition, data of ELISAs with group 1 IAV HA were subjected to linear regression to estimate their statistical significance (Table 1). With respect to linearity, both antibodies showed high R^2 values: for 1G5, R^2 was 0.9936 ± 0.0113, and for 2C12, R^2 was 0.9937 ± 0.0104. Detailed results are summarized in Table 1. In conclusion, there was a high degree of correlation between the quantity of egg-derived HA antigen and ELISA response.

In terms of sensitivity, the LOD values of HAs (except H9 subtype) were lower than ~0.05 μg/ml. However, slight differences were observed among HA antigens from different strains. Again, the affinity to H9 subtype was much lower than that of other HAs, extending the results from recombinant HAs (Supplemental Table 4).

Regarding the reproducibility, The CV was 3.962 ± 0.824% for 1G5 and 4.268 ± 1.004 for 2C12 (Table 1), indicating high reproducibility. Thus, 1G5 and 2C12 are group 1 IAV HA-specific, without cross-reactivity to HAs belonging to group 2 IAV or IBV, showing high degrees of linearity, sensitivity, and reproducibility. Considering that these egg-derived HA antigens are currently used as standard reagents for SRID assay, the present results imply that both mAb, 1G5 and 2C12, can be directly applicable to the quantification of HAs.

3.7. ELISA with quadrivalent vaccine HA antigens

Finally, the ELISA was extended to commercial influenza vaccine antigens (Fig. 6), with relevant statistical indicators (Supplemental Table 5). The tested vaccine components for quadrivalent influenza vaccines were derived from embryonated eggs and were supplied by Green Cross Pharma (Yongin-si, Korea). Both 1G5 and 2C12 bound to group 1 HA A/Singapore/GP1908/2015 IBR-180 (H1N1). For 1G5, R^2 was 0.9991, and for 2C12, R^2 was 0.9949. The CV values were ~4.1%, and LODs were <0.02 μg/ml. However, both antibodies failed to bind to HA of A/Hong Kong/4801/2014 (NYMC X-263B) (H3N2), B/Phuket/3073/2013 (Yamagata-like), and B/Brisbane/60/2008 (Victoria-like). The group 1 specificity of the mAbs, as proven for recombinant HAs (Supplemental Figs. S4 and S5), egg-derived standards (Supplemental Figs. S6 and S7), and commercial vaccines (Fig. 6), strongly suggests their utility in an ELISA-based assay for the quantitation of HA in influenza vaccines.

3.8. Comparison between SRID and ELISA

A test antigen (standard HA antigen of A/Singapore/GP1908/2015 IBR-180) was quantified by SRID, currently the recommended standardized assay, and the ELISA using the universal mAbs introduced in this study, for comparison (Fig. 7). The antigens were treated with the Zwittergent 3-14 detergent. The SRID assay was conducted as described in Section 2.7. For the ELISA, the antigen was treated with pretreatment buffer (pH 5.0, 200 mM DTT, 200 mM sodium acetate buffer), and ELISA was conducted as described in Section 2.8. The concentrations of antigens were calculated using slope-ratio model with two replicates, following the guidelines from European pharmacopoeia 5.0 [17].

The concentration of HA as quantified by SRID was 36.53 ± 2.97 μg/ml. The concentration quantified by ELISA using 1G5 was 39.92 ± 1.98 μg/ml and that using 2C12 was 38.44 ± 1.60 μg/ml. The concentrations determined by SRID and ELISA were similar, but those from ELISA were slightly higher than those from SRID. Of note, error ranges from ELISA were smaller than those of SRID. In summary, subjected to the limited availability of commercial sample materials, the quantitative ELISA using antibodies against the consensus stalk domain is found comparable to SRID with respect to its determined concentration. However, the ELISA can produce more accurate and reliable result than SRID method.

