

# Confronting the next pandemic—Workshop on lessons learned from potency testing of pandemic (H1N1) 2009 influenza vaccines and considerations for future potency tests, Ottawa, Canada, July 27–29, 2010

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## Introduction

The 2009 H1N1 influenza pandemic created an urgent need to expedite vaccine manufacture, bringing to light some of the challenges associated with the methods and processes used in the regulatory release of influenza vaccines. The release of vaccines on a lot-by-lot basis is required by most national regulatory authorities to confirm that distributed product meets pre-approved specifications. The single radial immunodiffusion (SRID) assay has been the standard assay to measure the potency of influenza vaccines for decades. In the context of a public health emergency, the suitability of the SRID assay has been questioned, and during the 2009 H1N1 influenza pandemic, alternative approaches were used in some cases at the beginning of the campaign for product release.

A workshop was jointly organized by Health Canada, the United States Food and Drug Administration (FDA), and the World Health Organization (WHO) in July 2010 with the specific goals to exchange knowledge and experience gained in vaccine release around the world throughout the 2009 influenza pandemic; formulate plans to address gaps in our knowledge about the use of alternative approaches to assess the potency of influenza vaccines; and identify ways forward on possible incorporation of such assays into pandemic and seasonal influenza vaccine regulatory decision making.

Scientists from national regulatory agencies, national control laboratories, research institutes, academia, WHO, and the vaccine industry were invited to attend. The meeting was structured as a series of short presentations followed by open discussion. Day three consisted of a closed session that excluded individuals with conflicts of interest.

This publication contains the collective views of meeting participants and does not necessarily represent policies or recommendations of the WHO.

## Lessons learned from the pandemic (H1N1) 2009 experience

### Testing for potency

Dr. Robert Webster opened the session with an overview of the advantages and shortcomings of influenza vaccine characterization, highlighting the need for surveillance of influenza in swine to identify new strains with pandemic potential. Dr. Othmar Engelhardt (National Institute for Biological Standards and Control), Dr. Rajesh Gupta (FDA), Dr. Michael Pfeleiderer (European Medicines Agency), Dr. Junzhi Wang (National Institute for the Control of Pharmaceutical and Biological Products (NICPBP)), and Mr. Tony Colegate (International Federation of Pharmaceutical Manufacturers and Associations) then presented on lessons learned from potency testing of H1N1 2009 pandemic influenza vaccines using SRID and alternative methods.

The SRID assay is based on diffusion of virus antigen through agarose gel containing hemagglutinin (HA)-specific antibodies and subsequent formation of an antigen–antibody precipitate, with the area of the zone of precipitation proportional to antigen concentration. An overview of this method and its history is included in a review by Williams.<sup>1</sup> In 1979, the WHO recommended that SRID be used to standardize influenza vaccine potency and it has been used ever since.

Advantages of the SRID assay are that it measures the quantity of antigenic HA and has been shown to correlate with HAI titer and vaccine efficacy, although it is unclear whether correlation is maintained with all strains.<sup>2–4</sup> The assay is simple and cheap and is type and subtype specific. Disadvantages of SRID include the time required to produce the reference reagents (6–8 weeks). In addition, the assay is unsuitable for certain adjuvanted vaccines, is not sensitive enough for very low-dose vaccines, and does not lend itself to automation.

Generation and calibration of SRID reference reagents is a complex and time-consuming process and was a challenge to accomplish in a short time frame during the H1N1 2009 pandemic. In addition, there were difficulties in the purification of HA from the 2009 H1N1 virus that is used to immunize sheep as a source of reference antiserum. This was further complicated because the initial candidate vaccine virus had a low yield. A new strain had higher yield; however, strain-specific reference reagents were not immediately available. While homologous reference antigen is important for specificity of the assay,<sup>5</sup> the FDA allowed the use of heterologous reagents that were appropriately re-calibrated for the new strain to expedite vaccine availability. When homologous antigen became available, it was demonstrated that similar results were obtained with this standard and the re-calibrated reagents.

