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ORIGINAL RESEARCH PAPER

Application of recombinant hemagglutinin proteins as alternative antigen standards for pandemic influenza vaccines

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Abstract

Objectives The single radial immunodiffusion (SRID) assay, used to quantify hemagglutinin (HA) in influenza vaccines, requires reference reagents; however, because centralized production of reference reagents may slow the emergency deployment of vaccines, alternatives are needed.
Results We investigated the production of HA proteins using recombinant DNA technology, rather than a traditional egg-based production process. The HA proteins were then used in an SRID assay as a reference antigen. We found that HA can be quantified in both egg-based and cell-based influenza vaccines when recombinant HAs (rHAs) are used as the reference antigen. Furthermore, we confirmed that rHAs obtained from strains with pandemic potential, such as H5N1, H7N3, H7N9, and H9N2 strains, can be utilized in the SRID assay. The rHA production process takes just one month, in contrast to the traditional process that takes three to four months.
Conclusions The use of rHAs may reduce the time required to produce reference reagents and facilitate timely introduction of vaccines during emergencies.

Keywords Alternative antigen production ·
Pandemic influenza vaccine · Recombinant HA ·
Reference antigen

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Introduction

Influenza viruses cause influenza and evade the immune systems of hosts by antigenic drift, which occurs as a result of mutations that alter the surface antigens, hemagglutinin (HA), and/or neuraminidase (NA) (WHO 2016). The best way to prevent influenza virus infection is by vaccination. In particular, timely production of vaccines can decrease the number of influenza patients during a pandemic. Hence, WHO promotes research on increasing the speed of production and deployment of pandemic vaccines, including

the preparation of reagents to test vaccine potency (WHO 2004). In 2009, in an influenza A (H1N1) pandemic, the time required for vaccine development in China was shortened by 1 month by measuring the HA content of the bulk vaccine by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) rather than waiting for international reference materials to be distributed (Li et al. 2010).

Vaccine potency is determined by the HA content of a vaccine, which in turn is determined by the single radial immunodiffusion (SRID) assay (Wood et al. 1977; Quinnan et al. 1983). The SRID assay requires a reference antigen and reference antiserum, which require approximately 3–4 months for production and distribution by WHO Essential Regulatory Laboratories (ERLs) (WHO 2013a) after strain recommendation by the WHO. Because the SRID assay is essential to the manufacturing process and quality control of influenza vaccines, this results in a considerable delay in vaccine availability (Wen et al. 2015; Minor 2015).

Recently, an alternative method of influenza vaccine production that relies on the use of a baculovirus expression vector system (BEVS) was developed (Krammer and Grabherr 2010; McPherson 2008). FluBlock (Protein Sciences Corporation, USA), which contains recombinant HA (rHA) proteins produced in insect cells, was approved by the U.S. Food and Drug Administration (FDA) in 2013, after demonstration of its safety, efficacy, and immunogenicity (Treanor et al. 2006, 2011).

We investigated whether rHA proteins produced through a BEVS could be used as reference antigens in the SRID assay. We produced rHAs using an influenza virus strain with pandemic potential (WHO 2013b) and compared the HA content of vaccines containing rHAs and egg-based international reference antigens.

Materials and methods

Preparation of cDNA

We obtained HA nucleotide sequences from influenza strains A/California/07/2009 (H1N1), A/Anhui/1/2005 (H5N1), A/mallard/Netherlands/12/2000 (H7N3), A/duck/Anhui/SC702/2013 (H7N9), and A/chicken/Hong Kong/G9/1997 (H9N2) from the National Center for Biotechnology Information (NCBI) and synthesized 1.7-kb cDNAs (Cosmogenetech,

Seoul, Korea). For vector cloning with restriction enzymes, NotI (GCGGCCGC) and XhoI (CTCGAG) sequences were added to the 5′ and 3′ ends, respectively. GenBank accession numbers for these respective sequences are GQ214335.1, DQ371928.1, KF695239.1, CY147060.1, and KF188366.1.

Construction of expression vector

HA genes from each subtype of influenza virus were PCR-amplified from cDNA and cloned at the NotI-XhoI restriction site of the pFastBac vector. The resulting recombinant donor plasmids were transformed into DH10Bac *Escherichia coli* cells, and recombinant bacmid DNA was purified according to the manufacturer's recommendations. The whole sequences of HA genes were verified using DNA sequencing.