4. Discussion

Timely distribution of vaccines is important in preventing influenza infection among humans. The current standard method for HA quantification, the SRID assay, represents a hurdle for timely vaccine licensing because of its intrinsic limitations. Here, we introduced a novel ELISA using antibodies that can bind to HA of various strains. The mAbs against the consensus stalk domain

Table 1
Validation of linearity, sensitivity and reproducibility of the ELISA with standard HAs (NIBSC). Calibration curves determined by four-parameter linear regression using data from five replicates.

			mAb							
			1G5				2C12			
Type	HA subtype	Strain	Response	Linearity (R^2)	Sensitivity (LOD (μg/ml))	Reproducibility (%CV)	Response	Linearity (R^2)	Sensitivity (LOD (μg/ml))	Reproducibility (%CV)
A group 1	H1	A/Michigan/45/2015 (NYMC X-275)	+	0.997	0.030	4.224	+	0.995	0.028	5.720
		A/Singapore/GP1908/2015 (IVR-180)	+	0.999	0.018	3.521	+	0.998	0.018	4.497
		A/California/7/09 (NYMC-X181)	+	0.999	0.015	3.779	+	0.998	0.012	2.705
		A/Puerto Rico/8/34	+	0.998	0.042	3.332	+	0.999	0.050	3.437
		A/Brisbane/59/2007 (IVR-148)	+	0.999	0.010	3.236	+	0.998	0.012	3.289
	H2	A/Singapore/1/57	+	0.999	0.017	3.946	+	1.000	0.016	4.244
	H5	A/Anhui/1/05 IBCDC-RG-6	+	0.997	0.036	3.075	+	0.999	0.042	4.153
	H9	A/chick/Hong Kong/G9/1997 NIBRG-91	+	0.963	0.322	5.850	+	0.965	0.451	5.933
A group 2	H3	A/Hong Kong/4801/2014 (NYMC X-263B)	–				–			
		A/Switzerland/9715293/2013 (NIB88)	–				–			
		A/Texas/50/2012 (NYMC X-223A)	–				–			
B		B/Brisbane/60/2008 (NYMC BX-35)	–				–			
		B/Phuket/3073/2013	–				–			
		B/Massachusetts/02/2012	–				–			

Response: '+' (positive); '–' (negative).

exhibited group-specific universality against various subtypes within group 1 IAVs, without cross-reactivity to group 2 IAVs or IBVs. The present approach could be extended to HAs of group 2 IAVs as well as to IBVs for the quantitation of HA antigens of multivalent seasonal influenza vaccines.

As an alternative to SRID, various HA quantification assays using mAbs have been developed [26–28]. However, most of these methods rely on strain-specific mAbs corresponding to the test antigen of interest. One study used a mAb targeting the fusion peptide of HA that universally bound to various HAs [29]. However, the assay could not differentiate between groups 1 and 2, and thus was unable to quantify each HA component in multivalent vaccine formulations. The present approach is based on the recognition of group-specific consensus sequence of the HA stalk domain, and thus allows quantitation of a single component (group 1 HA in this case, regardless of drift or shift strains) in multi-component influenza vaccines.

The group-specificity of the mAbs used in this study was tested among various subtypes, including H1, H2, H5, and H9. Among these, H1 subtype virus has been circulating in the human population over the last century [30]. H2 subtype strains were responsible for the pandemic outbreak in 1957 [31], and had circulated for a decade before replacement by H3N2 in 1968 [32]. However, there remains a possibility of re-introduction of H2 subtype strains by zoonotic transmission from birds and swine [33,34]. The H5 subtype can cause fatal infection in humans as exemplified by the avian-human transmission of the highly pathogenic avian influenza H5N1 [35,36]. A rare case of human-to-human transmission has been documented [37], and multiple mutations that would enable circulation among mammals have been identified [38]. In this regard, a group-specific universal antibody-based HA quantification method, primarily designed for the current seasonal influenza vaccine, may, in the future, be extended to pandemic vaccines, if necessary.

It is desirable to use a conserved domain to elicit antibodies with cross-reactivity. HA is composed of a highly variable HA1 globular domain and a relatively conserved HA2 stalk [39]. Our approach was therefore to use the stalk domain, and to enhance cross-reactivity further, a consensus sequence deduced from multiple HA subtypes within group 1 IAVs was employed as immunogen (Fig. 1B). Yet, we observed differences in the magnitudes of ELISA responses of the mAbs against various subtypes of HA within the same group (Figs. 6 and Supplemental Fig. S4–S7). There are several possible explanations. First, there could be slight sequence variation in the test HA antigens, which may affect the mAb binding affinity. Second, there may exist structural differences within HA2, where the HA stalk undergoes pH-dependent conformational transition [40]. Stalk domain exposure is essential to ensure full mAb binding and a subsequent ELISA response. As such, pretreatment at low pH to induce the extended alpha-helical conformation and with DTT to dissociate and remove the HA1 domain was necessary (Figs. 4 and 5). The pH dependence of coiled-coil structural

