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Qualitative and quantitative analyses of virtually all subtypes of influenza A and B viral neuraminidases using antibodies targeting the universally conserved sequences

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ABSTRACT

Neuraminidase-induced immune responses are correlated with protection of humans and animals from influenza. However, the amounts of neuraminidase in influenza vaccines are yet to be standardized. Thus, a simple method capable of quantifying neuraminidase would be desirable. Here we identified two universally conserved sequences in all influenza A and B neuraminidases, one representing a novel finding of nearly 100% conservation near the enzymatically active site. Antibodies generated against the two highly conserved sequences bound to all nine subtypes of influenza A neuraminidase and demonstrated remarkable specificity against the viral neuraminidase sequences without any cross-reactivity with allantoic and cellular proteins. Importantly, employing these antibodies for the analyses of vaccines from eight manufacturers using the same vaccine seeds revealed marked variations of neuraminidase levels in addition to considerable differences between lots from the same producer. The reasons for the absence or low level of neuraminidase in vaccine preparations are complex and could be multi-factorial. The antibody-based assays reported here could be of practical value for better vaccine quality control.

1. Introduction

Two types of surface proteins are present in influenza viruses, i.e., hemagglutinin (HA) and neuraminidase (NA) [1]. While HA is well characterized as the receptor-binding and membrane fusion glycoprotein of the virus in addition to the main target for infectivity-neutralizing antibodies [2], the neuraminidase is also known to play important roles in the viral life cycle and induce protective immune responses [3]. Specifically, NA exerts its functional role by enzymatically removing the sialic acid, which is the cell receptor for influenza virus, from the infected cells or the viruses themselves, thereby facilitating the release of the virus particles [4,5]. Indeed, NA-defective virus or wild type viruses in the presence of NA inhibitors are found to form aggregations on the apical surface of the cells [5–8]. Recently, NA has also been implicated in facilitating the entry of the virus [9,10], but the mechanisms are not as well understood as that underlying the release of the viral particles.

The current annual influenza vaccines typically contain two subtypes of influenza virus type A and one of influenza type B derived from the strains predicted to circulate in the upcoming flu season. HA, the major surface protein that induces protective immune responses, is routinely used as the vaccine potency marker [11]. Noticeably, NA-specific immune responses have also been reported to protect the host from influenza [12]. In experimental studies, animals immunized with NA antigen can be protected from influenza; NA-specific antibodies alone are sufficient to afford protection from lethal challenges [12–19]. In humans, NA-specific immune responses have also been correlated to protection of subjects from viral infection [20–25]. It is of note that the levels of NA-specific antibodies are modest in people immunized with the standard influenza vaccine which usually contains 15 μg of the hemagglutinin of each strain. But an eight-fold increase in the levels

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of NA-specific antibody was observed when 4 times concentrated standard vaccines were used, suggesting an increased quantity of the NA antigen in vaccine can ensure a better immune response to NA [25]. These data reinforce the notion that the NA antigens in the influenza vaccines could be increased and better controlled so as to elicit a balanced immune response [26–28]. However, the neuraminidase in the current influenza vaccines is not quantified but only verified for the presence of enzymatic activity. Moreover, the activity of neuraminidase as a potential potency marker is questionable since the enzymatic activity levels vary in the vaccine preparations or could be very low or even missing in some of them [28–30]. The low level of enzymatic activity in vaccine preparations is likely due to the labile nature of the protein during various chemical treatments and storage [29–31], rendering the measurement of enzymatic activity unlikely to be accepted as a means for accurate quantification of NA in vaccines [32].

Given the problems associated with enzymatic activity measurements in the vaccine preparations, alternative approaches have been sought to quantify NA in vaccines. Gerentes et al. used strain specific antibodies in an ELISA to quantify NA antigens in vaccine [29]; Tanimoto et al. estimated the NA content of the split-product (SP) vaccine (virus treated with ether, then formalin) by an immunochromatography based on monoclonal antibodies (mAbs) to viral NA for A/Panama/2007/99 (A/Pa) (H3N2), B/Shangdong/7/97 (B/S) or A/New Caledonia/20/99 (A/NC) (H1N1) viral strains [31]. Yet, these antibodies are strain specific and need to be made for the viral NA of each strain, which could present a significant challenge because of the frequently changing nature of the NA. In fact, while physico-chemical methods have been successful in quantifying the HA proteins without the need of antibody reagents [33–42], none has reported the detection of the elusive NA proteins in vaccines. Clearly, lack of appropriate reagents or a suitable analytical method is the bottle-neck for the NA quantification in vaccines [32]. We therefore hypothesized that a simple immunoassay using antibodies targeting the most conserved sequences of all neuraminidases would be of practical importance for the analyses of NA antigens in vaccine preparations. In this communication, we report the development and characterization of two mono-specific and monoclonal antibodies targeting the two most conserved sequences in all neuraminidases and their application to qualitative and quantitative determination of neuraminidase antigens. Specifically, both antibodies were able to bind to all nine subtypes of type A influenza viral neuraminidases, with one of them also binding to the influenza B viral neuraminidase. Moreover, both antibodies demonstrated remarkable specificity against the viral NA sequences with no cross-reactivity to allantoic or MDCK cellular proteins. With these antibodies, we found significant variations of NA in vaccines from different manufacturers using the same seed strain; variations of NA were also found between lots of vaccines from the same producer, revealing the daunting necessity for NA quantification in vaccines. We also report the details for the assay conditions and discuss the relevant implications for NA quantifications.

2. Materials and methods

2.1. Viruses, recombinant proteins and vaccines

Table 1 lists the viruses, recombinant neuraminidase proteins (rNA) or human vaccines used in the characterization of the antibodies. The influenza virus strains listed are derived from the national inventory at the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP), Beijing, China. All the virus strains have been confirmed with reference sera according to internationally accepted criteria [43]. The viruses were propagated in embryonated chicken eggs. The recombinant NAs were purchased from Proteins Sciences Corporation (Meriden, CT). The pandemic influenza vaccines (H1N1) were obtained from eight manufacturers, which produced them by the routine vaccine production process using the same strain NYMC X-179A (A/California/7/09(H1N1)v-like strain) provided by New York Medical College.

Table 1
Viruses or recombinant neuraminidase antigens used in the studies.

Subtype	Strains
Influenza A virus	
H1N1	A/duck/Shantou/1734/2003
H7N2	A/duck/Hong Kong/A 47/1976
H3N3	A/duck/Shantou/708/2000
H8N4	A/turkey/Ontario/6118/1968
H12N5	A/duck/Hong Kong/838/1980
H4N6	A/duck/Siberia/378/2001
H7N7	A/EQ/Prague/1/56
H2N8	A/duck/Shantou/992/2000
H5N9	A/turkey/Wisconsin/68
Subtype (type)	**Donor strains**
Human vaccine	
H1N1	A/Solomon Island/3/06
H1N1	A/California/7/09
H3N2	A/Wisconsin/67/2005
Influenza B	B/Malaysia/2506/2004
H5N1	A/Vietnam/1194/2004
rNA	**Donor strains**
Recombinant neuraminidase	
A/N1	A/New Caledonia/20/1999
A/N4	A/Wisconsin/67/2005

The pH1N1 vaccine 2009 reference antigen and antibody standards were kindly provided by the National Institute for Biological Standards and Control (NIBSC), UK and Centre for Biologics Evaluation and Research (CBER), FDA, U.S.A. Influenza A strains A/Puerto Rico/8/34 (H1N1) was kindly provided by Dr. Jim Robertson at the National Institute for Biological Standards and Control (Potters Bar, UK). Influenza B/Singapore/222/97 virus was provided by Dr. Kathryn Wright (University of Ottawa, Canada).

2.2. Preparation of peptides and production of mono-specific polyclonal and monoclonal antibodies

A bioinformatics approach was used to locate the universally conserved regions of influenza neuraminidase. Influenza types A and B were analyzed separately. All publicly available non-identical full-length neuraminidase protein sequences (except laboratory strains) were retrieved from the NCBI influenza virus resource (http://ww.ncbi.nih.gov/genomes/FLU/FLU.htm), with the last confirmatory verification conducted on April 21, 2010. In total 7486 sequences were retrieved for influenza A; 911 for influenza B. Each dataset was then filtered in such a way that only sequences representing a unique combination of host, location, subtype, and collection year were selected in order to reduce overrepresentation of strains collected in a given region and time. Sequences containing ambiguous amino acid (a.a.) codes (BJOUXZ) were also removed. The final set of sequences was reduced from 7486 to 723 for influenza A and from 911 to 45 for influenza B, respectively. Each dataset was multiply aligned using CLUSTALW-MPI [44] on a 256-processor Linux cluster. The Shannon entropy for each position of amino acid of the identified consensus sequences was then calculated to determine the degree of variation [53]. Two sequences were identified because of the high degree of conservation (for details of

the analyses, see Section 3). The two peptides: ILRTQESEC (positions 222–230, N2 numbering; designated as HCA-2) and MNPNQKIITIGS (positions 1–12, N2 numbering; designated as HCA-3) were synthesized, modified with a 6-aminocaproic-lysine-lysine-cysteine linker and then conjugated in procedures described previously [43]. The peptide-carrier conjugates were then used to immunize rabbits for the production of mono-specific polyclonal antibodies as described previously [43]. For the production of rabbit monoclonal antibodies, the spleens from immunized rabbits were collected and then sent to Epitomics, Inc. (Burlingame, CA) for the production of customized hybridomas and monoclonal antibodies.

