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Immunocapture isotope dilution mass spectrometry in response to a pandemic influenza threat

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Abstract

As a result of recent advances in mass spectrometry-based protein quantitation methods, these techniques are now poised to play a critical role in rapid formulation of pandemic influenza vaccines. Analytical techniques that have been developed and validated on seasonal influenza strains can be used to increase the quality and decrease the time required to deliver protective pandemic vaccines to the global population. The emergence of a potentially pandemic avian influenza A (H7N9) virus in March of 2013, prompted the US public health authorities and the vaccine industry to initiate production of a pre-pandemic vaccine for preparedness purposes. To this end, we evaluated the feasibility of using immunocapture isotope dilution mass spectrometry (IC-IDMS) to evaluate the suitability of the underlying monoclonal and polyclonal antibodies (mAbs and pAbs) for their capacity to isolate the H7 hemagglutinin (HA) in this new vaccine for quantification by IDMS. A broad range of H7 capture efficiencies was observed among mAbs tested by IC-IDMS with FR-545, 46/6, and G3 A533 exhibiting the highest cross-reactivity capabilities to H7 of A/Shanghai/2/2013. MAb FR-545 was selected for continued assessment, evaluated by IC-IDMS for mAb reactivity against H7 in the H7N9 candidate vaccine virus and

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compared with/to reactivity to the reference polyclonal antiserum in allantoic fluid, purified whole virus, lyophilized whole virus and final detergent-split monovalent vaccine preparations for vaccine development. IC-IDMS assessment of FR-545 alongside IC-IDMS using the reference polyclonal antiserum to A/ Shanghai/2/2013 and with the regulatory SRID method showed strong correlation and mAb IC-IDMS could have played an important role in the event a potential surrogate potency test was required to be rapidly implemented.

Keywords

Influenza; Mass spectrometry; Hemagglutinin; Quantitation; Antibodies; Immunocapture; Pandemic; Vaccine; H7N9; Potency assay

1. Introduction

Influenza type A viruses are highly transmissible respiratory pathogens that can cause severe morbidity and mortality in the course of annual seasonal epidemics and sporadic pandemics [1]. Vaccination is considered the most cost-effective means of preventing seasonal influenza epidemics and mitigating pandemics. The influenza A viruses are classified into multiple subtypes, based on the antigenic characteristics of the two major surface proteins (hemagglutinin (HA) and neuraminidase (NA)), which are the most important targets for acquired protective humoral immunity in the population. HA is the critical antigenic component of inactivated and recombinant influenza vaccines.

Pre-existing immunity to antigenically similar viruses that have been circulating in the environment for some time can exist through direct exposure to the virus, cross-protection, or through vaccination. However, antigenic drift causes these viruses to genetically change and evolve gradually over time and these drift mutations result in antigenically different viruses that escape immunologic protection elicited by previously circulating viruses. For this reason, the seasonal influenza vaccine composition must be continually reviewed and updated in order to reduce and control influenza infection effectively.

Antigenic shift results from the replacement of HA, or more infrequently NA, with novel subtypes that have not circulated in humans for a long time [2]. Consequently, when this shift causes a new or dramatically altered subtype to emerge in humans most people have little to no protection against the new virus and the strain is considered to be potentially pandemic. If this variant virus further evolves and adapts to person to person transmission, an influenza pandemic can occur, in which the virus starts spreading more broadly than seasonal influenza with the likelihood of more serious disease in the outbreak because there will be no preexisting immunity to the new virus. Influenza B viruses have no animal reservoirs of antigenically novel HA and are not likely to cause pandemics, as opposed to Influenza A strains that are found in large animal reservoirs such as aquatic birds, poultry, and domestic swine, thus presenting a higher probability of genetically reassorting to become infectious to humans [3]. An influenza pandemic is a rare but recurrent event. During the last 100 years, four pandemics have occurred; “Spanish H1N1 influenza” in 1918, “Asian H2N2 influenza” in 1957, “Hong Kong H3N2 influenza” in 1968, and “Swine-origin H1N1 influenza” in 2009. While increased preparedness, surveillance, and response

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measures can slow the progression of a pandemic and greatly reduce the severity of its impact, mass vaccination, which induces protective immunity from disease by antigenically similar viruses, is still considered the first line of defense for protecting global populations.

Regardless of a seasonal or pandemic influenza response, achieving the benefits of vaccination is dependent on developing, producing, and delivering vaccine as early as possible [4]. Currently, inactivated seasonal influenza vaccines contain components from two influenza A virus subtypes, A(H1N1) and A(H3N2), and influenza B viruses from one or both B/Yamagata and B/Victoria lineages. These trivalent or quadrivalent influenza vaccines provide protection primarily by eliciting the production of neutralizing antibodies to HA, with the final formulation requiring a minimum amount of 15 mg HA from each subtype (H1, H3, B/Yamagata, and B/Victoria) in each 0.5 mL dose. In the case of pandemic vaccines, final formulations will be dependent on variables such as emerging pandemic strain, vaccine dose regime needed for protection, necessity of adjuvant, expected vaccine effectiveness, and information on vaccine uptake among different populations [4].

The final formulation and filling of vaccine prior to delivery to the public depends on testing the bulk product by single radial immunodiffusion (SRID), the required regulatory method for quantification of the HA antigen in vaccines [5–7] to assess potency [8]. However the timeline for producing calibrated SRID reagents is generally 2–3 months with potentially pandemic strains historically taking even longer to produce [9,10], which could delay vaccine availability to mitigate the emerging pandemic.