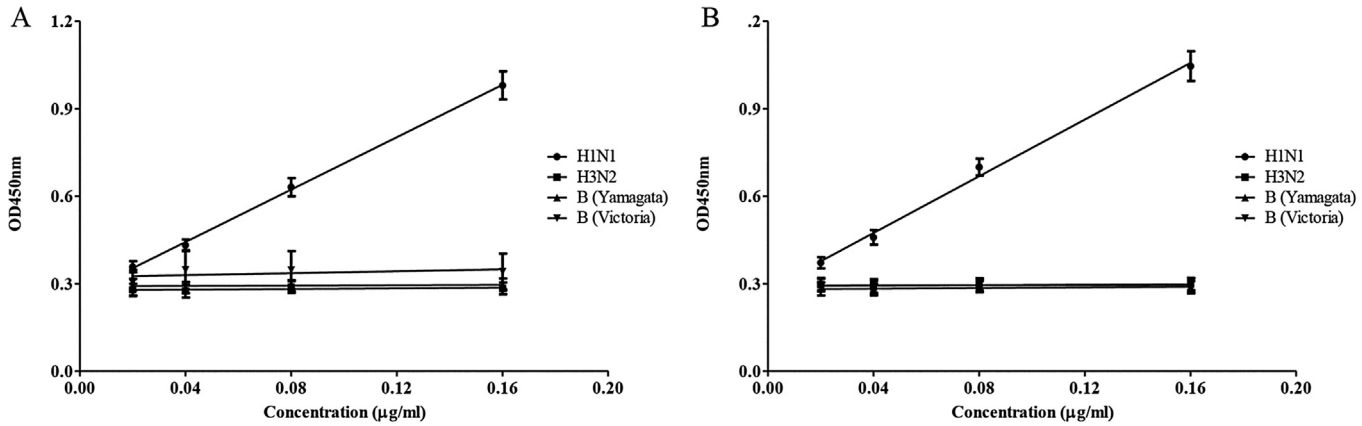

Fig. 6. ELISA with quadrivalent influenza vaccine antigens (GC flu). Influenza vaccine antigens were supplied by Green Cross Pharma (Yongin-si, Korea). Coated antigens were HA of A/Singapore/GP1908/2015 IBR-180 (H1N1), A/Hong Kong/4801/2014 X-263B (H3N2), B/Phuket/3073/2013 (Yamagata-like), and B/Brisbane/60/2008 (Victoria-like). Calibration curves were determined by four-parameter linear regression. Error bars indicate the standard deviation in five replicates. (A) 1G5 (B) 2C12.

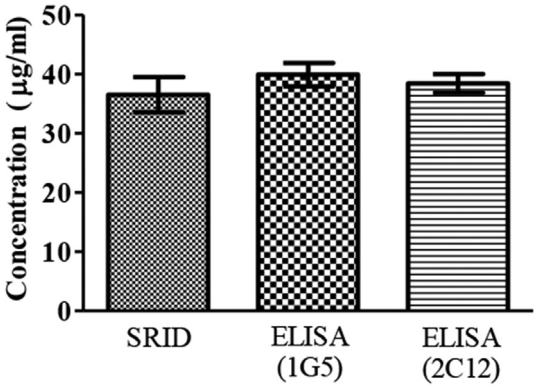

Fig. 7. HA concentrations determined by SRID using polyclonal anti-HA serum and ELISA using 1G5 and 2C12. Standard HA antigen was A/Singapore/GP1908/2015 HA (NIBSC) and test antigen was A/Singapore/GP1908/2015 HA (GC Pharma). Concentrations were calculated based on the slope-ratio model. Error bars indicate 95% confidence intervals.

transition at the HA2 region is influenced by the pKa value of amino acids in the inter-connecting peptide [40]. Third, secondary modification (e.g., glycosylation) of HA depends on the production host (mammalian, insect cells, or embryonated chicken eggs) [41], and the quality or extent of glycosylation, especially in the vicinity of the HA2 domain, may affect ELISA reactivity. Finally, variations among HA quantification assays of different vendors cannot be excluded. Notably, the quantification of international standards from NIBSC is based on quantitative SDS-PAGE [42]. Other methods have been developed independently, including mass spectrometry [43], high-performance liquid chromatography [44], and antibody-based assays [26,27,29,45], and should be compared for robustness of quantification and user friendliness.