2.3. GST-HCA-2 and HCA-3 fusion proteins

Synthetic forward and reverse oligonucleotides (HCA-2: Fw: 5′ *GATCC*-ATCCT GCGTA CCCAA GAAAG CGAGT GCTAA-*C* and Rev: 5′ *TCGAG*-TTAGC ACTCG CTTTC TTGGG TACGC AGGAT-*G*, HCA-3: Fw: 5′ *GATCC*-ATGAA TCCGA ACCAG AAAAT TATCA CCATT GGCAG CTAA-*C* and Rev 5′ *TCGAG*-TTAGC TGCCA ATGGT GATAA TTTTC TGGTT CGGAT TCAT-*G*) corresponding to the sequence of the mature polypeptides (ILRTQESEC for HCA-2 and MNPNQKIITIGS for HCA-3) and the appropriate restriction sites were annealed and inserted into the pET19b-GST vector using the *BamH1/Xho1* restriction sites. Vector pET19b-GST is a modified version of pET19b (Novagen, San Diego, CA) to which a glutathione S-transferase (GST) fusion partner has been inserted between the poly-histidine tag and the multiple cloning site, along with a thrombin recognition cleavage site. The constructs were sequenced using an ABI 3130xl Genetic Analyzer.

Plasmids pET19b-GST-HCA-2 and pET19b-GST-HCA-3 were transformed in *Escherichia coli* BL21(DE-3) competent cells (Stratagene, Kirkland, WA). Expression was carried out by incubating a freshly transformed colony in LB media at 37 °C until a cell density measure at OD600 of 0.8 was obtained. Protein expression was induced with the addition of 1 mM isopropyl thiogalactoside (IPTG) and allowed to proceed for 3 h at 27 °C. Cells were harvested by centrifugation and purified using the Qiagen standard protocol for poly-histidine tagged proteins.

2.4. Western blot and Slot blot

The NA antigens serving as positive controls are rNA obtained from Protein Sciences Corporation (Meriden, CT) or NA antigens in pH1N1 reference antigens from CBER/FDA. The concentration of rNA is determined by Lowry assay while the NA content in the vaccine reference standards was determined by the standard protocols for antigen references using SDS-PAGE in conjunction with densitometry scanning as originally reported by Harvey et al. [45] with slight modification as described previously [52].

The specificities of the antibodies were determined by Western blot (W.B.) using a procedure as described previously [43]. In brief, allantoic fluids directly from eggs inoculated with influenza viruses were fractionated on sodium dodecyl sulfate (SDS)-12% polyacrylamide gels (PAGE), followed by transferring the samples to PVDF membrane (Millipore). The membrane was then blocked with 5% skim milk in PBS at 37 °C for 1 h. Following incubation of filters for 1 h at 37 °C with rabbit antisera against NA as described above, peroxidase-conjugated goat anti-rabbit immunoglobulin (IgG) (Sigma, Oakville, Canada) was added for an additional incubation of 1 h at room temperature, followed by chemiluminescent detection (ECL, Amersham Pharmacia Biotech, Piscataway, NJ). Western blot for infected Madin-Darby canine kidney (MDCK) cells (ATCC: CCL-34) was performed as described above with the exception of using cell lysates derived from virus-infected MDCK cells.

Slot blot was conducted as previously described [46], with the exception that the vaccines were not treated with urea but diluted in 0.01% Zwittergent (Sigma, Oakville, Ontario) in Tris buffered saline (TBS) buffer (Invitrogen, Burlington, ON).

2.5. ELISA

Indirect ELISA was performed as described before [43]. In summary, 4 μg/ml of NA protein or 1 μg/ml of peptides was coated onto 96-well plates (Nunc/VWR, Mississauga, ON) at 40 °C overnight. The wells were then washed five times with PBS-T, followed by the addition of blocking buffer comprised of PBS-T and 5% skim milk. After incubation at 37 °C for 1 h, the blocking buffer was removed and the primary antibodies were then added. The plates were incubated again at 37 °C f or 1 h. Afterwards, secondary antibodies (peroxidase-conjugated goat anti-rabbit immunoglobulin) were added at concentrations recommended by the supplier (Cedarlane Labs, Burlington, On). Following an additional incubation at 37 °C for 1 h, the plates were washed five times before tetramethylbenzidine (TMB) substrate (Rockford, IL) was added for colorimetric development. The cut-off value was defined as mean of five negative samples (from pre-bleed or hybridoma supernatant controls) plus two SD, with triplicate samples assayed at the same time. Unless specified, the O.D. 450 values obtained with the use of the antibodies have been subtracted by those from the pre-bleed or culture medium negative control.

2.6. Separation and identification of NA using size-exclusion high performance liquid chromatography (SE-HPLC) in conjunction with NA universal antibodies

SE-HPLC was conducted for the separation of major influenza viral proteins as described previously [42]. In brief, the analytical HPLC system consisted of a Waters Alliance 2695 chromatograph equipped with an autosampler with a sample cooling device, and coupled to a Waters 2996 Photo Diode Array Detector with a 10 mm pathlength flow cell (Waters, QC, Canada). Data acquisition and integration were performed with Empower Pro Software from Waters. The column was TSKgel G4000SWXL 300 mm × 7.8 mm, 8-μm particles, 450 Å pore size (TosoHaas, Montgomeryville, PA, USA). The optimized elution conditions were: 0.1 M sodium phosphate, 200 mM NaCl and 0.05% SDS at pH 7.

Fractions were collected from consecutive 100 μl injections (90 μg HA on column) of vaccine and pooled fractions were concentrated by centrifugation on Centricon MWCO 10 K membranes, washed twice with d.d.H20 and concentrated to a volume of 50–75 μl. The collected samples were subjected to W.B. analyses using NA universal antibodies.

2.7. Neuraminidase activity measurements

The neuraminidase activity in vaccine samples was measured using a chemiluminescent neuraminidase assay as described by Buxton et al. [47] with slight modification [8]. In brief, equal amounts of vaccine samples (normalized by HA contents) were incubated with MUNANA [2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid] at 37 °C for 60 min, followed by the addition of Stop Solution comprised of 0.14 M NaOH in 83% ethanol and measurements of the fluorescent product using an excitation of 360 nm and emission of 448 nm.

2.8. Data analyses

Unless specified, results are presented as mean ± SD with $n = 3$ and the currently accepted 4-parameter logistic (4-PL) model was employed for the calibration curve fitting in the immunoassays [46].