Following the 2009 A(H1N1)pdm09 pandemic, a 2010 World Health Organization (WHO) workshop concluded that an alternative influenza vaccine potency assay was a necessary priority to speed up the vaccine delivery process in future responses to pandemic influenza and in seasonal vaccine release [11]. Answering this need, our laboratory has taken concerted action by developing several isotope dilution mass spectrometry (IDMS) methods for accurate quantitation of HA, neuraminidase (NA), and other influenza proteins [12–15]. IDMS involves enzymatic digestion of viral proteins and detection of evolutionarily conserved target peptides for accurate quantitation. Specifically, IDMS alone quantifies total HA protein regardless of whether or not it is in an antigenically correct conformation. Recently we described an immunocapture-IDMS (IC-IDMS) “potency” method that measures HA that binds to antibodies with high accuracy, specificity, precision, and sensitivity. In that work, the IC-IDMS protocol utilized SRID regulatory polyclonal antibodies (pAbs) provided by the U.S. Food and Drug Administration (FDA), to capture and mass spectrometry (MS) to quantify immunoreactive HA subtypes in seasonal influenza vaccine [15]. IC-IDMS was developed with the intent to mimic SRID antibody-antigen by incorporating an immunoaffinity selection step using identical SRID polyclonal antibodies to isolate intact HA that binds to the antibodies and quantifies the amount of HA bound to the antibodies and the amount that remains unbound by IDMS.

In March of 2013, an A(H7N9) virus was first reported to have infected humans in China [16]. H7, a serotype of Influenza A normally circulating in avian populations, has been known to occasionally infect humans [17–19]. Although several candidate vaccine strains for the H7 subtype (H7N7, H7N1, H7N2) had been developed previously [20–22]; they were

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antigenically different from the emerging A(H7N9) viruses [23]. As part of a collaborative effort among the U.S. Centers for Disease Control and Prevention (CDC), the FDA, and the U.S. Biomedical Advanced Research Development and Authority (BARDA) to prepare and rapidly respond to the A(H7N9) emerging pandemic threat, our laboratory expanded our IDMS and IC-IDMS methodologies to quantify total and immunocaptured H7 in various A(H7N2), A(H7N9) and A(H7N7) influenza vaccine matrices [14]. Additionally, we adapted ICIDMS to a monoclonal antibody (mAb) immunocapture platform as a risk-mitigation alternative. We evaluated 13 anti-HA monoclonal antibodies (mAbs) by IC-IDMS for their ability to immunocapture H7 from A/Shanghai/2/2013 and observed significant differences in their affinities to this novel strain. We determined mAb FR-545 as an ideal candidate for A(H7N9) immunocapture prior to enzymatic digestion and IDMS analysis and conclude that IC-IDMS could be rapidly implemented in the event a surrogate potency test was required to speed up the vaccine production timeline.

2. Materials and methods

2.1. Influenza antibodies and viral matrices

MAbs procured for screening to assess potential cross-reactivity to A(H7N9) were obtained from various sources. Anti-A/ Netherlands/219/2003 (H7N7) mouse mAbs FR-543, FR-544, FR545, FR-546, and FR-547 were made available through CDC's International Reagent Resource (IRR) (Manassas, VA). Anti-A/chicken/ Malaysia/94 (H7N1) mAbs 62 and 98 were obtained from Dr. He Fang of the College of Animal Sciences at Zhejiang University (Hangzhou, China). Anti-A/Vietnam/1203/2004 (H5N1) mAb H5.31, was isolated at the Vanderbilt Vaccine Center of Vanderbilt University Medical Center (Nashville, TN). A533 human IgG1 was isolated by antibody phage display (Mehta I/II) library panning against trimeric H7 protein from the A/Netherlands/219/2003 strain that was modified to introduce a N-glycan at position N158 in the globular head whereas F105 human single-chain variable fragment (scFv) IgG1 Fc fusion protein (scFv-Fc) was isolated by panning the same library against a glycan variant of trimeric H3 protein of A/Aichi/2/1968 (H3N2) strain that was modified by removal of N38 (G1) and addition of N158 (G3) and were provided by Dr. Wayne Marasco of the Department of Medicine at Harvard Medical School and the Department of Cancer Immunology & Virology Dana-Farber Cancer Institute (Boston, MA) (manuscript in preparation). A/Seal/Massachusetts/1/1980 (H7N7) clones 46/6, 55/2, and 58/2 were procured from Dr. Richard Webby and Mr. Scott Krauss of the Department of Infectious Disease at St. Jude Children's Research Hospital (Memphis, TN) with clones 46/6 and 58/2 now publicly available through BEI Resources (Manassas, VA). Polyclonal reference antiserum (sheep anti-hemagglutinin, A/Shanghai/2/2013 (H7N9), CBER Lot H7-Ab-1320 (herein pAbH7), b-propiolactone (BPL)-inactivated A/Shanghai/2/2013 lyophilized whole virus reference reagent ((H7N9) Ref. #78, 60 mg/mL H7) and A/Shanghai/2/2013 detergent-split monovalent vaccine preparations were provided by FDA's Center for Biologics Evaluation and Research (CBER) (Silver Springs, MD). Recombinant influenza H7 HA protein (recH7) from A/ Netherlands/219/2003 and BPL-inactivated A/Shanghai/2/2013 allantoic fluid and purified whole virus preparations were obtained from the National Center for Immunization and Respiratory Diseases (NCIRD), Influenza Division, CDC (Atlanta, GA). With the exception

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of H5.31, all mAbs were stored at 80 C until thawed prior to analysis. Purified whole virus, monovalent vaccine preparations, H5.31 and lyophilized pAb-H7 antiserum were stored at 4 C. Allantoic fluid, recH7 and lyophilized whole virus preparations were stored at 80 C. Prior to analysis lyophilized pAb-H7 antiserum and lyophilized whole virus reagent were reconstituted in deionized H_2O.