As an alternative method, the present ELISA is easy to perform, is substantially less time-consuming than SRID (hours vs. days) and can be automated. Being less subject to the experience and skill of the experimenter, the results are more reproducible, resulting in a lower error range. Furthermore, the quantitative ELISA we developed shows improved sensitivity and repeatability (Tables 1, Supplemental Table 4 and 5). LOD values for all ELISAs were lower than that for SRID, which are usually 3–5 μg/ml, and CVs were less than 5%. The concentrations of HA determined by ELISA were similar to those determined by SRID (Fig. 7).

We expect our ELISA to be useful for the evaluation of new types of influenza vaccines, either licensed or under development. Of note, recombinant subunit vaccine derived from insect cells has been licensed [46], and the current SRID method, optimized for egg-based influenza vaccines, may not be suitable for recombinant vaccines [47]. The universal antibodies used in this study performed equally efficiently for traditional egg-derived vaccines and recombinant HAs produced from eukaryotic expression systems (Supplemental Figs. S4 and S5), underscoring their utility for the quantification of newly developed recombinant subunit vaccines. Moreover, the ELISA is suitable for a universal vaccine that would provide cross-protection against multiple influenza viruses of either seasonal or pandemic nature. The current approach for cross-protection is to redirect the antibody responses from the variable HA globular domain to the conserved HA stalk domain [48–50]. Traditional SRID based on polyclonal anti-serum with strain-specific immune responses targeting to the immunologically dominant HA globular domain is unable to validate these vaccines. If stalk-based universal vaccines are ever developed and licensed, the present quantitative ELISA using stalk-targeting universal mAbs would provide a proper choice for potency validation.

In summary, we developed a HA quantitative ELISA using broadly reactive group-specific universal mAbs. Based on a limited set of experiments, this method was proven to be more sensitive, reliable, accurate, and convenient than the current SRID method. To render it amenable to high-throughput testing, validation of the method as an alternative for the current SRID assay is needed. The assay is currently being extended to group 2 IAV and IBVs, and if successful, would serve as the evaluation of the potency of trivalent and quadrivalent influenza vaccines.

Acknowledgements

This research was supported by grants from the Ministry of Food and Drug Safety, Republic of Korea (16172MFDS199 and 18172MFDS252) and the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (HI13C0826). We thank Dr. Dong-wook Kim (Department of Statistics, Sungkyunkwan University, Seoul, Korea) for statistical analyses of data. The authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.01.068.