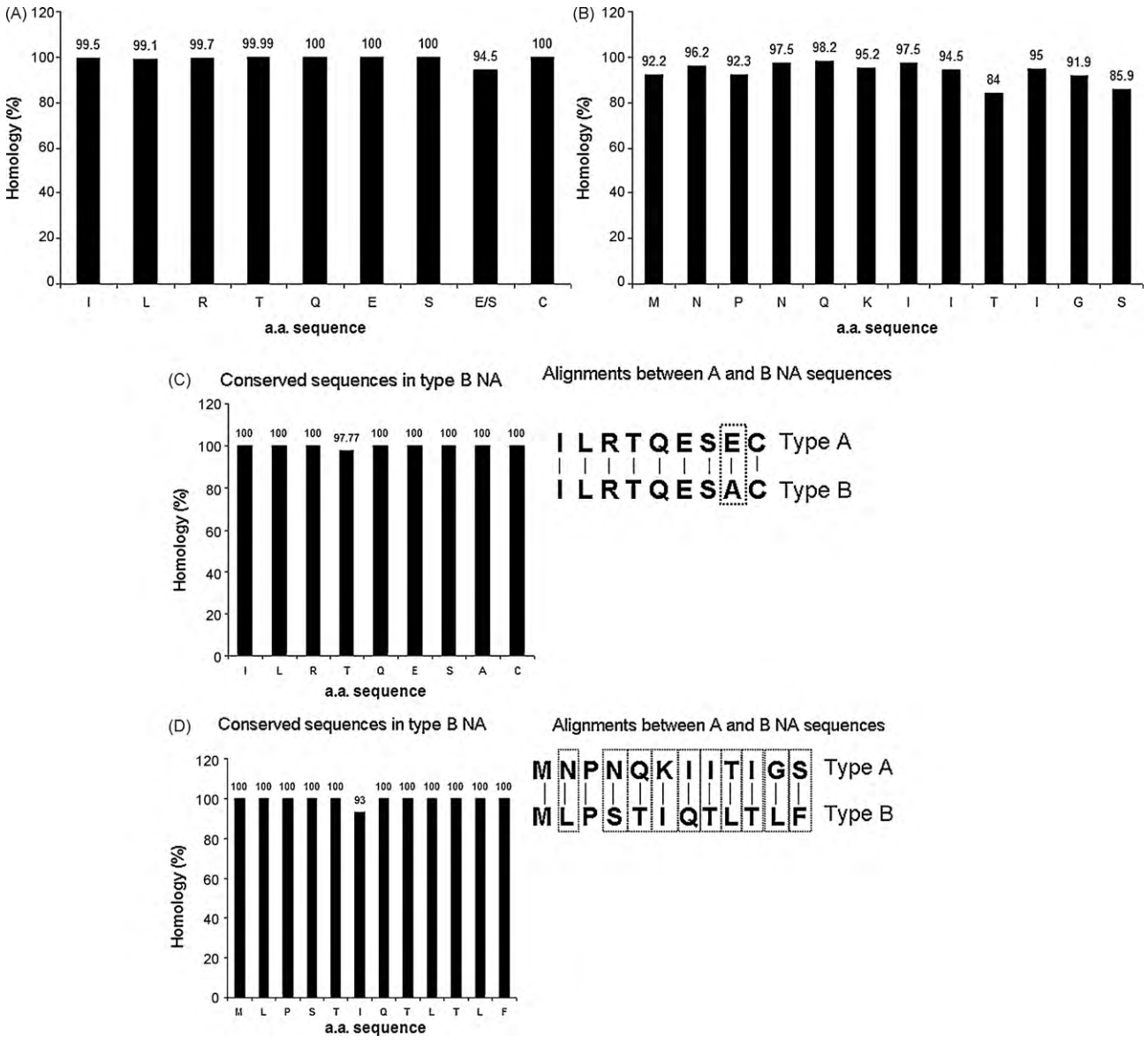

Fig. 1. Sequence homology of influenza A and B viral NA. A total of 8813 and 911 full-length of NA proteins for influenza A and B were retrieved, respectively, from the public database (NCBI influenza virus resource database), including Pandemic (H1N1) 2009 viruses, and Flu Project sequences, with the latest confirmatory analyses conducted on March 20, 2010. Shannon's entropy was calculated for each position of amino acid of the identified consensus sequences to determine the degree of variation (for details see Section 2). Panel A represents the universally conserved epitope (designated as HCA-2) near the enzymatically active site of all NA enzymes of influenza A. The a.a. position is located at 222-230 in the NA (N2 numbering) [54]. Panel B depicts the second highly conserved epitope (denoted as HCA-3) located at the N-terminus of influenza A viral NA proteins. The a.a. position is located at 1-12 in the NA (N2 numbering) [54]. Panel C represents the comparison between type B and A NA protein sequences near the enzaymatically active site of all available influenza viral NA proteins. The left side of the figure shows the conserved sequence in type B NA proteins. The a.a. position is located at 222–230 in the NA (N2 numbering) [51,54], while the right side depicts the alignment of the conserved sequences between type A and B NA sequences. Panel D represents the comparison between type B and A NA protein sequences at the N-terminus of all available influenza NA proteins. The left side of the figure shows the conserved sequence in type B NA proteins, while the right side depicts the alignment of the conserved sequences between type A and B NA sequences.

3. Results

3.1. Selection of peptides for the generation of antibodies against diverse strains of influenza viruses

Following a comprehensive analysis of the publicly available neuraminidase sequences (see also Section 2), two stretches of amino acid sequence were found to be universally conserved among all reported neuraminidase sequences of influenza A viruses (Fig. 1). One of them (ILRTQESEC), denoted as HCA-2, a novel finding reported here, is positioned close to the enzymatic site of the neuraminidase, e.g., located at a.a. positions 222–230 in the NA (N2 numbering) [54] (Fig. 1, panel A). The other sequence (MNPNQKIITIGS), designated as HCA-3, is located at the N-terminus of the neuraminidase (the cytoplasmic tail) and is also very conserved (Fig. 1, panel B) among influenza A sequences, an observation consistent with that made by Air and co-workers [49,50]. Both HCA-2 and HCA-3 are highly conserved, especially HCA-2, which is close to 100% conserved among all influenza type A and B viral NA sequences we analyzed (Fig. 1, panel A and C). More interestingly, with respect to HCA-2 epitope, similar positions in the type B viral NAs are also very conserved and very similar to the HCA-2 sequence, with only one a.a. difference ($E/S \rightarrow A$) (Fig. 1C). In contrast, there is little similarity between influenza A and B NA proteins in the N-terminus (Fig. 1D), although the N-terminal 1–12 a.a. sequences are very conserved in their respective type. These anal-

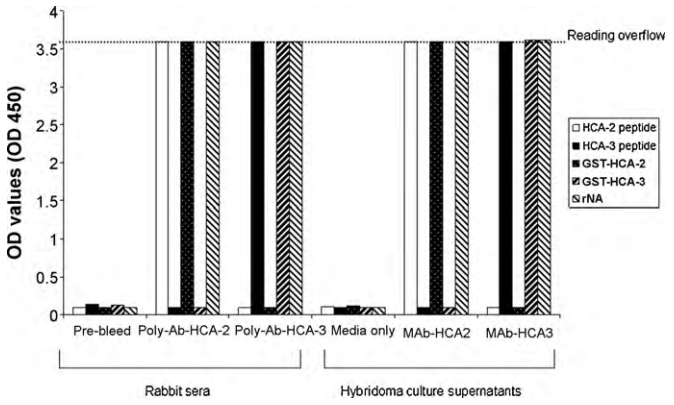

Fig. 2. Binding of mono-specific and monoclonal antibodies to the HCA-2 and HCA-3 peptides in direct ELISA. HCA-2 or HCA-3 free peptides, GST-peptide or recombinant NA (rNA) were coated onto 96-well plates, followed by incubation with the antibodies and subsequently goat anti-rabbit IgG peroxidase conjugates. The left side of the figure represents the reactions with mono-specific polyclonal antibodies and their pre-bleed controls while the right side of the figure describes the reactions with monoclonal antibodies (MAbs) derived from the hybridoma supernatants and their culture medium controls. The data show that the HCA-2 or HCA-3 mono-specific antibodies (1:4000 dilution) or MAbs (undiluted hybridoma supernatants) bind to their respective epitopes in the free peptides, GST-peptides or rNA. Same results in relation to binding specificity to the targeted peptides were obtained from affinity-purified antibodies (not shown).

yses led us to predict that antibodies against the HCA-2 epitope should bind to the NA proteins from both A and B virus (Fig. 1A and C), while antibodies against the HCA-3 epitope should bind to the type A neuraminidases only.

To elicit high levels of antibody production in animals, the two peptides were first linked with a spacer (6-aminocaproic acid) and then modified prior to being conjugated to KLH carrier [43]. The peptide-conjugates were injected into NZW rabbits, followed by boosting every two weeks. The antibody titres were measured in sera and the derived hybridoma cultures by an indirect ELISA using HCA-2 and HCA-3 peptides. As shown in Fig. 2, we found that both mono-specific polyclonal and monoclonal antibodies can bind to their respective peptides, GST-peptide fusion proteins or the recombinant NA while neither the pre-bleeds nor the cell culture media were found to bind the two conserved epitopes; it is also clear that the same results were observed between mono-specific polyclonal antibodies and monoclonal antibodies (Fig. 2). Thus, we used them interchangeably for our subsequent experiments unless specified for purified MAbs (see below).