2.21. Synthesis of native and labeled peptides

Custom synthetic peptides for H7 were synthesized at a 1–5 mg scale by Midwest Bio-Tech, Inc. (Fishers, IN) as recently described [14]. The target peptide sequences are listed in Table 1. Briefly, the lyophilized peptides were reconstituted in formic acid, dispensed into 200-μL aliquots in 1.5-mL vials using a Biomex NXP Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA), and then lyophilized again and stored at 80 °C until use. The peptide standards were quantified by an in-house isobaric-tagged isotope dilution mass spectrometry (IT-IDMS) method for amino acid analysis (AAA) [24] that uses NIST-certified amino acid standards to assure peptide contents' accuracies. Labeled analogs of the target peptides FVNEEALR and VNTLTER in H7 were synthesized by incorporating the leucines with ^{13}C and ^{15}N, producing peptides with a 7 Da increase in mass over the unlabeled peptides. For STQSAIDQITGK the isoleucine at the ninth position was ^{13}C and ^{15}N-labeled, also producing a peptide with a 7 Da increase in mass over the corresponding unlabeled peptide.

2.3. Preparation of working stock, calibration, and labeled solutions

All labeled and unlabeled peptide standards were reconstituted with 100 mL of 10% (v/v) formic acid and diluted with 0.1% (v/v) formic acid to yield a 5-pmol/μL stock solution. For each peptide, working stock solutions of 0.5 pmol/μL were made from the 5pmol/μL stock solution by diluting with 0.1% formic acid. This working stock solution was used to make the calibration standards. Seven 0.5-mL stock calibration standards, ranging from 2 to 90 fmol/μL, were prepared by adding 2, 5, 10, 30, 50, 70 and 90 μL of each of the unlabeled peptides, 50 μL of the labeled peptides, and 0.1% formic acid to make the final volume 0.5 μL. The 0.5–pmol/μL spiked solutions of the labeled peptides were used for the internal standards. Mean area ratios (unlabeled/labeled) were plotted against concentrations for each standard. Linear regression without weighting was applied to the data sets and calibration curves were generated for each peptide. Regression analysis of the calibration curves showed a linear relationship with R^2 values exceeding of 0.99 for each H7 peptide.

2.4. Covalent IgG immobilization on protein G magnetic beads

Improvements and minor modifications of the immobilization method described previously [15] were made to reduce incubation times and to accommodate the various antibodies' IgG concentrations for optimal binding of the Dynabeads® Protein G (Life Technologies, Carlsbad, CA) [25]. For bead preparation, A DynaMag™15 magnetic particle concentrator (Life Technologies) was used for magnetic separation. Briefly, each antibody's concentration was adjusted to be approximately twice that of the 8 μg IgG/mg bead binding capacity to achieve complete saturation and binding to the magnetic beads. Dynabeads® were resuspended, aliquoted into 15-mL Eppendorf™ conical tubes (Eppendorf, Hauppauge, NY), and washed twice with 10 mL of 0.1 M PBS. H7 antibodies (Ab-H7) were added

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directly to the washed beads, diluted in 3 mLs of 0.1 M PBS and incubated at room temperature (RT) for 4 h with gentle rotation, allowing antibody attachment. Following incubation, the antibody-bead slurry was washed three times with 10 mL of 0.1 M PBS followed by two washes with 10 mL of 0.2 M triethanolamine pH 8.2. Protein G beads with bound Ab-H7 were then resuspended in 3 mL of freshly prepared cross-linking solution (20 nM dimethyl pimelimidate dihydrochloride in 0.2 M triethanolamine, pH 8.2) and incubated at RT for 30 min with gentle rotation. The supernatant was discarded, the Ab-bead complexes were resuspended in 1 mL of 50 mM Tris, pH 7.5 and incubated for 15 min at RT with gentle rotation to stop the crosslinking reaction. The beads with covalently attached IgG were washed twice with a 10 mL 0.1 M PBS/0.05% Tween-20 solution followed by a final 10 mL wash in 0.1 M PBS/0.01% Tween-20. Final Ab-H7-bead preparations were resuspended to their initial starting volume in 0.1 M PBS/0.01% Tween-20 buffer and stored at 4 C for 1 week.

2.5. H7 Immunocapture

With the exception of the allantoic fluid samples, all influenza vaccine preparations tested were diluted with deionized water to a starting concentration of 30 μg/mL H7 and used without further purification. Undiluted allantoic fluid was used due to the low concentration of H7 present in the crude matrix. Test samples were pretreated in 1% (w/v) Zwittergent 3–14 detergent (Calbiochem Behring, La Jolla, CA), which is assumed to solubilize the viral HA proteins anchored in the influenza virion lipid envelope [26,27]. Prior to introduction of the Ab-H7-bound Protein G beads, ten microliters of 10% Zwittergent 3–14 and 10 μL of each H7 influenza preparation (30 μL allantoic fluid preparation) was pipetted into separate wells of a 96-well plate, diluted with 0.1 M PBS to a final volume of 100 μL, and incubated for 30 min at RT. A Kingfisher™ Flex 96 magnetic particle processor (Thermo Scientific Corp., Waltham, MA) was used to isolate H7 from the virus and vaccine preparations. The Kingfisher™ Flex was programmed for automatic sample handling and optimized to accurately and reproducibly immunocapture conformationally active H7 in vaccine and viral preparations. The profile included a “fast” mixing step of the Zwittergent-treated vaccine preparations with the bead-Ab complex (50 μL) (antibody-antigen binding) followed by a gentle wash in 200 μL of 0.1 M PBS/0.01% Tween-20 and elution in 200 μL of 50 mM ammonium bicarbonate. The total run time was 37 min. For pAb-H7 immunocapture, multiple binding events were necessary for complete affinity capture of all active H7 using the polyclonal reference antiserum. Therefore, following the first incubation, second, third, and fourth consecutive binding events, identical to the first, were performed with the Zwittergent-treated samples and eluted into the final 200 μL 50 mM Ammonium bicarbonate solution. For mAb immunocapture, only one binding event was necessary to reproducibly quantify the extent of each mAb’s cross-reactivity to active H7 and subsequent binding events performed did not isolate additional immunoreactive H7. Following elution, the 200 μL–slurries were transferred to 0.5 mL Eppendorf™ LoBind microcentrifuge tubes and all magnetic sample purification steps were performed using a DynaMag™-2 magnetic particle concentrator (Life Technologies). The bead-Ab-Ag complexes were then magnetically separated from the 200 μLs ammonium bicarbonate and the elution buffer was pipetted off. The samples were briefly centrifuged to further isolate any residual supernatant from the