In some countries, H1N1 vaccine clinical trial materials were produced by the end of June 2009, while the H1N1 virus-specific reference reagents only became available at the end of July 2009. Alternative methods such as reverse-phase (RP) HPLC or SDS-PAGE were used in some cases to quantify HA for early lot release and immunogenicity studies. SRID is indicative of native HA structure<sup>6</sup> and preliminary studies show that methods which measure the absolute quantity of HA, such as HPLC or SDS-PAGE, do not accurately measure antigenicity and stability and do not distinguish between native and denatured forms of HA.

European Medicines Agency (EMA) guidelines for pandemic influenza vaccines encourage the development of alternative methods for antigen standardization to bridge the phase when no such reagents are available. During the development of H5N1 mock-up vaccines, several companies presented concepts for alternative potency testing. However, the availability of SRID reagents, absence of guidance, and lack of coordination to develop new methods decelerated or stopped these research initiatives, and all pivotal H5N1 trials have been performed based on SRID potency data. Only one clinical trial of monovalent pandemic H1N1 vaccine was initiated based on potency determination using HPLC as an alternative method in Europe. Retesting with standard reagents demonstrated an underestimation of HA content, so there were doubts whether these data should be used to recommend a single dose regimen. All other trials started after SRID reference reagents became available.

In China, the State Food and Drug Administration (SFDA) first granted regulatory registration to pandemic (H1N1) 2009 vaccines in August 2009. This was approximately 1 month earlier than any other country. Early approval was because of the use of alternative methods that were established as part of H5N1 vaccine development efforts, including virus strain evaluation using RT-PCR and determination of HA content by SDS-PAGE and total

protein measurement. All ten manufacturers of pandemic (H1N1) 2009 influenza vaccine in China applied SDS-PAGE to measure HA content. Results generated from SDS-PAGE achieved 88–120% consistency with those from traditional SRID methods.<sup>7</sup> China may use SDS-PAGE in a future emergency situation, although it is recognized this method has limitations because it is not indicative of native protein structure.

While SDS-PAGE was proven valuable in the initial assessment of monovalent pandemic vaccine lots, it is not suitable for trivalent vaccines because of the inability of the method to distinguish between types and subtypes.

### Testing for stability

Observations from multiple manufacturers and regulators suggest that the pandemic (H1N1) 2009 influenza strain is less stable than most seasonal strains. The root cause of the poor stability is unknown and investigations are ongoing. Potential contributing factors include the inherent structural properties of the A/California/07/2009-like HA[8]\* the formation of aggregates unique to this strain, or the potential effect of total protein concentration, inactivating agents, or vaccine preservatives on stability.

In Canada, real-time stability data supporting an 18-month shelf life for an H5N1 mock-up vaccine (GSK) were used to justify a preliminary 18-month shelf life for the Arepanrix H1N1 antigen, with a post-market commitment to provide ongoing accelerated and real-time testing data to confirm the shelf life. Analysis of field samples showed HA levels below the level predicted for an 18-month shelf life. No irregularities in cold chain management or sample handling were detected and the issue did not appear to be lot-specific. The impact of transportation has not been ruled out as a potential cause of the loss of potency. Based on data collected post-authorization, Health Canada determined that the shelf life should be revised to 6 months. Collection of real-time stability data is ongoing.

In the United States, strain change supplements to existing seasonal influenza vaccine licenses were approved for the pandemic (H1N1) 2009 monovalent formulations. Data to support vaccine stability were not required for approval, and shelf life was expected to be the same as the seasonal licensed vaccine. However, problems with vaccine stability resulted in various recalls or field corrections of some inactivated as well as live attenuated pandemic (H1N1) 2009 influenza vaccine lots between December 2009 and February 2010.

Potential instability of pandemic (H1N1) 2009 monovalent vaccine raises concern regarding stockpiled H5N1 vaccines. Long-term stability results for stockpiled H5N1 pandemic influenza vaccines in the United States suggest

\*Data published online January 2011.

that there are product- and strain-specific differences in stability. A study to compare an enzyme-linked immunosorbent assay (ELISA) with SRID as potency assay is ongoing in the laboratory of Dr Jerry Weir (FDA) and will evaluate whether these methods correlate with *in vivo* measures of immunogenicity.