3.2. Binding of the NA antibodies to the diverse strains of the influenza viruses

Experiments were next performed to determine whether the antibodies could bind to diverse strains of influenza. To this end, nine subtypes of influenza A viruses available at our institutions were propagated in embryonated eggs. Allantoic fluids containing the viruses were used directly to test the specificity of antibodies against the viral sequences and any cross-reactivity to the allantoic fluid proteins. As shown in Fig. 3, both HCA-2 (panel A) and HCA-3 (panel B) antibodies were found to bind all nine subtypes, with no significant cross-reactivity to allantoic fluid proteins in Western blot. Fig. 3(panel C) shows the detection of the NP proteins using NP-specific antibodies as described [43]. Similar results were obtained in MDCK cells infected with influenza viruses, in which both HCA-2 and HCA-3 antibodies bind to NA protein from influenza A strains A/Puerto Rico/8/34 (H1N1), while only HCA-2 antibody binds to NA protein from influenza B/Singapore/222/97 virus (Fig. 4), consistent with the results from bioinformatics anal-

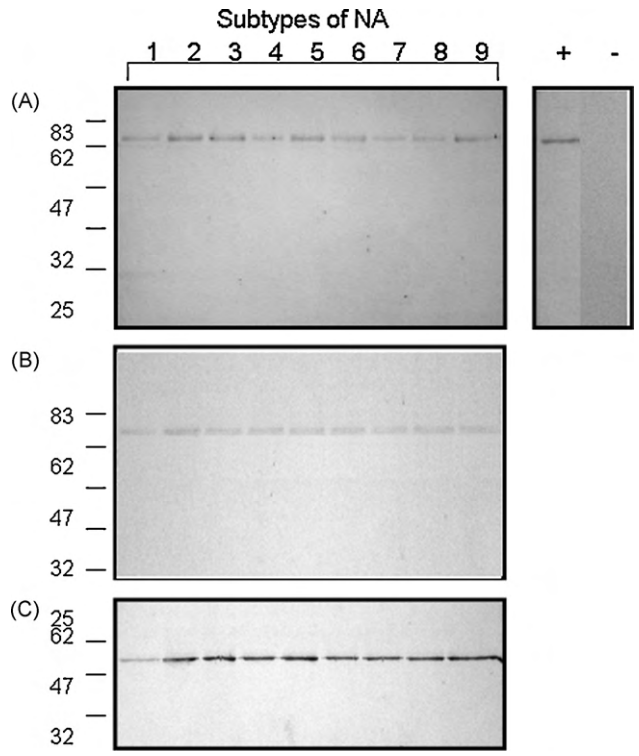

Fig. 3. Binding of HCA-2 and HCA-3 Antibodies to 9 subtypes of NA proteins. Allantoic fluids of nine NA subtypes of influenza viruses propagated in embryonated eggs were fractionated in SDS-PAGE, followed by detection of the NA proteins using the HCA-2 (panel A, upper panel) and HCA-3 antibodies (panel B, middle panel). Rabbit polyclonal anti-NP proteins of influenza viruses were used as another control (panel C, lower gel panel). Allantoic fluids spiked with rNA1 of A/New Caledonia/20/99 reacting with the corresponding anti-NA antisera represents the positive control (+), while the negative controls (−) were allantoic fluids from uninfected eggs.

yses showing that the N-terminus of influenza B does not share the same homology as that of influenza A viral neuraminidases (Fig. 1). Finally, no detectable cross-reactivity to cellular or parainfluenza viral proteins was observed (Fig. 4), suggesting that these antibodies are highly specific for the viral NA sequences.

3.3. Application of the antibody-based assays to the detection of neuraminidase in human vaccine (A/California/7/09) from various vaccine manufacturers

Having determined that the antibodies could detect all nine subtypes of NA (Figs. 1 and 3), we next set out to use W.B. to analyze samples from eight vaccine manufacturers using the same seed strain (A/California/7/09) for the production of the 2009 pandemic influenza vaccine. To this end, 450 ng of various pH1N1 vaccines (pre-determined by SRID using WHO reference reagents), were loaded on SDS-PAGE and then probed with anti-NA antibodies. As shown in Fig. 5, the levels of NA proteins varied amongst the vaccines from different manufacturers (Fig. 5, panel A); the varied NA proteins detected by the antibodies were largely consistent with the levels of the enzymatic activities (Fig. 5, panel B), with vaccines from manufacturers #3, #5 and #7 demonstrating the lowest levels of NA proteins and enzymatic activities. Interestingly, the levels of the nucleoprotein (NP) were also low in #5 and #7.

Given that the samples were loaded onto SDS-PAGE based on equal amounts of HA (450 ng), we were also interested to find out whether any difference in HA forms could be observed by W.B. For this purpose, the samples were subjected to W.B. analyses using universal antibodies targeting the fusion peptide of HA [43]. The universal antibodies (denoted as Uni-1) can bind to unprocessed

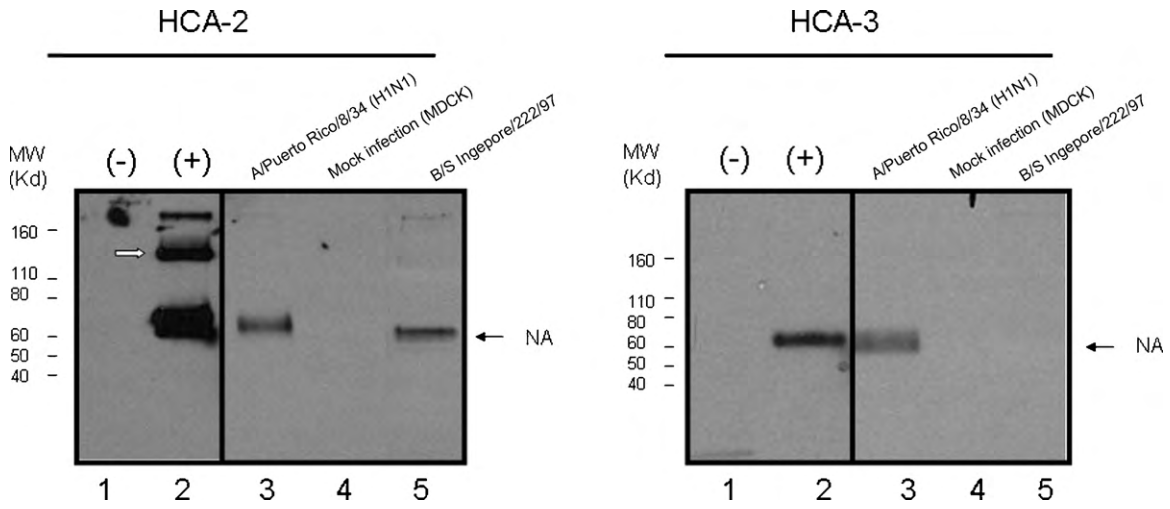

Fig. 4. Detection of NA proteins from MDCK cells infected with type A and type B influenza virus. Influenza A virus (A/Puerto Rico/8/34 (H1N1) and influenza B virus (B/Singapore/222/97) respectively were used to infected MDCK cells. Total cell lysates were then subjected to W.B. using the HCA-2 and HCA-3 antibodies respectively. MW: denotes protein molecular weight markers. Lane 1: negative control (−) represents cell lysates from HEK cells infected with parainfluenza virus. Lane 2: positive control (+) represents commercial rNA4 (Protein Sciences Corporation, Meriden, CT). Note the upper band (indicated by open arrow) may be largely due to aggregated forms of NA obtained from commercial vendor. Lane 3: cell lysates from MDCK cells infected with A/Puerto Rico/8/34 (H1N1). Lane 4: cell lysates from MDCK cells uninfected with virus. Lane 5: cell lysates from MDCK cells infected with influenza B virus (B/Singapore/222/97). Both HCA-2 and HCA-3 antibodies bind to the NA protein in cells infected with the A/Puerto Rico/8/34 (H1N1). No detectable cross-reaction to cellular proteins was observed with both antibodies. Note that HCA-2 Ab also bound to NA in cells infected with type B influenza virus (B/Singapore/222/97), consistent with our data from the bioinformatics analyses (Fig. 1A and C) since the HCA-2 is conserved in both type A and B influenza viral NA, while HCA-3 epitope is only conserved in type A influenza viral NA.