Transfection of insect cells and production of recombinant HA proteins

One hour after seeding of Sf9 cells into a six-well plate (8×10^5 cells/well), bacmid DNA (2 μg) containing HA genes from each subtype of influenza virus was transfected into insect cells using Cellfectin Reagent. Four hours later, the culture medium was replaced with fresh medium, and the cells were incubated for an additional 4 days at 28 °C. By repeated infection of the viral supernatants to Sf9 cells, high titer viral stock was prepared (1×10^8 PFU/mL or higher). The Sf9 cells were infected with this viral stock at an MOI of 5, and the recombinant HA proteins were produced by 4 days of incubation at 28 °C.

Western blotting

The Sf9 cells were lysed with 1% Triton X-100 in phosphate-buffered saline (PBS) and centrifuged at $12,000 \times g$ for 10 min to collect the supernatant. Quantified proteins were then separated on a 4–12% native SDS PAGE gel and detected by western blotting using an Influenza A virus HA antibody.

SRID assay

Agarose was diluted to 1% in PBS, melted, and mixed with an international reference HA antibody. The reference HA antigen and vaccines were treated with

Zwittergent solution (10%) at a 9:1 ratio for 30 min. The solution was diluted 3:4, 2:4, and 1:4 in PBS, and then, 20 µL of the mixed solution was loaded into each well and incubated at room temperature for 16–24 h. After washing, agarose gels were stained with Coomassie Brilliant Blue R and then destained for viewing of stained rings, and zone sizes were measured using ProtoCol (Synbiosis, USA)

Vaccines

To measure the HA content of an H1N1 strain, trivalent or quadrivalent inactivated influenza vaccines were used. We tested GC Flu (an egg-based, split-virion vaccine) from Green Cross Corp., and SKY Cell Flu (a cell-based, surface antigen vaccine) from SK Chemicals Corp., in five lots each.

Results

First, we synthesized a cDNA encoding the HA segments of influenza virus strain A/H1N1/California/7/2009, which was responsible for a pandemic in 2009, and prepared a recombinant bacmid for the expression of recombinant HA (rHA) gene in Sf9 cells. We validated the recombinant bacmid clone by PCR analysis (Supplementary Fig. 1) and DNA sequencing (data not shown). Based on a Western blot assay, rHA proteins expressed in Sf9 cells showed molecular weight similar to the international reference antigen (Fig. 1a). In fact, the HA band of international reference antigen showed slower migration.

Considering that we did not treat the international reference antigen with PNGase F, HA protein was likely not separated from the nucleoprotein (Li et al. 2010). When we performed the SRID assay, rHAs showed stained rings similar to those of reference antigen (Fig. 1b).

Next, we performed a SRID assay to investigate whether rHA could be used as a reference antigen to quantify HA proteins in vaccines. To determine the rHA concentration, the band density of the HA protein (%) relative to that of the total protein was calculated using SDS-PAGE and Coomassie Blue straining. Then, we multiplied the calculated rHA contents by 0.3 to establish the HA contents of the antigen reference for SRID. Because strain A/H1N1/California/7/2009 strain was included among the influenza vaccine strains for the 2016–2017 Northern Hemisphere influenza season, H1N1 rHA was used as a reference antigen to quantify H1N1 HA. We used egg-based and cell-based influenza vaccines in our experiments, both of which have been approved in Korea. An SRID assay was performed in five lots for each of these vaccines. The HA contents of the vaccines were compared with those of the international reference antigen or rHA as shown in Supplementary Table 1.

Next, we tested other influenza strains defined as having pandemic potential by the WHO. We selected four strains from this list (H5N1, H7N3, H7N9, and H9N2), synthesized each cDNA from the HA gene of the viruses, and then cloned the cDNAs into bacmids. After transfection of Sf9 cells with recombinant HA bacmids, total DNAs were extracted and subjected to PCR analyses. The specific HA gene was identified in
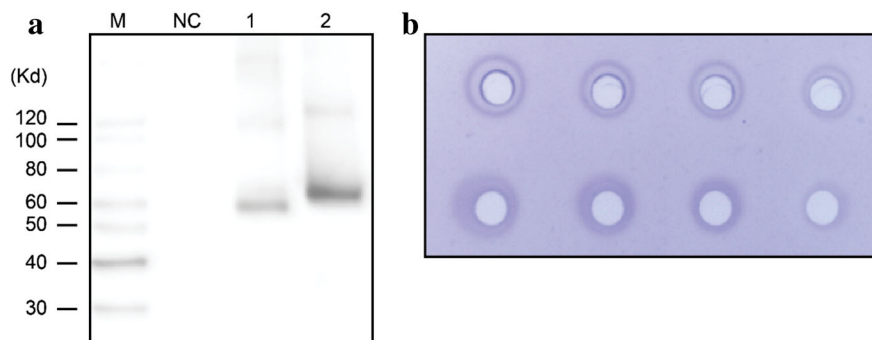

Fig. 1 Expression and analysis of Sf9 cell-based recombinant H1N1 HA protein. **a** Western blot analysis of the HA protein. M: marker, NC (negative control): Sf9 cell proteins, *1* Proteins of Sf9 cells expressing recombinant HA, *2* reference antigen (NIBSC 12/168), **b** SRID assay using reference antiserum NIBSC 14/310. *Upper* reference antigen (NIBSC 12/168), *Lower* recombinant HA protein

each strain without cross-reactivity among the strains (Fig. 2).