## Alternative assays for evaluation of quality

### Biological/immunological assays

Antisera used to evaluate vaccine composition are usually strain and subtype specific to facilitate the analysis of trivalent vaccine formulations. In contrast, pandemic vaccines are likely to be monovalent, as was the case for the (H1N1) 2009 vaccine. Evaluation of the quality and total concentration of HA or NA in these vaccines may be expedited through the use of antibodies to conserved regions of these antigens. A collaborative study between Health Canada, Canada's National Microbiology Laboratory, and China's SFDA/NICPBP was conducted to generate antibodies to conserved HA and NA peptides that can easily be applied to analyze vaccine content. Antisera and monoclonal antibodies generated in response to immunization with conserved peptides bound to virtually all subtypes of HA and NA and could be used in a variety of immunoassays, such as ELISA, slot blot, and Western blot.<sup>9,10</sup> The immunoassays allow the quantification of the total amount of either HA or NA in a vaccine sample but do not distinguish between different subtypes nor necessarily distinguish between biologically active and inactive antigen. Assays with these cross-reactive antibodies have practical value for quality control testing of in-process and monovalent bulk samples because there is no need to generate new strain-specific reagents.

Antibodies that inhibit NA activity reduce virus titers and disease symptoms in animal models of highly pathogenic viruses.<sup>11,12</sup> Even though clinical studies have demonstrated NA-inhibiting (NI) titers correlate with vaccine efficacy,<sup>13,14</sup> routine measurement of antibodies with this specificity is often not performed as the traditional NA inhibition assay, the thiobarbituric acid (TBA) method,<sup>15</sup> is not practical for routine use. To consider NA as a relevant vaccine antigen from a regulatory perspective, a practical assay to measure responses to NA has to be developed. A miniaturized TBA method has been developed as well as an enzyme-linked lectin assay (ELLA).

Currently, there is no requirement to determine the amount of NA in vaccines. While licensed vaccines contain NA, recent data show that the amount present in vaccines from different manufacturers using the same vaccine seeds may not be the same and can even vary among different lots from the same manufacturer.<sup>16</sup> Multiple factors may

account for low levels of NA in split-virion-inactivated vaccine preparations, including the viral strain, and different manufacturing processes. A method to measure the potency of NA is therefore also needed. This method should quantify native conformation of NA because correct tertiary structure is required for the induction of NA-inhibiting antibodies. Potency assays that are being considered include the measurement of enzyme activity or quantitation of NA with native conformation by antibody-independent mass spectrometry (MS)-based or antibody-dependent ELISA methods.

### Biophysical analysis

At Health Canada, work is ongoing to further evaluate the potential of HPLC for the assessment of influenza vaccines and to provide qualitative and quantitative measurements for specific constituents. Size-exclusion (SE) HPLC provides a profile of protein components in vaccines according to protein size and has been used by Health Canada for research purposes to analyze seasonal vaccines. Size-exclusion-HPLC of multiple lots provides an indication of the production process reproducibility. The method is highly reproducible and can be applied to bulks and vaccines directly without modification of samples.

RP-HPLC provides a profile of protein components in vaccines according to hydrophobicity. This method has been used to provide accurate absolute amounts of HA in both monovalent and trivalent formulations. RP-HPLC still needs a reference antigen to quantitate HA. RP-HPLC can distinguish HA1 from different strains as these have different elution profiles.

Two-dimensional (2D) HPLC combines SE and RP chromatography, providing the highest level of selectivity and quantification of HA1.<sup>17</sup> During the 2009 H1N1 pandemic, Health Canada monitored total HA concentrations in vaccine using a combination of SE-HPLC and RP-HPLC in conjunction with SRID during investigations of product instability.

MS-based methods can be used for characterization and quality control of influenza vaccines. Health Canada is working on the development of proteomics-based MS methodology to identify proteins present in influenza vaccines. Although standard HPLC and MS-based methods do not provide assurance of correct folding or antigenic structure of HA, these tests provide important analysis of product identity and quality<sup>18,19</sup> but do not indicate whether the product is stable or potent.