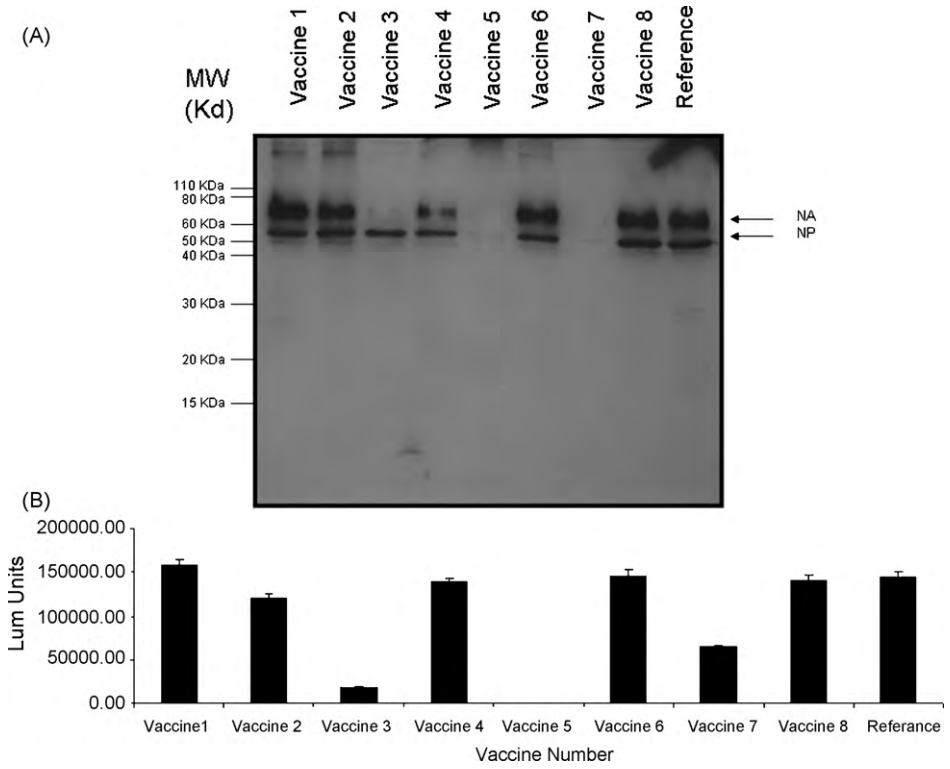

Fig. 5. Detection of NA antigens in vaccine samples by universal NA antibodies in W.B. and NA enzymatic activities using chemiluminescent neuraminidase assay. Panel A: W.B. analysis of NA antigens was conducted using NA universal antibodies. Samples of pH1N1 vaccine (equivalent to 450 ng of HA) from eight manufacturers were fractionated by SDS-PAGE and then transferred onto PVDF for a W.B. analysis using the procedure as described [43]. Reference antigen (HA) was obtained from CBER/FDA. The antibodies used for the detection of NA shown in this figure were HCA-3 antibodies. Similar results were obtained with the other universal NA antibodies (HCA-2) (not shown in the figure); membrane was then re-probed with anti-NP antibodies. Note: identification of the NP and NA proteins has also been confirmed by mass spectrometry analyses using a procedure as described previously [52]. Panel B: NA enzymatic activities in the eight vaccine samples were analyzed using a chemiluminescent neuraminidase assay as described by Buxton et al. [47] with slight modification [8].

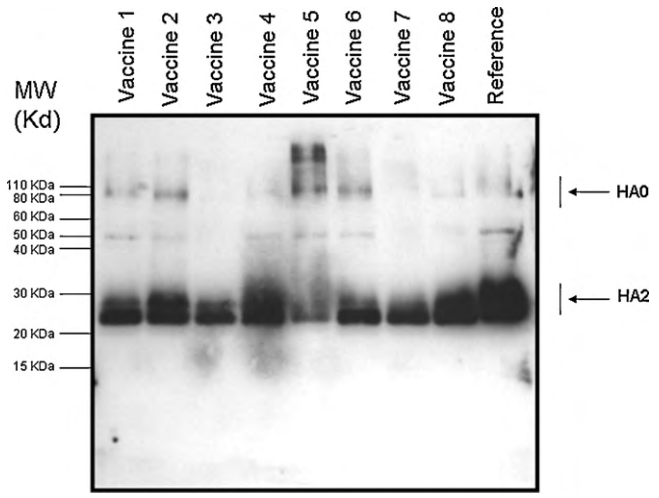

Fig. 6. Detection of HA proteins for the same vaccine samples analyzed in Fig. 4 using Uni-1 antibodies. All eight vaccine samples were subjected to W.B. analyses using Uni-1 antibodies, which target the fusion peptide of HA [43]. In brief, equal amounts of vaccines (equivalent to 450 ng of HA) were loaded onto SDS-PAGE and probed with Uni-1 antibodies to detect the HA. Most of the vaccine samples appear to have fully processed HA proteins as revealed by the presence of HA2 (pointed to by lower arrow). The higher arrow points to the HA0 precursor detected by the Uni-1 antibodies [43].

precursor of HA (HA0) and the processed product (HA2) [43]. As shown in Fig. 6, the presence of HA0 and decreased levels of HA2, especially for #5 vaccine, suggest that the HA precursor (HA0) was not completely processed, co-incident with the decreased levels of NA antigens and enzymatic activities (Fig. 5). Interestingly, in #2 and #6 vaccines, the presence of HA0, albeit much less compared with #5 vaccine, was not accompanied with deceased levels of NA. Thus, these results collectively suggest that reasons for the low level of NA could be multi-factorial (see below for more discussion).

3.4. Antibody-based assay for NA quantification

We next investigated the feasibility of developing a quantitative assay similar to that we developed for HA [46]. Towards this end, we first determined the condition for the sample treatment in Slot blot as pre-treatment of sample can greatly affect the sensitivity of antigen detection in vaccine preparations [46]. As shown in Fig. 7(panel A), treatment of vaccine with Zwittergent at 0.01% significantly improved the sensitivity while higher concentrations of the detergent resulted in decreased intensities of detection signal, an observation which could be ascribed to the detergent at high concentrations preventing the proteins from being bound to the membrane. Moreover, 4 M urea, known to facilitate exposure of HA fusion peptide and used to quantify HA antigen in Slot blot [46], had negligible effect on assay sensitivity (Fig. 7, panel A). Several other detergents/solvents including SDS, Triton and BugBuster were also tested but failed to improve assay sensitivity (see Fig. S1). We therefore chose 0.01% Zwittergent (final concentration) as the sample diluent to develop a standard curve for the quantification of NA. As shown in Fig. 7(panel B), the concentration of the antigen was proportional to the signal intensity developed in Slot blot in the 4-PL curve fitting model (Fig. 7, panel C), suggesting this assay condition could be used to quantify the amounts of NA in vaccine preparation.

Considering that the levels of NA in vaccine products from various manufacturers vary (Fig. 5A and B), we next studied whether there was any variation in the NA amounts in different lots produced by the same company using the quantitative Slot blot

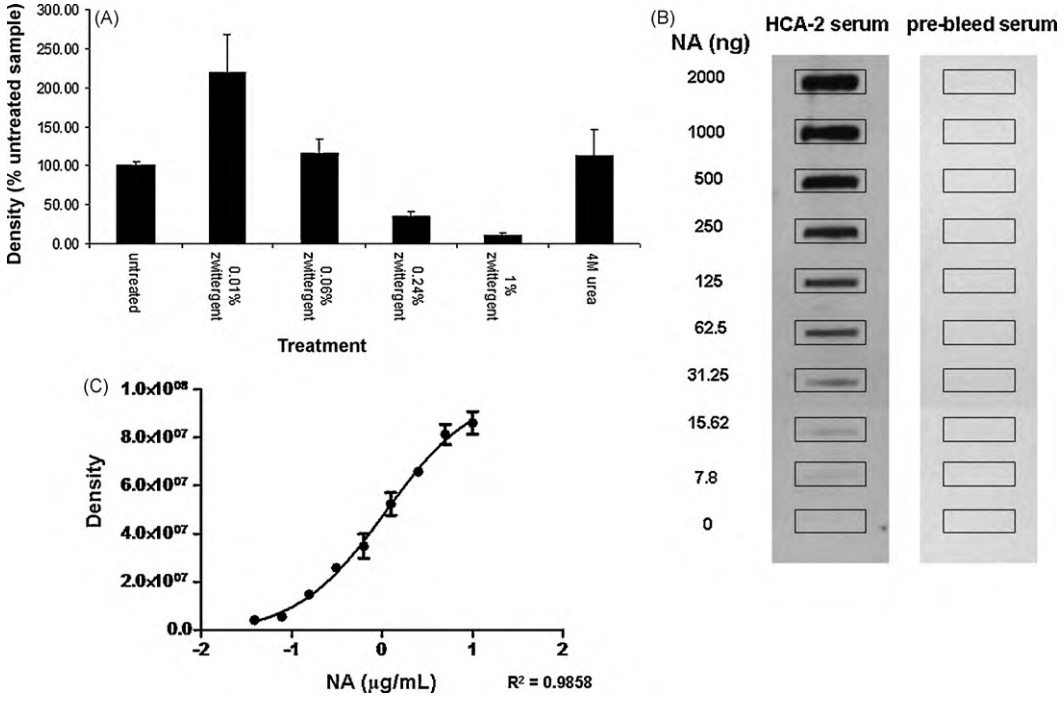

Fig. 7. Quantification of NA by Slot blot. Slot blot was conducted in a similar procedure as described previously [46]. Panel A: the effects of detergent on the sensitivity of Slot blot: the vaccine samples (125 ng of NA pre-determined using SDS-PAGE densitometry [45,52]) were treated with 4 M urea (Li et al. [46]) or Zwittergent at various concentrations (0.01–1%). The antibodies used were HCA-2 MAb (1 μg/ml). The figure shows that 0.01% Zwittergent is the optimal concentration to enhance the detection signal. Panel B: quantitative detection of NA using antibodies against NA: the NA antigens at various concentrations were diluted in PBS-containing 0.01% Zwittergent (final concentration) and blotted onto PVDF membrane. The membrane was then incubated with the universal NA antibodies (HCA-2), followed by detection with anti-rabbit IgG peroxidase conjugate. Similar results were obtained with HCA-2 MAb or with the use of commercial recombinant NA antigens obtained from Protein Sciences Corporation (Meriden, CT) (not shown in the figure). Panel C: standard curve for the quantification of NA: the currently accepted 4-parameter logistic (4-PL) model was employed for the calibration curve fitting in the immunoassays as described [43,46]. The curve fits well as suggested by $R^2 = 0.9858$.