References

[1] World Health Organization. Influenza (seasonal) fact sheet; 2018.
[2] Knossow M, Gaudier M, Douglas A, Barrere B, Bizebard T, Barbey C, et al. Mechanism of neutralization of influenza virus infectivity by antibodies. Virology 2002;302:294–8.
[3] La Montagne JR, Noble GR, Quinnan GV, Curlin GT, Blackwelder WC, Smith JI, et al. Summary of clinical trials of inactivated influenza vaccine - 1978. Rev Infect Dis 1983;5:723–36.
[4] Hobson D, Curry RL, Beare AS, Ward-Gardner A. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. J Hygiene 1972;70:767–77.
[5] Williams MS. Single-radial-immunodiffusion as an in vitro potency assay for human inactivated viral vaccines. Vet Microbiol 1993;37:253–62.
[6] Hardy S, Eichelberger M, Griffiths E, Weir JP, Wood D, Alfonso C. Confronting the next pandemic–workshop on lessons learned from potency testing of pandemic (H1N1) 2009 influenza vaccines and considerations for future potency tests, Ottawa, Canada, July 27–29, 2010. Influenza Other Respir Viruses 2011;5:438–42.
[7] Wood JM, Weir JP. Standardisation of inactivated influenza vaccines-Learning from history. 2018;12:195–201.
[8] Kim YS, Son A, Kim J, Kwon SB, Kim MH, Kim P, et al. Chaperna-mediated assembly of ferritin-based middle east respiratory syndrome-coronavirus nanoparticles. Front Immunol 2018;9:1093.
[9] Yang SW, Jang YH, Kwon SB, Lee YJ, Chae W, Byun YH, et al. Harnessing an RNA-mediated chaperone for the assembly of influenza hemagglutinin in an immunologically relevant conformation. FASEB J: Off Publ Federation Am Soc Exp Biol 2018;32:2658–75.
[10] Thomsen MC, Nielsen M. Seq2Logo: a method for construction and visualization of amino acid binding motifs and sequence profiles including sequence weighting, pseudo counts and two-sided representation of amino acid enrichment and depletion. Nucl Acids Res 2012;40:W281–7.
[11] Combet C, Blanchet C, Geourjon C, Deleage G. NPS@: network protein sequence analysis. Trends Biochem Sci 2000;25:147–50.
[12] Guex N, Peitsch MC, Schwede T. Automated comparative protein structure modeling with SWISS-MODEL and Swiss-PdbViewer: a historical perspective. Electrophoresis 2009;30(Suppl 1):S162–73.
[13] Comeau SR, Gatchell DW, Vajda S, Camacho CJ. ClusPro: a fully automated algorithm for protein-protein docking. Nucl Acids Res 2004;32:W96–9.
[14] Shrake A, Rupley JA. Environment and exposure to solvent of protein atoms. Lysozyme and insulin. J Mol Biol 1973;79:351–71.
[15] Kim P, Jang YH, Kwon SB, Lee CM, Han G, Seong BL. Glycosylation of hemagglutinin and neuraminidase of influenza A virus as signature for ecological spillover and adaptation among influenza reservoirs. Viruses 2018;10.
[16] National Research Council Committee on Methods of Producing Monoclonal A. The National Academies Collection: Reports funded by National Institutes of Health. Monoclonal Antibody Production. Washington (DC): National Academies Press (US) National Academy of Sciences; 1999.
[17] 5.3 Statistical analysis of results of biological assays and tests. European pharmacopoeia 7th edition. Strasbourg: Council Of Europe: European Pharmacopoeia Commission; 2010. p. 551–79.
[18] Soria-Guerra RE, Nieto-Gomez R, Govea-Alonso DO, Rosales-Mendoza S. An overview of bioinformatics tools for epitope prediction: implications on vaccine development. J Biomed Inform 2015;53:405–14.
[19] Choi SI, Han KS, Kim CW, Ryu KS, Kim BH, Kim KH, et al. Protein solubility and folding enhancement by interaction with RNA. PLoS ONE 2008;3:e2677.
[20] Vera A, Gonzalez-Montalban N, Aris A, Villaverde A. The conformational quality of insoluble recombinant proteins is enhanced at low growth temperatures. Biotechnol Bioeng 2007;96:1101–6.
[21] Skehel JJ, Waterfield MD. Studies on the primary structure of the influenza virus hemagglutinin. PNAS 1975;72:93–7.
[22] Harrison SC. Viral membrane fusion. Nat Struct Mol Biol 2008;15:690–8.
[23] Lu X, Shi Y, Gao F, Xiao H, Wang M, Qi J, et al. Insights into avian influenza virus pathogenicity: the hemagglutinin precursor HA0 of subtype H16 has an alpha-helix structure in its cleavage site with inefficient HA1/HA2 cleavage. J Virol 2012;86:12861–70.
[24] Leikina E, Ramos C, Markovic I, Zimmerberg J, Chernomordik LV. Reversible stages of the low-pH-triggered conformational change in influenza virus hemagglutinin. The EMBO J 2002;21:5701–10.
[25] Congyu K, Wujuan S, Qunzheng Z, Xindu G. Refolding of urea-denatured alpha-chymotrypsin by protein-folding liquid chromatography. Biomed Chromatogr: BMC 2013;27:433–9.