In addition, rHA proteins of four influenza strains were expressed in Sf9 cells (Fig. 3a). The three bands observed might indicate monomers, dimers, and trimers of the HA proteins (Feshchenko et al. 2012); HAs are normally present as trimers within cells. Next, we performed an SRID assay using the newly expressed rHA proteins. International reference standards distributed from the National Institute for Biological Standards and Control (NIBSC) were used in parallel in the assay. The results are shown in (Fig. 3b–e). We observed the formation of stained rings because of the reaction between rHA and the HA antiserum for each strain. Therefore, we confirmed that rHAs synthesized from the HA genes of various strains of influenza virus could be produced and used as reference materials for the SRID assay.

Discussion

Although various quantitative assays have been and are currently being developed for use with influenza vaccines, none has been approved by NRA (National Regulatory Authorities) (Minor 2015). WHO also recommends that the SRID assay be used during a pandemic as long as reagents are available (WHO 2011).

Furthermore, although it is permissible for an antiserum to show cross-reactivity with a strain being used, the reference antigen must be of the same strain as the vaccine (Minor 2015). Therefore, the time-consuming process of producing and distributing international reference materials has slowed the development of vaccines. Moreover, one of the manufacturers usually supplies the regulatory authorities with one of their first batches of antigen in a new vaccine campaign for use as the SRID antigen. In a pandemic situation, vaccine manufacturers would be under enormous pressure to meet orders in time and may find it difficult to supply the SRID antigen (WHO 2011).

Here, we showed that rHA proteins can be used as reference antigens. It is likely that the use of these proteins rather than international reference materials will shorten vaccine production time during a pandemic. In addition, rHA proteins are advantageous in that they can be used for quality control of not only bulk but also final products.

However, there are a number of limitations to overcome before rHA can be used in the SRID assay. Because rHA was mixed with other proteins in a total protein solution obtained via lysis of Sf9 cells, the purity of the HA proteins was low. Therefore, additional research on ways to increase the purity of HA proteins is necessary. In addition, understanding of the challenges associated with scale-up of rHA production for use as a reference antigen, including storage and stability of rHA, is required. Lastly, validation of the SRID assay with rHA as a reference antigen in experiments using a sufficient number of vaccine lots