Table 2
NA contents from 14 lots of pH1N1 vaccines from one manufacturer.

Sample	Average μg NA/ml vaccine ± SD	Average % NA vs HA ± SD
Lot A	3.29 ± 0.26	21.94 ± 1.72
Lot B	5.28 ± 1.36	35.18 ± 9.09
Lot C	0.73 ± 0.18	4.88 ± 1.20
Lot D	4.47 ± 0.57	29.81 ± 3.81
Lot E	2.25 ± 0.03	15.03 ± 0.18
Lot F	3.84 ± 0.22	25.61 ± 1.44
Lot G	2.28 ± 0.01	15.2 ± 0.04
Lot H	3.22 ± 0.47	21.46 ± 3.12
Lot I	3.26 ± 0.22	21.72 ± 1.49
Lot J	3.28 ± 0.94	21.84 ± 6.29
Lot K	3.8 ± 1.05	25.33 ± 6.97
Lot L	3.54 ± 0.95	23.59 ± 6.35
Lot M	3.73 ± 0.02	24.89 ± 0.12
Lot N	5.08 ± 0.67	33.84 ± 4.46

described in Fig. 7. The availability of multiple sample lots from one manufacturer allowed us to test 14 lots of pH1N1 vaccine. As shown in Table 2, the ratio of NA compared to HA ranged from 4% to 35% in the 14 lots analyzed, with about half of the lots displaying a ratio of NA/HA being around 20–25%. Taken together, these preliminary results show marked differences in the NA levels between manufacturers or lots from the same vaccine producer.

3.5. Identification of neuraminidase in vaccine samples fractionated by HPLC

A variety of analytical methods including reversed-phase HPLC for the quantification of hemagglutinin and size-exclusion HPLC in conjunction with mass spectrometry for fast protein profiling have been developed recently [33–42]. Yet, none of these physicochemical methods has thus far described the successful detection of neuraminidase in vaccine samples fractionated by chromatography. We therefore determined whether the antibodies could identify the elusive neuraminidase in vaccine preparations. For this purpose, seven (7) fractions collected from a size-exclusion HPLC injection were subjected to W.B. analysis using the antibodies. As shown in Fig. 8, NA was detected in fraction 2. The apparent molecular weight of components in this particular fraction ranges between 90 and 500 kDa [42], which is in line with the presence of NA as a tetramer of approximately 240 kDa. NP was also found to co-elute in fraction 2, an observation in agreement with the presence of NP oligomers and hetero-oligomers with other proteins from the vaccine. We are currently using the antibodies to detect the NA antigens in fractions collected from other types of chromatographic separation methods, which could better separate NA from other viral components in vaccines (Girard M. et al., unpublished data). Nevertheless, the data show here that the antibody can be used to detect the presence of NA from fractions collected from separating methodologies.

4. Discussion

As one of the two surface proteins, NA antigen can induce protective immune responses in humans; indeed, anti-NA-specific antibody has been shown to correlate with immunity to influenza in humans and animals [20–26,32,48]. Yet, the NA contents in the currently licensed vaccines remain to be standardized largely due to the labile nature of the proteins, low amounts and/or lack of international reference standards [28–31]. Indeed, measurements of neuraminidase activities as a marker for neuraminidase antigens have been questioned as a valid means since the enzymatic activity levels vary in the vaccine preparations or could be very low or even missing in some of them [28–31]. Because of the problems associated with enzymatic activity measurements, alternative approaches have been explored to quantify the amounts of the NA antigens in vaccine preparations [32]. Specifically, ELISA

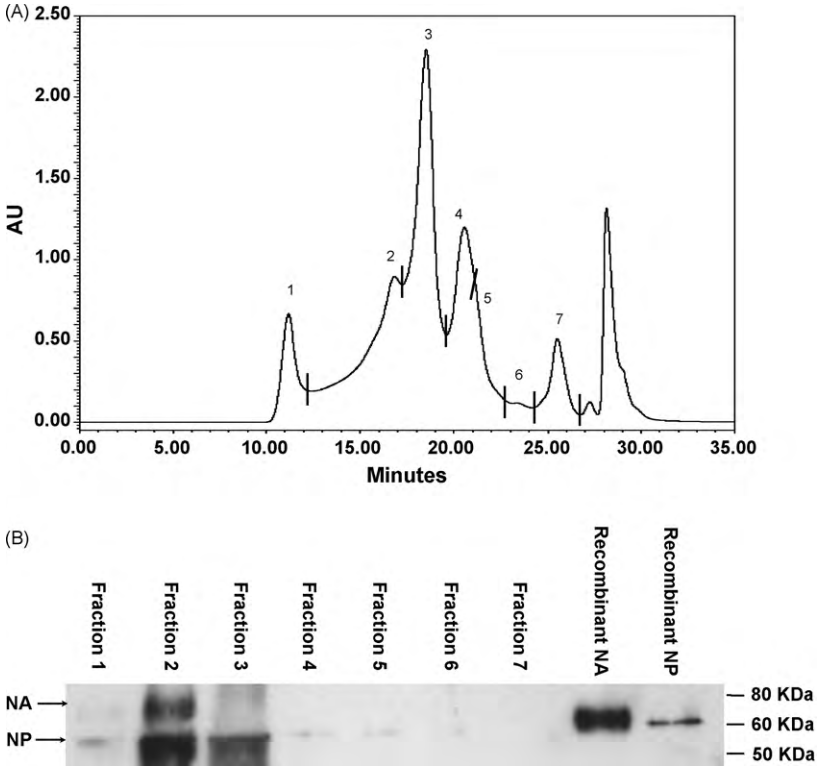

Fig. 8. Detection of NA by size-exclusion HPLC (A) in conjunction with the W.B. using anti-NA (B). Monovalent vaccine samples (A/Solomon Island/3/06) were fractionated as previously described [42]. Numbers in the chromatogram correspond to the collected fractions shown in the W.B. HCA-3 antibody was used to probe the NA presence in a W.B. The figure shows that NA was eluted at peak 2 together with NP.

and antibody-based immunochromatography have been reported [29,31]. Yet, these antibodies are strain specific and must be made for each viral NA strain. Physico-chemical methods such as RP-HPLC or HPLC with tandem mass spectrometry have been reported to quantify the HA. It is unclear as to how or whether they could be used for NA quantification. Additionally, technical training for the staff and sophisticated instruments are also required for such analytical methods. These previous observations underscore the challenge that the bottle-neck for NA quantification is largely due to a lack of appropriate reagents or suitable analytical/biological assay [32]. We therefore thought that simple immunoassays using antibodies targeting the most conserved sequences of all neuraminidases would be of practical use for the analyses of NA antigens. Indeed, the two regions we identified using bioinformatics analyses were universally conserved (Fig. 1). The HCA-3 epitope (Fig. 1B), comprised of the first 12 a.a. from the N-terminus, was highly conserved, an observation consistent with findings reported by Air and co-workers [49,50]. We found this HCA-3 epitope was not present in the influenza B viral NA proteins (Fig. 1D). Moreover, we identified for the first time another novel epitope (HCA-2) which proved to be even more conserved, i.e., close to 100% in both influenza A and B viral neuraminidases. Specifically, this epitope, located near the enzymatically active site of the NA proteins (Fig. S2), shares remarkable homology between influenza type A and type B viral NA (Fig. 1A and C), enabling us to succeed in generating universally reactive antibodies against these two conserved but separated regions in the NA molecules. Indeed, the data from subsequent antibody binding assays are consistent with the results from bioinformatics, i.e., HCA-2 antibodies can bind to both the influenza A and B NA while HCA-3 antibodies bind to influenza A NA but not to influenza B viral NA. Importantly, the abilities of the two antibodies to detect all nine subtypes of NA of type A viruses with little cross-reactivity to allantoic proteins or cellular proteins (Figs. 3 and 4) demonstrate that the antibodies are highly specific for the viral NA sequences.