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bead complexes, and then liquid-extracted by pipette again to ensure complete removal of the buffer from the on-bead sample preparations.

2.6. Preparation of viral digests and IDMS quantification

Bulk H7 influenza vaccine preparations not subjected to anti-body capture were used to quantify total H7 content. For these samples, 30-μL aliquots of allantoic fluid and 10-μL aliquots of purified whole virus, whole virus reference antigen, and monovalent vaccine preparations were diluted in 10 μL of a 0.2% solution of sodium 3-[(2-methyl-2-undecyl-1,3-dioxolan-4-yl)methoxyl]-1-propanesulfonate (*Rapigest*™ SF Surfactant) (Waters Corporation, Milford, MA) in 50 mM ammonium bicarbonate to solubilize proteins and improve tryptic protein digestion [28,29]. The samples were heated for 5 min at 100 °C and allowed to cool to RT. After cooling, 86 pmol of sequence grade trypsin (Promega, Madison, WI) was added to each vial, and samples were incubated at 37 °C for 2 h to achieve complete in-solution digestion. Digests were allowed to cool, 10 μL of a 0.45 M HCl solution was added, and digests were incubated at RT for 30 min to reduce the pH and cleave the acid-labile surfactant. After incubation, 10 μL of the 0.5-pmol/μL labeled H7 internal standard (ISTD) working stock solution was added to each sample, and a 0.1% formic acid solution (aq) was used to dilute the final sample volume to 100 μL. The digested samples were mixed, centrifuged, and transferred to LC autosampler vials for analysis.

Immunocaptured on-bead trypsin digests were treated similarly to the above in-solution digest preparation with the following exceptions. (1) Immunocaptured collections were reconstituted in 20 μL or 30 μL of 50 mM ammonium bicarbonate, for mAbs and pAbs, respectively, prior to *Rapigest* dilution; (2) A 0.4% solution of *Rapigest* was used to solubilize proteins; (3) 10 μL of trypsin (172 pmol) was used; (4) 37 °C incubated samples were mixed gently for 2 h to achieve complete on-bead digestion of our peptides of interest and (5) 15 μL of 0.45 M HCl solution was used to reduce the pH.

2.7. IDMS Instrumentation parameters

An Agilent 1200 series LC system (Agilent Technologies, Inc., Santa Clara, CA) was configured for alternating column regeneration to increase sample throughput. A dual column, dual pump system coupled to an Agilent 1200 Series 2 position/10 port valve allowed simultaneous analysis of one column eluent while a second identical column was flushed and equilibrated. The analytical columns utilized were 150 mm ×1 mm i.d. Symmetry300™ reverse phase C_{18} (3.5 μm particle size, Waters Corporation, Milford, MA). The injection volume was 5 μL, and a 2-μL full loop injection with three-time loop overfill was utilized for injections. The aqueous mobile phase (A) consisted of 0.1% formic acid in HPLC-grade water, while the organic mobile phase (B) was 0.1% formic acid in acetonitrile. A gradient profile for the analysis column was utilized at a flow rate of 75 μL min^1. Initially, the mobile phase consisted of 98% A and 2% B. At 3 min the gradient was stepped to 80% A and 20% B over the next 7 min. After 10 min, the gradient was stepped to 75% A and 25% B over the next 5 min and then held constant for 2 min. After 17 min total the gradient was stepped to 2% A and 98% B for 7 min to clean the column, then stepped to 98% A and 2% B for the next 2 min to begin equilibrating the column to initial conditions. The isocratic gradient for the regeneration column utilized a 50 μL min^1 flow rate and consisted

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of a constant eluent composition of 98% A and 2% B. The total analysis run time was 36 min.

The column eluent was introduced into a Thermo Scientific Vantage TSQ triple quadrupole tandem mass spectrometer with an electrospray interface (Thermo Scientific). The instrument was operated in positive ion multiple reaction monitoring mode. The precursor → fragment ion quantitative transitions were *m/z* 416.7 → 619.3, m/z 489.3 → 731.4, and m/z 624.8 → 774.4 for the native peptides and m/z 420.2 → 626.4, m/z 492.8 → 738.4, and m/z 628.3 → 781.5 for the corresponding isotopically labeled peptides. For each peptide, one additional transition was monitored for confirmation purposes (Table 1). Instrument parameters were as follows: spray voltage 4000 V, sheath gas 4, auxiliary gas 2, capillary tube temperature 270 °C, and collision gas pressure of 1.5 mTorr. Collision energies and tube lens settings were optimized for each peptide. Instrument control was performed via the Thermo Scientific Xcalibur software.