MS-based methods are also being investigated in the United States to improve the current reagent calibration process, which currently takes several weeks. This process requires the determination of the HA concentration in a primary liquid standard by determining total protein concentration and determination of the proportion of HA

present in the standard by densitometry of protein bands separated by SDS-PAGE. Within the United States, a multi-center collaboration (National Center for Environmental Health, CDC, and FDA's Center for Biologics Evaluation and Research (CBER), and Center for Food Safety and Applied Nutrition) has been initiated to evaluate the use of isotope-dilution MS to determine the absolute quantity of HA in primary influenza standards.<sup>20</sup> If correlated with current methods, the time necessary for calibration could be reduced from weeks to days.

MS-based methods to evaluate vaccine stability or potency tests are also being developed. The US CDC has used reference antiserum bound to beads as a means to extract HA with native conformation from vaccine. The amount of HA bound to these complexes can then be measured by isotope-dilution MS. Other MS-based methods that do not require the use of HA-specific antiserum to determine the relative amount of folded and unfolded HA present in vaccine are being developed as potential stability-indicating assays. These methods as well as other platforms such as ELISA and surface plasmon resonance are being investigated as alternative potency assays at CBER, FDA, and other institutions.

In addition to expediting calibration of reference antigen as described earlier, other approaches to improve the SRID assay were discussed during the workshop. This includes generating HA-specific antiserum by preparing antigen for immunization of sheep in a manner that does not require purification of the antigen from whole virus. Antiserum generated in response to DNA and recombinant vaccinia was recently tested.<sup>21</sup> Alternatively, a panel of monoclonal antibodies could be used as a source of reference antibodies, with the expectation that a specific antibody combination would be relevant for several seasons.

Overall, different methods may be useful for different purposes. Manufacturers encouraged the standardization/harmonization of SRID as a first step, urging regulators to agree to use one set of reagents and the same method regardless of the market.

## Conclusions and recommendations

The workshop concluded with the formulation of several recommendations regarding improved assessment of vaccine potency by SRID, the development and use of alternative approaches to assess potency of influenza vaccines, and how new assays might be incorporated into influenza vaccine regulatory decision making. The influenza vaccine industry is looking to national regulatory authorities and Essential Regulatory Laboratories (ERLs) for direction in these areas. New methods are more likely to be adopted by industry if they are low cost, not labor intensive, high throughput (preferably automated), high specificity, stabil-

ity indicating, and indicative of antigenic structure and vaccine efficacy.

In some cases, alternative methods were used during the 2009 H1N1 pandemic until SRID reference reagents became available or as part of special investigations of product quality and stability. While knowledge and technologies are expanding, more research and confidence building are needed before these alternative approaches can be incorporated into routine decision making.

As a first step, it was recommended that improvements be made to the SRID assay. This can potentially be accomplished by using one assay method and one set of reagents for each strain, by improving the method used to calibrate reagents, and by harmonizing SRID calibration across markets and continents. This may require generating larger amounts of reference antigen, potentially through a contract laboratory. It was recommended that at the next ERL meeting (scheduled for January 2011), the participants explore ways to improve reference antigen production, calibration, and quality control.

Various promising alternative *in vitro* methods are being explored, including several types of HPLC, ELISA, SDS-PAGE, MS, and surface plasmon resonance. Continued research is needed to determine how these techniques are best used, for example, as alternatives to SRID, as supplemental techniques, as in-process controls or for stability testing. The advantage of some of these methods is that they are antibody independent and thus can be used without delays owing to reagent preparation. It was recommended that priority be given to continuing research and development of these and other promising approaches. In the context of supporting implementation of new potency assays, it may be possible to use SRID to bridge new assays that measure antigenicity of HA; however, novel assays may require testing in animal models and clinical trials to demonstrate that results correlate with immunogenicity.

To keep momentum toward development of alternative assays and to better coordinate and monitor research and development efforts, participants recommended that venues be identified for the purpose of communicating progress on the evaluation of alternative methods.

The focus of the workshop was on potency assays for licensed inactivated influenza vaccines; however, research and development of new assays should also address the need for the assessment of the potency of new generation vaccines, such as adjuvanted vaccines, cell culture-derived vaccines, recombinant protein-based vaccines, and virus-like particles.

This workshop was an important step toward developing a global understanding of alternative methods for potency testing that could potentially expedite the availability of seasonal and pandemic influenza vaccines and for identifying ways to move forward collaboratively.

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