The two antibodies targeting two highly conserved but separate regions could be used interchangeably for the type A NA detection assays reported in this communication; no marked difference in terms of specificity was found between the mono-specific polyclonal antibodies and monoclonal antibodies (data not shown). As the antibodies bind to separate regions, they may also be tested for the suitability as capturing and detecting antibodies in a sandwich ELISA or as pooled antibodies to enhance the detection signals.

Tests conducted so far for these antibodies suggest that the antibodies could be used for ELISA, W.B. and Slot blot (Figs. 2–4, 6 and 7). Of particular interest are the conditions for the Slot blot assay. While 4 M urea has been found to substantially increase the sensitivity for the detection of the HA fusion peptide epitope [43,46], it failed to enhance the sensitivity for the detection of NA (Fig. 7A); neither did SDS, Triton or BugBuster at concentrations ranging from 0.008–1% (Fig.S1). Interestingly, Zwittergent at a final concentration of 1%, used in SRID for influenza vaccine potency testing (HA quantification), greatly reduced detection signal while it significantly improved NA detection at 0.01% (Fig. 7A). This observation, albeit not completely understood, could be due to higher concentration of Zwittergent (a detergent) preventing absorption of the proteins onto the PVDF, while lower concentrations of the detergent might facilitate exposure of the epitopes for the antibodies to bind. Under aforementioned conditions for Slot blot, it is unclear as to how close to the "native conformation" the NA protein resemble to a native NA protein although the antibodies do bind very well to recombinant NA in indirect ELISA at neutral pH (PBS buffer) (Fig. 2), a condition which might not be deemed harsh enough to totally denature the NA proteins. It would be interesting in the future to explore, however, whether alternative method such as sandwich ELISA would be suitable as another type of assay for NA quantification, in which one of the antibodies could be used as capturing antibody while the other would be serving as detecting antibodies. Also of note is that as far as any monoclonal antibody is concerned, these two NA-specific monoclonal antibodies reported here bind to their respective single epitope; thus, they are not suitable for the detection of potentially "damaged" a.a. sequences or altered conformations in the non-conserved regions which might arise during the manufacturing process. However, the availability of these antibodies did allow us to reveal marked difference in the "physical contents" of the NA proteins in vaccines from different manufacturers or even absence of the NA proteins in addition to lot-to-lot difference from the same producer (Figs. 5–7 and Table 2). Understandably, substantial loss of NA could resulted in a reduced immune responses [12,14,17,18,25,26,28].

In our analyses of NA proteins in vaccine samples, we chose to normalize the input amount of vaccine samples based on the same amounts of HA pre-determined by SRID, given that SRID is the standard for vaccine potency testing. The rationale behind this choice is that we intended to know what the variations in NA are when the vaccines are released by SRID, which has been successfully playing a critical role in the quantification of HA, but is not designed to analyze viral proteins other than HA. In the subsequent analyses of samples from various manufacturers, the variations of NA detected by the antibodies (Fig. 5A and B) are remarkable. Similar variations were also observed if we normalize the input sample amounts by total proteins (data not shown). The low levels of NA from certain producers (#3, #5 and #7) may largely reflect the difference(s) in the manufacturing process and/or in-process control amongst the manufacturers. However, the exact reasons are complex and could be multi-factorial. Interestingly, the complete loss of NA antigen and enzymatic activities in one of the products (#5 vaccine) appeared to be accompanied by the incomplete cleavage of the HA0 precursor and loss of NP protein (Figs. 5 and 6). On the other hand, the presence of un-cleaved HA0 in #2 and #6 vaccines, albeit much less so in terms of the amounts compared to that in vaccine #5, did not appear to be co-incidental with a loss of NA proteins. Furthermore, it is noteworthy that variable amounts of NA were also detected in different lots from the same manufacturer (Table 2), even if the HA0 was completely processed (data not shown). These data collectively suggest that the final products may vary in NA content, underscoring the daunting challenges of in-process quality control of NA antigens. Obviously, more studies are required to better understand the variations of NA antigens in the vaccines including analyses of additional lots from more than one manufacturer and collection of samples from each step of the manufacturing processes to quantify the NA antigens, which are currently being investigated in our laboratories.

Despite the demonstrated advantages, such as their versatilities for application to NA detection in vaccines from various manufacturers by simple and inexpensive immunoassays, the methods described here have certain limitations. One of the drawbacks is that they are designed to detect virtually all NA proteins and not to distinguish different NA strains/subtypes, a disadvantage shared by the measurement of NA enzymatic activities routinely used by many vaccine producers. Thus, they are more suitable for in-process control and/or analyses of monovalent vaccine preparations. Clearly, alternative methods should be considered for NA quantification when strain identification is required or NAs in multivalent vaccines are to be determined.

In conclusion, two highly conserved regions have been identified through extensive bioinformatics analyses of all available sequences reported so far. The antibody targeting the N-terminus of NA (HCA-3) has been known to be conserved among influenza A viruses, while the antibody targeting the region near the enzymatic active site (HCA-2) is even much more conserved or universally conserved not only in the nine NA subtypes of influenza A viruses

but also in influenza B viruses, a novel finding reported in this communication. The antibodies targeting the two conserved sequences could be used in simple immunoassays without the need for expensive and sophisticated instruments. The practical values of these antibody-based immunoassays were demonstrated in the analyses of vaccine samples from 8 manufacturers producing pH1N1 vaccine (2009) using the same seed strain. The marked variations in the levels of NA in vaccines between producers or even in different lots from the same which we presented in this report may largely reflect the labile nature of the NA antigens and/or different manufacturing processes. Understandably, the universally reactive antibody-based immunoassays still require vigorous international validations to determine the suitability as another means for routine NA quantifications in production processes. Nevertheless, given mounting evidences from both, human and animal studies, that HA as well as NA are contributing to protection against influenza, additional assays for NA quantification are badly needed. Moreover, in light of the fact that vaccines are produced within a limited time period, simple, fast and reproducible assays should be further explored.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2010.06.075.