2.8. Data analysis

In general, two independent IC-IDMS digest preparations were analyzed in duplicate alongside two independent IDMS digest preparations, also analyzed in duplicate. General practice for selecting peptides and peptide standards for protein quantitation by mass spectrometry and the standard rules and guidance for optimization of proteolytic MS-based assays have been described by others [30]. Three unique peptides were quantified independently to ensure completeness of digestion in our targeted HA regions [31]. Data acquired on the triple quadrupole mass spectrometer was analyzed and processed by Thermo Scientific Xcal-ibur software. Typical ICIS peak integration settings were smoothing width of seven points, area noise factor of 10, peak noise factor of 5, and a tailing factor of 2. Peak integrations were reviewed manually, and transitions from analyte peptides were confirmed by having the same retention times of the heavy stable isotope-labeled peptides. Linear regression was performed without weighting in Xcalibur on the native:labeled peak area ratio versus expected HA concentration to construct response curves. The most abundant transition for each peptide was selected as the quantitative transition to be used in quantitative and statistical analyses. The values obtained using the three peptides' quantitative responses were then averaged. Typical results are presented in Table 2. Percent immunocapture recoveries for HA are calculated as mean immunocaptured H7 concentration divided by total H7 concentration multiplied by 100 (IC-IDMS/IDMS* 100). Percent coefficient of variation is expressed as percent relative standard deviation (standard deviation divided by the mean multiplied by 100).

3. Results and discussion

Immediately after avian influenza A(H7N9) virus infections in people were reported in China, and the US public health authorities initiated pre-pandemic vaccine manufacture, we rapidly launched an effort to support vaccine potency testing and formulation. To this end, we procured mAbs against other H7 influenza strains that would likely be cross-reactive to H7 HA in the newly circulating A/ Shanghai/2/2013 (H7N9) virus. We then evaluated their abilities to affinity capture active H7 in the A/Shanghai/2/2013 virus by ICIDMS. Previous

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characterization by CDC's Influenza Division of mouse mAbs (FR-543, FR-544, FR-545, FR-546, FR-547) to recH7 (A/Netherlands/219/2003) (H7N7) demonstrated reactivity with the globular head of HA1 by indirect ELISA, suggesting the epitopes are located on the HA1 domain (data not shown) [32]. A/Chicken/ Malaysia/94 (H7N1) mAbs 62 and 98, neutralizing mouse mAbs, display high hemagglutination inhibition (HI) activity as well as strong neutralizing activity against H7 avian influenza viruses [33]. Neutralizing mAb H5.31, a human hybridoma IgG mAb directed against the HA head domain, was generated from an A/Vietnam/1203/2004 H5N1 vaccine trial donor [34]. Mab H5.31 broadly binds to group 1 and group 2 influenza HAs and is also cross-reactive with recombinant H7 HAs from A/Netherlands/219/2003 (H7N7). Human antibody A533 (IgG1) bound to H7 strains A/Netherlands/219/2003 (H7N7), A/Shanghai/2/2013 (H7N9) and A/Anhui/1/2013 (H7N7) whereas F105 (scFv-Fc) bound to several H3 strains as well as A/Netherlands/213/2003 (H3N2) by ELISA. Neutralizing H7N7 mouse mAbs 46/6, 55/2, and 58/2 were generated against A/Seal/Massachusetts/1/1980, the first documented avian influenza virus to replicate in and cause disease in mammals [35], with clones 55/2 and 58/2 both binding to an overlapping antigenic site and 46/6 binding to a different antigenic site [35]. These anti-H7N7 mAbs display high binding affinities, exhibit HI activity in intact purified virus, allantoic fluid and isolated HA, and have been found to be cross-reactive to A(H7N3) and A (H7N8) strains of avian and equine origin as well as to A/Singapore/1/1957 (H2N2) [35–37].

Following mAb procurement and prior to receiving A/Shanghai/2/2013 vaccine preparations, our laboratory preliminarily screened the mAb panel against purified recH7 from A/Netherlands/219/2003 (H7N7). In April of 2013, several weeks following the initial human cases, screening of HA from allantoic preparations allowed our laboratory to immediately assess the mAb panel against the variant H7 virus. In May of 2013, upon availability of inactivated A/Shanghai/2/2013 purified whole virus preparations, the mAb panel performance was reassessed. Conserved tryptic peptides from both HA1 and HA2 subunits were selected as stoichiometric representatives of the HA protein and quantified against the corresponding spiked isotopologue internal standard to yield a measure of protein concentration under the assumption that one mole of peptide equals one mole of protein. Table 3 presents mean percent immunocaptured recoveries by viral preparation as analyzed during early H7N9 onset, summarizing the ability of each mAb to immunocapture H7 in the available relevant matrices. A broad range of H7 HA capture efficiency was observed among mAbs, spanning from <1% to 67% for A/Netherlands/219/2003 (H7N7) recombinant HA preparations, 4% to 83% for A/Shanghai/2/2013 allantoic fluid preparations, and 2% to 88% for A/Shanghai/2/2013 purified virus preparations. Trends were observed among antibodies' particular affinities to recH7 HA from A/Netherlands (H7N7) and to A/Shanghai/2/2013's allantoic fluid and purified virus matrices (H7N9). For example, mAbs 46/6, G3 A533, and 55/2 that recovered the highest percentages of H7 in recH7 HA from A/Netherlands (H7N7) also recovered high percentages of H7 HA in A/Shanghai/2/2013's allantoic fluid and purified virus matrices (H7N9). Similarly, mAbs FR-543, FR-544, FR-546, and H5.31 that captured low amounts of H7 HA in recH7 HA from A/Netherlands also isolated minimal H7 HA from A/Shanghai/2/2013 allantoic fluid and purified virus matrices. These data suggest that select mAb responses to A/Shanghai/2/2013 (H7N9) can potentially be inferred by their

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performance to A(H7N7) A/ Netherlands. Further examination of emerging H7N9 strains is warranted to determine if A/Netherlands's reactivity is in any way predictive of H7N9 viral responses. Of interesting note, mAbs 62 and 98 to A/chicken/Malaysia/94 (H7N1), mAbs 46/6, 55/2, and 58/2 to A/Seal/Massachusetts/1/1980 (H7N7) and mAb G3 A533 (H7N7), all exhibited higher capture recoveries to A/Netherlands recH7 than was elicited by the corresponding A(H7N7) strain-matched mAbs FR-543, FR-544, FR-545, FR-546, and FR-547.