[26] Bodle J, Verity EE, Ong C, Vandenberg K, Shaw R, Barr IG, et al. Development of an enzyme-linked immunoassay for the quantitation of influenza haemagglutinin: an alternative method to single radial immunodiffusion. Influenza Other Respir Viruses 2013;7:191–200.
[27] Schmeisser F, Vasudevan A, Soto J, Kumar A, Williams O, Weir JP. A monoclonal antibody-based immunoassay for measuring the potency of 2009 pandemic influenza H1N1 vaccines. Influenza Other Respir Viruses 2014;8:587–95.
[28] Gravel C, Elmgren C, Muralidharan A, Hashem AM, Jaentschke B, Xu K, et al. Development and applications of universal H7 subtype-specific antibodies for the analysis of influenza H7N9 vaccines. Vaccine 2015;33:1129–34.
[29] Chun S, Li C, Van Domselaar G, Wang J, Farnsworth A, Cui X, et al. Universal antibodies and their applications to the quantitative determination of virtually all subtypes of the influenza A viral hemagglutinins. Vaccine. 2008;26:6068–76.
[30] Suryadevara M, Domachowske JB. Quadrivalent influenza vaccine in the United States. Human Vacc Immunotherap 2014;10:596–9.
[31] Nabel GJ, Wei CJ, Ledgerwood JE. Vaccinate for the next H2N2 pandemic now. Nature 2011;471:157–8.
[32] Chen GL, Lamirande EW, Yang CF, Jin H, Kemble G, Subbarao K. Evaluation of replication and cross-reactive antibody responses of H2 subtype influenza viruses in mice and ferrets. J Virol 2010;84:7695–702.
[33] Ma W, Vincent AL, Gramer MR, Brockwell CB, Lager KM, Janke BH, et al. Identification of H2N3 influenza A viruses from swine in the United States. Proc Natl Acad Sci USA 2007;104:20949–54.
[34] Jones JC, Baranovich T, Marathe BM, Danner AF, Seiler JP, Franks J, et al. Risk assessment of H2N2 influenza viruses from the avian reservoir. J Virol 2014;88:1175–88.
[35] Poovorawan Y, Pyungporn S, Prachayangprecha S, Makkoch J. Global alert to avian influenza virus infection: from H5N1 to H7N9. Pathogens Global Health 2013;107:217–23.
[36] Alexander DJ. An overview of the epidemiology of avian influenza. Vaccine 2007;25:5637–44.
[37] Yang Y, Halloran ME, Sugimoto JD, Longini Jr IM. Detecting human-to-human transmission of avian influenza A (H5N1). Emerg Infect Dis 2007;13:1348–53.
[38] Imai M, Watanabe T, Hatta M, Das SC, Ozawa M, Shinya K, et al. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. Nature 2012;486:420–8.
[39] Khanna M, Sharma S, Kumar B. Protective immunity based on the conserved hemagglutinin stalk domain and its prospects for universal influenza vaccine development. 2014;2014:546274.
[40] Carr CM, Kim PS. A spring-loaded mechanism for the conformational change of influenza hemagglutinin. Cell 1993;73:823–32.
[41] Gambaryan AS, Marinina VP, Tuzikov AB, Bovin NV, Rudneva IA, Sinitsyn BV, et al. Effects of host-dependent glycosylation of hemagglutinin on receptor-binding properties on H1N1 human influenza A virus grown in MDCK cells and in embryonated eggs. Virology 1998;247:170–7.
[42] WHO. Generic protocol for the calibration of seasonal and pandemic influenza antigen working reagents by WHO essential regulatory laboratories. Sixty-second report of the Expert Committee on Biological Standardization, Annex 5. WHO Technical Report Series 979; 2013.
[43] Williams TL, Pirkle JL, Barr JR. Simultaneous quantification of hemagglutinin and neuraminidase of influenza virus using isotope dilution mass spectrometry. Vaccine 2012;30:2475–82.
[44] Roh HS, Song HM, Yun BR, Kang HK, Choi KS, Park YJ, et al. A hemagglutinin quantification method for development of an influenza pandemic vaccine using size exclusion high performance liquid chromatography. Mol Med Rep 2015;11:2819–24.
[45] Li C, Jaentschke B, Song Y, Wang J, Cyr TD, Van Domselaar G, et al. A simple slot blot for the detection of virtually all subtypes of the influenza A viral hemagglutinins using universal antibodies targeting the fusion peptide. Nat Protoc 2010;5:14–9.
[46] Cox MM, Hollister JR. FluBlok, a next generation influenza vaccine manufactured in insect cells. Biologicals: J Int Assoc Biol Standard 2009;37:182–9.
[47] Weir JP, Gruber MF. An overview of the regulation of influenza vaccines in the United States. Influenza Other Respir Viruses 2016;10:354–60.
[48] Yassine HM, Boyington JC, McTamney PM, Wei CJ, Kanekiyo M. Hemagglutinin-stem nanoparticles generate heterosubtypic influenza protection. Nat Med 2015;21:1065–70.
[49] Impagliazzo A, Milder F, Kuipers H, Wagner MV, Zhu X, Hoffman RM, et al. A stable trimeric influenza hemagglutinin stem as a broadly protective immunogen. Science (New York, NY) 2015;349:1301–6.
[50] Wohlbold TJ, Nachbagauer R, Margine I, Tan GS, Hirsh A, Krammer F. Vaccination with soluble headless hemagglutinin protects mice from challenge with divergent influenza viruses. Vaccine 2015;33:3314–21.