References

[1] Webster RG, Bean Jr WJ. Genetics of influenza virus. Annu Rev Genet 1978;12:415–31.
[2] Skehel JJ, Wiley DC. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem 2000 2000;69:531–69.
[3] Air GM, Laver WG. The neuraminidase of influenza virus. Proteins 1989;6:341–56.
[4] Kilbourne ED, Laver WG, Schulman JL, Webster RG. Antiviral activity of antiserum specific for an influenza virus neuraminidase. J Virol 1968;2:281–8.
[5] Compans RW, Dimmock NJ, Meier-Ewert H. Effect of antibody to neuraminidase on the maturation and hemagglutinating activity of an influenza A2 virus. J Virol 1969;4:528–34.
[6] Liu C, Eichelberger MC, Compans RW, Air GM. Influenza type A virus neuraminidase does not play a role in viral entry, replication, assembly, or budding. J Virol 1995;69:1099–106.
[7] Palese P, Compans RW. Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA): mechanism of action. J Gen Virol 1976;33:159–63.
[8] Hashem AM, Flaman AS, Farnsworth A, Brown EG, Van Domselaar G, He R, et al. Aurintricarboxylic acid is a potent inhibitor of influenza A and B virus neuraminidases. PLoS One 2009;4:e8350.
[9] Ohuchi M, Asaoka N, Sakai T, Ohuchi R. Roles of neuraminidase in the initial stage of influenza virus infection. Microbes Infect 2006;8:1287–93.
[10] Su B, Wurtzer S, Rameix-Welti MA, Dwyer D, van der Werf S, Naffakh N, et al. Enhancement of the influenza A hemagglutinin (HA)-mediated cell-cell fusion and virus entry by the viral neuraminidase (NA). PLoS One 2009;4(12):e8495.
[11] Wood JM, Dunleavy U, Newman RW, Riley AM, Robertson JS, Minor PD, et al. The influence of the host cell on standardisation of influenza vaccine potency. Dev Biol Stand 1999;98:183–8.
[12] Sylte MJ, Suarez DL. Influenza neuraminidase as a vaccine antigen. Curr Top Microbiol Immunol 2009;333:227–41.
[13] Schulman JL, Khakpour M, Kilbourne ED. Protective effects of specific immunity to viral neuraminidase on influenza virus infection of mice. J Virol 1968;2:778–86.
[14] Johansson BE, Grajower B, Kilbourne ED. Infection-permissive immunization with influenza virus neuraminidase prevents weight loss in infected mice. Vaccine 1993;11:1037–9.
[15] Rott R, Becht H, Orlich M. The significance of influenza virus neuraminidase in immunity. J Gen Virol 1974;22:35–41.
[16] Webster RG, Laver WG, Kilbourne ED. Reactions of antibodies with surface antigens of influenza virus. J Gen Virol 1968;3:315–26.
[17] Sandbulte MR, Jimenez GS, Boon AC, Smith LR, Treanor JJ, Webby RJ. Cross-reactive neuraminidase antibodies afford partial protection against H5N1 in mice and are present in unexposed humans. PLoS Med 2007;4:e59.
[18] Takahashi Y, Hasegawa H, Hara Y, Ato M, Ninomiya A, Takagi H, et al. Protective immunity afforded by inactivated H5N1 (NIBRG-14) vaccine requires antibodies against both hemagglutinin and neuraminidase in mice. J Infect Dis 2009;199:1629–37.
[19] DiNapoli JM, Nayak B, Yang L, Finneyfrock BW, Cook A, Andersen H, et al. Newcastle disease virus-vectored vaccines expressing the hemagglutinin or neuraminidase protein of H5N1 highly pathogenic avian influenza virus protect against virus challenge in monkeys. J Virol 2010;84:1489–503.
[20] Murphy BR, Kasel JA, Chanock RM. Association of serum anti-neuraminidase antibody with resistance to influenza in man. N Engl J Med 1972;286:1329–32.
[21] Couch RB, Kasel JA, Gerin JL, Schulman JL, Kilbourne ED. Induction of partial immunity to influenza by a neuraminidase-specific vaccine. J Infect Dis 1974;129:411–20.
[22] Kilbourne ED, Couch RB, Kasel JA, Keitel WA, Cate TR, Quarles JH, et al. Purified influenza A virus N2 neuraminidase vaccine is immunogenic and non-toxic in humans. Vaccine 1995;13:1799–803.
[23] Beutner KR, Chow T, Rubi E, Strussenberg J, Clement J, Ogra PL. Evaluation of a neuraminidase-specific influenza A virus vaccine in children: antibody responses and effects on two successive outbreaks of natural infection. J Infect Dis 1979;140:844–50.
[24] Ogra PL, Chow T, Beutner KR, Rubi E, Strussenberg J, DeMello S, et al. Clinical and immunologic evaluation of neuraminidase-specific influenza A virus vaccine in humans. J Infect Dis 1977;135:499–506.
[25] Cate TR, Rayford Y, Niño D, Winokur P, Brady R, Belshe R, et al. High dosage influenza vaccine induced significantly more neuraminidase antibody than standard vaccine among elderly subjects. Vaccine 2010;28:2076–9.
[26] Johansson BE, Pokorny BA, Tiso VA. Supplementation of conventional trivalent influenza vaccine with purified viral N1 and N2 neuraminidases induces a balanced immune response without antigenic competition. Vaccine 2002;20:1670–4.
[27] Brett IC, Johansson BE. Immunization against influenza A virus: comparison of conventional inactivated, live-attenuated and recombinant baculovirus produced purified hemagglutinin and neuraminidase vaccines in a murine model system. Virology 2005;339:273–80.
[28] Gillim-Ross L, Subbarao K. Can immunity induced by the human influenza virus N1 neuraminidase provide some protection from avian influenza H5N1 viruses? PLoS Med 2007;4:e91.
[29] Gerentes L, Kessler N, Aymard M. A sensitive and specific ELISA immunocapture assay for rapid quantitation of influenza A/H3N2 neuraminidase protein. J Virol Methods 1998;73:185–95.
[30] Gulati U, Wu W, Gulati S, Kumari K, Waner JL, Air GM. Mismatched hemagglutinin and neuraminidase specificities in recent human H3N2 influenza viruses. Virology 2005;339:12–20.
[31] Tanimoto T, Nakatsu R, Fuke I, Ishikawa T, Ishibashi M, Yamanishi K, et al. Estimation of the neuraminidase content of influenza viruses and split-product vaccines by immunochromatography. Vaccine 2005;23:4598–609.
[32] Bright RA, Neuzil KM, Pervikov Y, Palkonyay L. WHO meeting on the role of neuraminidase in inducing protective immunity against influenza infection, Vilamoura, Portugal, September 14, 2008. Vaccine 2009;27:6366–9.
[33] Bucher DJ, Li SSL, Kehoe JM, Kilbourne ED. Chromatographic isolation of the hemagglutinin polypeptides from influenza virus vaccine and determination of their amino-terminal sequences. Proc Nat Acad Sci U S A 1976;73:238–42.
[34] Calam DH, Davidson J. Isolation of influenza viral proteins by size-exclusion and ion-exchange high-performance liquid chromatography: the influence of conditions on separation. J Chromatogr 1984;296:285–92.
[35] Phelan MA, Cohen KA. Gradient optimization principles in reversed-phase high-performance liquid chromatography and the separation of influenza virus components. J Chromatogr 1983;266:55–66.
[36] Kapteyn JC, Saidi MD, Dijkstra R, Kars C, Tjon JC, Weverling GJ, et al. Haemagglutinin quantification and identification of influenza A&B strains propagated in PER.C6 cells: a novel RP-HPLC method. Vaccine 2006;24:3137–44.
[37] Garcia-Cañas V, Lorbetskie B, Girard M. Rapid and selective characterization of influenza virus constituents in monovalent and multivalent preparations using non-porous reversed-phase high performance liquid chromatography columns. J Chromatogr A 2006;1123:225–32.
[38] Kapteyn JC, Porre AM, de Rond EJ, Hessels WB, Tijms MA, Kessen H, et al. HPLC-based quantification of haemagglutinin in the production of egg- and

MDCK cell-derived influenza virus seasonal and pandemic vaccines. Vaccine 2009;27:1468–77.
[39] García-Cañas V, Lorbetskie B, Bertrand D, Cyr TD, Girard M. Selective and quantitative detection of influenza virus proteins in commercial vaccines using two-dimensional high-performance liquid chromatography and fluorescence detection. Anal Chem 2007;79:3164–72.
[40] Williams TL, Luna L, Guo Z, Cox NJ, Pirkle JL, Donis RO, et al. Quantification of influenza virus hemagglutinins in complex mixtures using isotope dilution tandem mass spectrometry. Vaccine 2008;26:2510–20.
[41] Luna LG, Williams TL, Pirkle JL, Barr JR. Ultra performance liquid chromatography isotope dilution tandem mass spectrometry for the absolute quantification of proteins and peptides. Anal Chem 2008;80:2688–93.
[42] Garcia-Cañas V, Lorbetskie B, Cyr TD, Hefford MA, Smith S, Girard M. Approach to the profiling and characterization of influenza vaccine constituents by the combined use of size-exclusion chromatography, gel electrophoresis and mass spectrometry. Biologicals 2010;38:294–302.
[43] 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.
[44] Li KB. ClustalW-MPI: ClustalW analysis using distributed and parallel computing. Bioinformatics 2003;19:1585–6.
[45] Harvey R, Wheeler JX, Wallis CL, Robertson JS, Engelhardt OG. Quantitation of haemagglutinin in H5N1 influenza viruses reveals low haemagglutinin content of vaccine virus NIBRG-14 (H5N1). Vaccine 2008;26:6550–4.
[46] 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.
[47] Buxton RC, Edwards B, Juo RR, Voyta JC, Tisdale M, Bethell RC. Development of a sensitive chemiluminescent neuraminidase assay for the determination of influenza virus susceptibility to zanamivir. Anal Biochem 2000;280:291–300.
[48] Deroo T, Jou WM, Fiers W. Recombinant neuraminidase vaccine protects against lethal influenza. Vaccine 1996;14:561–9.
[49] Blok J, Air GM, Laver WG, Ward CW, Lilley GG, Woods EF, et al. Studies on the size, chemical composition, and partial sequence of the neuraminidase (NA) from type A influenza viruses show that the N-terminal region of the NA is not processed and serves to anchor the NA in the viral membrane. Virology 1982;119:109–21.
[50] Air GM, Ritchie LR, Laver WG, Colman PM. Gene and protein sequence of an influenza neuraminidase with hemagglutinin activity. Virology 1985;145:117–22.
[51] Shaw MW, Lamb RA, Erickson BW, Briedis DJ, Choppin PW. Complete nucleotide sequence of the neuraminidase gene of influenza B virus. Proc Natl Acad Sci U S A 1982;79:6817–21.
[52] Li C, Shao M, Cui X, Song Y, Li J, Yuan L, et al. Application of deglycosylation and electrophoresis to the quantification of influenza viral hemagglutinins facilitating the production of 2009 pandemic influenza (H1N1) vaccines at multiple manufacturing sites in China. Biologicals 2010;38:284–9.
[53] Shannon CE. The mathematical theory of communication. 1963. MD Comput 1997;14:306–17.
[54] Colman PM, Hoyne PA, Lawrence MC. Sequence and structure alignment of paramyxovirus hemagglutinin-neuraminidase with influenza virus neuraminidase. J Virol 1993;67:2972–80.