For A(H7N9), the mAbs that exhibited the highest H7 antibody capture percentages in allantoic fluid preparations by IC-IDMS, FR545, 46/6, and G3 A533, also had the highest H7 antibody capture activities in the purified virus preparations (Table 3). These mAbs with the highest cross-reactive capabilities to H7 of A/Shanghai/2/2013 were considered to be the most suitable candidates in the event an urgent pandemic vaccine production was implemented, however, it was also well recognized that a large supply of vaccine and reagents would be necessary and would be needed quickly in the event an emergency process was authorized. For this reason, FR-545, which recovered the highest amounts of H7 in both the A/Shanghai/2/2013 allantoic fluid (83%) and A/Shanghai/2/2013 purified virus matrices (88%) by IC-IDMS and that was also available in sufficient quantities through CDC's IRR for wide distribution was selected for further testing. Previous characterization of available A/Netherlands IRR mAbs suggest that FR-545 exhibited the highest binding activity by indirect ELISA and the highest titers by hemadsorption inhibition assay (HADIA) [32], suggesting this mAb has a high binding affinity to the H7 receptor region, further supporting mAb candidacy. Further, to evaluate FR545's ability to specifically immunocapture functionally active H7 HA, we compared isolation of H7 from 'intact' A/Shanghai/2/2013 purified virus samples to A/Shanghai/2/2013 purified virus samples that had been subjected to forced heat degradation conditions. To model loss of potency, triplicate preparations of A/Shanghai/2/2013 virus (500 mL) were heated in a water bath for 24 h at 56 C, immunocaptured by FR-545, digested and quantified for H7 by IDMS. These stressed samples were then assessed alongside triplicate preparations of nonstressed A/Shanghai/2/2013 virus. FR-545 captured >90% (39.1 ± 2.6 mg/μL) of H7 in the unstressed viral samples, indicating the quality of the antibody and H7 protein. In contrast, loss of potency was apparent in the deliberately heat stressed samples, compromising the integrity of the H7 protein such that is was undetectable by our immunocapture method. Analytical sensitivity of the IC-IDMS method allows for HA levels as low as 1.5 mg/μL (≥standard 1; 2 fmol/μL) to be quantified by immunocapture.

Mean total digest concentrations, mean immunocapture digest concentrations, method standard deviations for two complete replicates, and percent immunocapture recoveries for HA peptides in all A/Shanghai/2/2013 matrices tested using FR-545 are presented in Table 4. While viral yields and thus H7 present in the allantoic fluid preparations were expectedly lower in concentration and more complex in composition than the vaccine preparations, we reliably quantified 3.0 μg/mL of total H7 without further purification with a mean H7 mAb capture recovery of 83% (2.5 μg/mL). In contrast to the allantoic fluid, the H7 concentration in the purified virus preparation was exceedingly high, with a total H7 concentration of 1103.3 μg/mL. Despite the wide concentration spread, FR-545's ability to immunocapture 88% of the H7 in the purified virus preparation (969.8 mg/mL) was

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noticeably similar to the percent recovered in allantoic fluid. Continued testing of FR-545 proved that the high antibody binding observed to H7 HA in the initial A/Shanghai/2/2013 (H7N9) viral matrices available for testing, allantoic fluid and purified virus preparations, also translated to subsequent Shanghai/2/2013 matrices, allowing predictions to be drawn early in the timeline based on the mAb's performance in allantoic fluid. In September of 2013, the A/Shanghai/2/2013 whole virus reference reagent required to perform SRID testing was made available by the FDA. By IDMS, the total H7 concentration in the whole viral reagent was found to be 55.5 μg/mL, with IC-IDMS showing high mAb affinity with 99% (55.2 μg/mL) of the H7 immunocaptured. Lastly, in February of 2014, a vaccine manufacturer released an A(H7N9) detergent-split monovalent vaccine bulk preparation which was determined to have a total H7 concentration of 232.9 μg/mL by IDMS, not unexpected as monovalent vaccine bulks are generally much more concentrated than final vaccines, with 92% immunoreactive H7 (213.8 μg/mL) correlating well with FR-545's aforementioned capture abilities.

In October of 2013, the strain-matched polyclonal antiserum necessary to perform the SRID method was released, allowing the regulatory potency assay to be implemented. Subsequently, for completeness, our laboratory reevaluated all A(H7N9) vaccine materials by IC-IDMS using the A(H7N9) reference polyclonal antiserum. Mean total digest concentrations, mean immunocapture digest concentrations, method standard deviations for two complete replicates, and percent immunocapture recoveries for all A/ Shanghai/2/2013 matrices tested by pAb-H7 are presented in Table 4. IC-IDMS results using FR-545 and the corresponding pAb-H7 are relatively similar and agree well, with an overall percent recovery for all matrices of 91% and 92%, respectively, with the largest discordance between FR-545 and pAb-H7 (11%) seen in the allantoic fluid preparation. Percent coefficients of variation between FR-545 and pAb-H7 for the balance of the intermediate vaccine preparations were 7%. Overall, pAb-H7 IC-IDMS results strongly correlate with FR-545 IC-IDMS results for all A(H7N9) vaccine materials, giving further confidence of mAb FR-545's capture selectivity being similar to that of the regulatory polyclonal antiserum's that is specific to active H7. IDMS, FR-545 IC-IDMS, and pAb-H7 IC-IDMS whole virus concentrations of 55.5, 55.2, and 54.1 mg/mL, respectively, all agree reasonably well with the published SRID reference value of 60 mg/mL [27]. By pAb-H7 IC-IDMS our 196.5 mg/mL split vaccine bulk concentration is particularly similar to the potency value of 193.7 μg/mL determined by SRID [38]. Additionally, FR-545 IC-IDMS also provided a comparable result of 213.5 μg/mL of HA. Achieving WHO and FDA approval of an alternative method that measures influenza vaccine potency will require correlation with the regulatory SRID method. Both the mAb IC-IDMS and pAb IC-IDMS results presented in this work demonstrate correlation with SRID and suggest that the H7 HA bound by the antibody-coated beads is representative of those involved in the formation of the measurable precipitation ring in the SRID assay. While continued evaluation is needed, these findings support IC-IDMS as an accurate and precise alternative potency assay to facilitate both pandemic and seasonal influenza preparedness and response.