Fig. 2 DNA analysis of Sf9 cells transfected with HA from each strain. a NC: Sf9 cell DNA. b H1N1-transfected cells. c H5N1-transfected cells. d H7N3-transfected cells. e H7N9-transfected cells. f H9N2-transfected cells. 1 H1N1 HA-specific primers, 2 H5N1 HA-specific primers, 3 H7N3 HA-specific primers, 4 H7N9 HA-specific primers, 5 H9N2 HA-specific primers. The primer sequences are indicated in the Supplementary methods
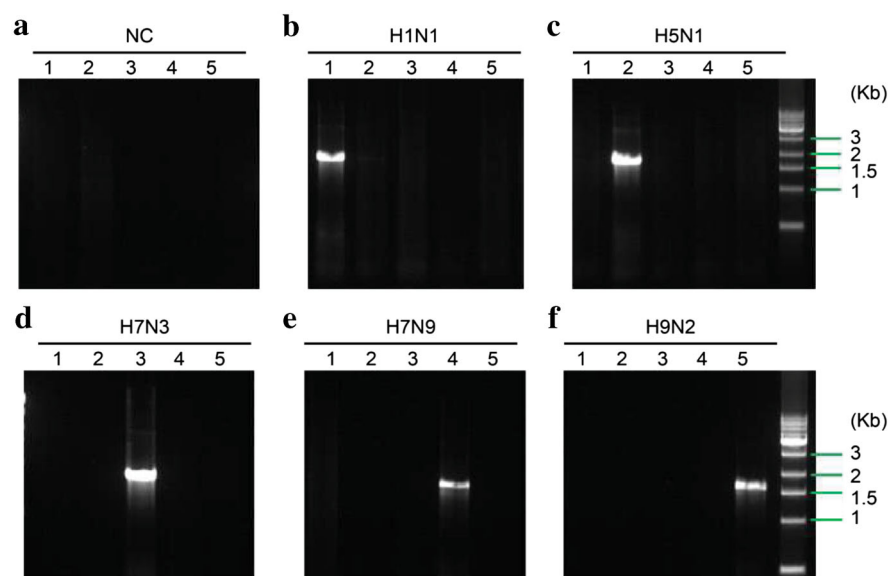
Fig. 3 Expression and analysis of Sf9 cell-based recombinant HA proteins. **a** Western blot assay for HA. M: Marker, NC: Sf9 cell proteins, *1* H5N1, *2* H7N3, *3* H7N9, *4* H9N2, (**b–e**). SRID assay, *upper* reference antigens, *lower* recombinant HA proteins. **b** H5N1, reference antigen (NIBSC 07/290), reference antiserum (NIBSC 07/338), **c** H7N3, reference antigen (NIBSC 07/336), reference antiserum (NIBSC 07/278). **d** H7N9, reference antigen (NIBSC 14/250), reference antiserum (NIBSC 13/180). **e** H9N2, reference antigen (NIBSC 08/228), reference antiserum (NIBSC 08/202)
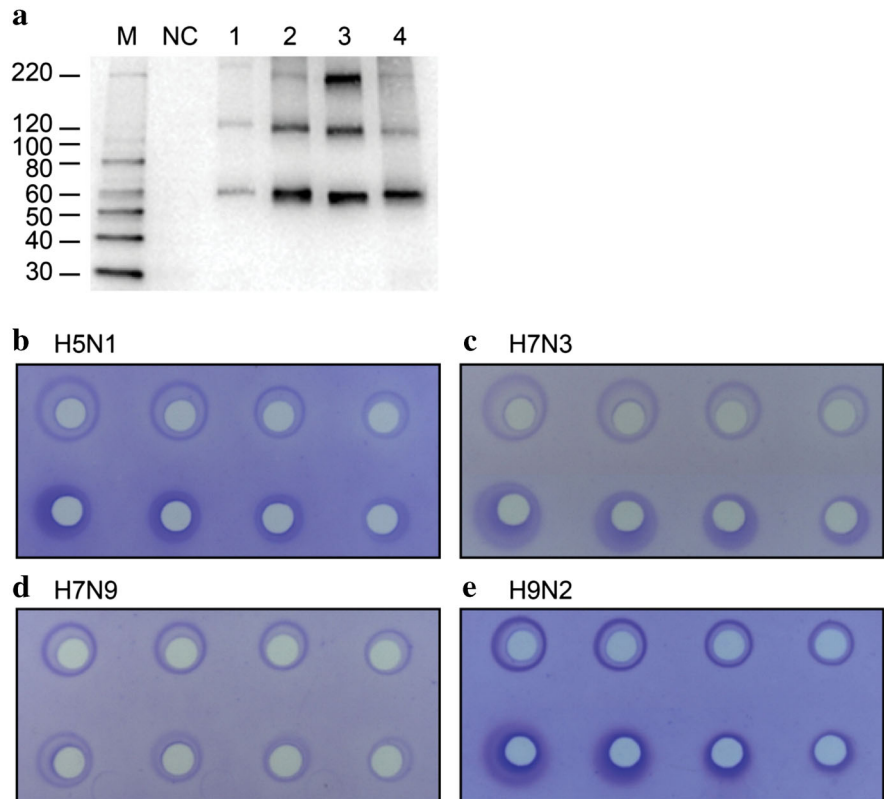
is needed. The rHA content differs between the Lowry method and SRID assay. Moreover, the rHA content measured by the SRID assay is only 30% of that measured by the Lowry method in the case of an H1N1 strain (Treanor et al. 2006); this discrepancy in HA content measured by different assays must be considered when using rHA as a reference antigen. In our study, we multiplied the rHA content by 0.3 to account for this discrepancy. This ratio should be determined for each vaccine strain.

Based on our findings, the use of rHA may reduce the time for production and distribution of vaccines during influenza pandemics. Although we only measured the HA content of commercial H1N1 vaccines, we may expand our data by conducting additional research on other pandemic vaccines as they are produced and become commercially available.

Data sharing

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Supporting information Supplementary Methods—Cloning and transformation, PCR analysis and sequencing, and Transfection of insect cells and amplification of viral titers.

Supplementary Figure 1—Analyses of recombinant bacmid (r bacmid) and Sf9 cell DNA.

Supplementary Table 1—Comparison of HA contents in five lots each of egg-based and cell-based influenza vaccines.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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