The advantages of quantifying total HA concentrations by IDMS alone have been previously described by our laboratory for seasonal vaccine, A(H5N1), A(H1N1)pdm09 and more recently in the case of A(H7N9) [12–14]. IDMS accurately, sensitively, and selectively

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quantifies total HA by choosing conserved HA peptides for quantification that can cover the majority of strains throughout a subtype. Prior to the A(H7N9) outbreak, our laboratory had identified conserved HA peptide sequences and prepared peptide standards to A(H7N7) and A(H7N2) strains that are known to have caused human disease in anticipation of future outbreaks. The H7 peptide standards previously chosen for HA quantitation were identified through the National Center for Biotechnology Information (NCBI) Influenza Virus Resource Database. As of July 24, 2013, the STQSAIDQITGK peptide was conserved in 81% of the H7 strains, the VNTLTER peptide was conserved in 76% of the H7 strains, while either the FVNEEALR or FTNEESLR sequence were present in 84% of the H7 strains. These HA peptides also were conserved and applicable for quantitation of HA in H7N9, adding another layer of preparedness contributing to a rapid response. However, in the event A/Shanghai/2/2013 or any novel circulating strain has a sequence variation, this variation can be easily detected and a new peptide standard, along with its corresponding internal standard, can be synthesized and ready for analyses in a matter of weeks, eliminating the current need for lengthy reagent production. By expanding the IDMS platform to include a mAb immunocapture step on the front-end, we provide a preliminary "potency" HA measurement similar to but potentially advantageous relative to SRID in that the mAb-based IC-IDMS methodology incorporates the strengths inherent to IDMS while also eliminating the need for pAb and reagent production as part of the current vaccine delivery process. The method can quantify H7 in various intermediate vaccine matrices as well as vaccine materials produced from different manufacturers. Further, IC-IDMS can quantify HA over a wide range of concentrations, has improved analytical sensitivity to SRID, and can also quantify immunocapture HA in multivalent formulations. Similar to SRID, IC-IDMS is an antibody-dependent method, thus identification of a cross-reactive mAb is central to achieving an abbreviated response. If a potentially pandemic strain emerges in which a cross-reactive mAb cannot be readily identified, our IC-IDMS timeline would be similar to that of SRID, dependent on the production of a suitable monoclonal or on the availability of CBER polyclonal antiserum. Here, several procured mAbs were good candidates for A(H7N9) IC-IDMS. FR-545 was chosen for further IC-IDMS testing based on its previous characterization, stock availability, and initial screening performance by ICIDMS. 46/6 and G3 A533 were also strong mAb candidates but their ability for immediate and wide-spread distribution was limited. Similarly, at the time of IC-IDMS analyses to the emerging pandemic threat, quantities of mAbs 62 and 98 were insufficient. However, the well-defined cross-reactivity of mAbs 62 and 98 to H7 subtypes makes them ideal candidates and a follow-up IC-IDMS study confirmed these mAbs had significantly high capture abilities to all A/Shanghai/2/2013 (H7N9) matrices (unpublished data). Recently, an important H7 antigenic site A was identified in A (H7N9) [39]. This antigenic site A was found to be structurally similar to H3 HA's antigenic site A, is relatively well-conserved among H7 virus isolates, and is suggested to be an important protective epitope with potential benefits of cross-reactive antibody responses [39]. The conservation of this site, the observed cross-reactive H7 antibody responses, the existing availability of monoclonal antibodies to A(H7N9) and recent advances in discovery or engineering of broadly neutralizing monoclonals [40,41] and the continued advancements in analytical technologies are all important advances in guiding H7 pandemic vaccine potency assay development and implementation.

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4. Conclusions

Prior to its detection in human respiratory disease cases in China, the novel A/Shanghai/2/2013 (H7N9) virus had not been previously seen in animals or humans. To date, human-to-human transmission of A(H7N9) has not been reported. Still, the high pathogenicity of A(H7N9) to humans, with a current case-fatality rate of approximately 37%, has the potential to greatly impact global health [42]. This threat continues to grow as a consequence of increases in the volume, speed, and reach of global travel [43]. Influenza vaccine production is a complex process that involves execution of several production steps and expert estimates indicate that it would take at least six months for initial vaccine to be released by standard methods [44]. For A(H7N9) preparedness, IC-IDMS was implemented and showed promise as a surrogate potency assay capable of providing "real-time" immunoreactive HA quantitation and eliminating the need for pAb and reagent production, which could provide an essential time-saving advantage as part of the current vaccine delivery process. Had an A(H7N9) influenza pandemic emerged or in cases of future pandemics or emerging pandemics, IC-IDMS proves to have the necessary attributes to be considered as an alternative method for rapid and accurate potency determination of the HA antigen in influenza vaccine preparations and to rapidly evaluate antibodies to be used as capture antibodies for other alternate potency assays.

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Table 1

Target H7 peptides employed for quantitation of hemagglutinin (HA).[a]

Target peptide	Influenza protein	Precursor ion m/z	Fragment ion (quantitation)	Fragment ion (confirmation)
VNTLTER	H7	416.7 (+2)	619.3 (y5)	733.4 (y6)
VNTL_TER	H7	420.2 (+2)	626.4 (y5)	740.4 (y6)
FVNEEALR	H7	489.3 (+2)	731.4 (y6)	830.4 (y7)
FVNEEAL_R	H7	492.8 (+2)	738.4 (y6)	837.5 (y7)
STQSAIDQITGK	H7	624.8 (+2)	774.4 (y7)	661.4 (y6)
STQSAIDQ_ITGK	H7	628.3 (+2)	781.5 (y7)	668.4 (y6)

[a]Underlined amino acids correspond to ^{13}C- and ^{15}N-labeled amino acids. H7, hemagglutinin of H7N7 and H7N9 influenza strains.

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Table 2

Amount of H7 Determined by mAb FR-545 IC-IDMS using three individual peptides from two lyophilized whole viral preparations with duplicate analytical replicates (fmol/μL and% HA immunocaptured).[a]

	A/Shanghai/2/2013 (H7N9)						
	H7 Peptide	**Prep 1/Run 1**	**Prep 2/Run 1**	**Prep 1/Run 2**	**Prep 2/Run 2**	**Average (fmol/μL)**	**Percent (%) H7 Recovered**
IC-IDMS	VNTLTER	58.7	60.5	57.3	49.0	66.7 ± 8.6	94%
	FVNEEALR	77.5	68.7	72.5	76.3		
	STQSAIDQITGK	66.4	71.3	68.7	72.9		
IDMS	VNTLTER	54.3	58.0	68.6	65.2	71.0 ± 8.8	
	FVNEEALR	81.0	75.9	79.8	80.2		
	STQSAIDQITGK	77.4	69.3	66.9	75.0		

[a]The average and standard deviation among the peptides are calculated using each peptide and each replicate run as independent measurements (n = 12 for IC-IDMS and n = 12 for IDMS).

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Table 3

Assessment of monoclonal performance to purified recombinant H7 HA from A/Netherlands/219/2003 (H7N7) and to H7 in A/Shanghai/2/2013 (H7N9) inactivated allantoic and purified virus preparations (% HA immunocaptured).

H7N7 rNetherlands/219/2003[a]			H7N9 A/Shanghai allantoic fluid[b]			H7N9 A/Shanghai purified virus[c]		
mAb	**Subtype**	**% H7 Captured**	**mAb**	**Subtype**	**% H7 Captured**	**mAb**	**Subtype**	**% H7 Captured**
46/6	H7N7	67	FR-545	H7N7	83	FR-545	H7N7	88
G3 A533	H7N7	57	46/6	H7N7	78	46/6	H7N7	66
55/2	H7N7	56	G3 A533	H7N7	68	G3 A533	H7N7	65
98	H7N1	56	55/2	H7N7	58	FR-547	H7N7	54
62	H7N1	54	58/2	H7N7	38	G1G3 F105	H3N2	41
58/2	H7N7	47	98	H7N1	25	FR-546	H7N7	33
FR-545	H7N7	33	62	H7N1	22	FR-544	H7N7	29
FR-546	H7N7	26	FR-547	H7N7	13	55/2	H7N7	26
FR-547	H7N7	25	H5.31	H5N1	10	58/2	H7N7	9
H5.31	H5N1	21	FR-544	H7N7	10	H5.31	H5N1	3
FR-543	H7N7	12	G1G3 F105	H3N2	8	FR-543	H7N7	2*
G1G3 F105	H3N2	11	FR-546	H7N7	8	98	H7N1	QNS
FR-544	H7N7	<1*	FR-543	H7N7	4*	62	H7N1	QNS

[a] Purified recombinant influenza H7 HA from influenza A virus (H7N7) Netherlands/219/2003.

[b] BPL-Inactivated influenza A virus (H7N9) Shanghai/2/13 in allantoic fluid.

[c] BPL-Inactivated influenza A/Shanghai/2/13 purified virus (H7N9).

* Below standard 1 (2 fmol/mL).

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Table 4

Amount of H7 immunocaptured using mAb FR-545 Anti-H7N7 and CBER pAb Anti-H7N9 in various A/Shanghai/2/2013 (H7N9) vaccine preparations (mg/mL and % HA immunocaptured).

Identifier	SRID value	mAb Anti-H7N7 IC-IDMS Digest (mean ± st dev)	pAb Anti-H7N9 IC-IDMS Digest (mean ± st dev)	IDMS Digest (mean ± st dev)	Percent (%)H7 Recovered mAb FR-545 Anti-H7N7	Percent (%)H7 Recovered CBER pAb Anti-H7N9
A/Shanghai/2/2013 (H7N9) allantoic fluid preparation	<LOD	2.5 ± 0.30	2.9 ± 0.64	3.0 ± 0.20	83%	97%
A/Shanghai/2/2013 (H7N9) purified whole virus preparation	QNS	969.8 ± 83.8	968.2 ± 115.9	1103.3 ± 29.3	88%	88%
A/Shanghai/2/2013 (H7N9) lyophilized whole virus preparation	60.0	55.2 ± 4.8	54.1 ± 5.8	55.5 ± 9.0	99%	97%
A/Shanghai/2/2013 (H7N9) split monovalent vaccine preparation	193.7	213.8 ± 18.8	196.5 ± 24.4	232.9 ± 32.3	92%	84%