Process of influenza vaccine virus selection and development

Seasonal

Collection of specimens and disease/epidemiological data

Diagnosis, virus isolation in MDCK, preliminary analysis

Ferret antisera production

Thorough antigenic and genetic analysis

Review and selection of candidate viruses for vaccine use

Classical reassortment of high-growth viruses for H1N1 & H3N2

Reassortment of high-growth viruses using reverse genetics (and full safety testing)

Antigenic and genetic characterization of reassortants

Development of standardized reagents for inactivated vaccines

Evaluation of growth property

Development of standardized reagents for inactivated vaccines

Antigenic and genetic characterization of reassortants

Availability of vaccine viruses and standardized reagents
Introduction

Influenza vaccine is the best available protection against the disease. Among all vaccines, however, the process of making influenza vaccines is considered uniquely complicated and difficult. One reason is that the constantly evolving nature of influenza viruses requires continuous global monitoring and frequent reformulation of the vaccine strains. Another reason is that the rapid spread of these viruses during seasonal epidemics, as well as the occasional pandemic, means that each step in the vaccine process must be completed within very tight time frames if vaccine is to be manufactured and delivered in time. In response to the realities imposed by influenza, a highly functional process has evolved over decades in which the public and private sectors work together to develop and produce influenza vaccine.

This paper, and the accompanying Diagram, describe many aspects of the influenza vaccine process including important similarities and differences in how seasonal human influenza viruses and H5N1 viruses are handled.

Background

The global monitoring of influenza viruses is essential for identifying influenza threats (risk assessment) and providing the information needed by countries to respond to these threat. In practice, the global link between risk assessment and risk response activities has long been the Global Influenza Surveillance Network (GISN), which continues to grow and currently includes 121 National Influenza Centres (NICs) in 92 countries and 5 WHO Collaborating Centres (WHO CCs). Together, this network processes an estimated 150,000 to 200,000 respiratory specimens per year with approximately 5000 viruses voluntarily shared by the NICs undergoing extensive antigenic and genetic characterization among the WHO CCs. The information provided by GISN through its participating institutions has identified new influenza threats, substantially helped define the epidemiology of influenza and the molecular evolution of the viruses, and formed the basis for selecting new influenza vaccine strains and for updating diagnostic tests. Moreover, the international infrastructure of GISN has provided a platform through which the world's best influenza expertise can be brought together quickly and coordinated to help countries and WHO address influenza.

Since 1971, WHO has provided formal recommendations for the composition of seasonal influenza vaccines based on the information provided by the GISN. In 1998, the WHO recommendations were increased in frequency from once to twice per year so that separate recommendations could be made and timed appropriately for the northern and southern hemisphere influenza seasons. Since the development and production of influenza vaccines requires several months, these recommendations precede the period of anticipated use by up to eight months. Some of the roles of GISN in this regard is to enable the pooling of worldwide...
information on influenza viruses and to provide the infrastructure for experts to examine all relevant antigenic, virological and immunological data. The results of that data examination in turn allow these experts to select candidate vaccine viruses that might be included -- if they pass additional testing -- in the following season's vaccines. While the process is difficult, complex and highly resource demanding, it remains manageable because countries and other entities have worked together collaboratively.

Before 2004, GISN primarily focused on seasonal influenza viruses for vaccine development but since 2004, has expanded its scope to include H5N1 viruses because this virus constitutes an unusually serious pandemic risk. Therefore, WHO now reviews the available antigenic and genetic data on animal and human H5N1 viruses in addition to the analyses of seasonal vaccine stains and has developed and made H5N1 candidate vaccine viruses available to vaccine producers so they can work on developing safe and effective human H5N1 vaccines for potential production. Some of these H5N1 candidate vaccine viruses have been used by manufacturers to produce human influenza H5N1 vaccines, some of which have been tested in clinical trials.

The entire process of influenza vaccine virus selection and development is possible and successful because it represents a true collaborative effort, involving all members in GISN as well as several other entities including three national regulatory laboratories in the Australian Therapeutics Goods Administration (TGA), the United Kingdom's National Institute of Biological Standards and Control, (NIBSC), and the United States' Food and Drug Administration (FDA), which provide essential input and services to the process, some contract laboratories that do highly specialized parts of the process for seasonal vaccines (but not H5N1 vaccines) and vaccine manufacturers.

The time constraints under which all these parties operate are great because of the epidemiology of influenza and steps that must be completed for vaccine production to take place. Once influenza viruses appear, either as seasonal outbreaks or, in the case of certain viruses possibly as a new pandemic, the spread of disease can be very quick. Given this reality, even short delays anywhere in the process can result in significant downstream delays in the availability of influenza vaccines.

Recent Development
In June 2007, a revised set of International Health Regulations (IHR) entered into force. The IHR creates a global, collective system to enable WHO to 1) identify public health threats; 2) assess their risk; and 3) put in place effective public health responses to mitigate their impact. By accepting to be bound by the IHR, Member States accept to participate and cooperate in this global system. The IHR is disease specific for only 4 diseases. One of them is human influenza caused by a novel virus (i.e., a virus that has the potential to cause a pandemic). As such, any case of human influenza by a virus such as H5N1, must be reported to WHO under the IHR. Each notification must then be followed up with sufficiently detailed information to allow WHO to thoroughly assess whether the new case signals the start of a pandemic. Under the IHR, WHO is responsible not only for the assessment of risk but also, importantly, to put in place a public health response. When it comes to influenza, a critical response is the development, production, availability and access to influenza vaccines. The rapidity with which vaccines can be developed will depend on access to the actual virus.
List of acronyms and abbreviations used in this report

BSL3 - Biosafety Level 3
CRADA - Collaborative Research And Development Agreement
CSL - CSL Limited
FDA - Food and Drug Administration, United States
HA - Hemagglutinin
MDCK - Madin-Darby Canine Kidney Cells (virus tissue culture)
NA - Neuraminidase
NIBSC - National Institute of Biological Standards and Control, United Kingdom
NIC - National Influenza Centres
NYMC - New York Medical College
PCR - Polymerase chain reaction
PR8 - A/Puerto Rico/8/34
TGA - Therapeutic Goods Administration, Australian Government
WHOCC - WHO Collaborating Centre on influenza

Step by step explanation of the flow diagram
In this section, each "step" corresponds to a number in the flow diagram.

Step 1. Collection of clinical specimens and disease/epidemiological data from patients - year round
- Conducted by health care workers, surveillance or National Influenza Centre staff

Public health relies on surveillance to identify and assess all types of important health risks. For influenza, surveillance consists of collecting specimens and the related epidemiological data, assessing the information to identify health threats such as the emergence of new seasonal virus variants or viruses that could cause a pandemic, and then providing the information so it can be used to respond to the threat. One critical use of the information derived from the study of the viruses and the related epidemiological data is to select influenza vaccine viruses.

Global influenza virus surveillance is a year round activity which begins with the collection of clinical specimens from patients with influenza symptoms. Within a country with influenza surveillance, some of these clinical specimens and the relevant clinical and epidemiological information are sent to a country's National Influenza Centre (NIC) or perhaps another national influenza reference laboratory. NICs are designated by national authorities and recognized by WHO. They have formal Terms of Reference with WHO for participating in GISN and are an integral and essential component of the Network.
Step 2. Diagnosis, virus isolation and preliminary analyses ~ hours - 3 weeks
- Conducted by NICs and other national influenza reference laboratories

As part of their seasonal influenza virus surveillance, NICs generally receive influenza viruses from other laboratories in the country or clinical specimens. NICs often conduct preliminary testing for influenza on clinical specimens and based on those findings, place the clinical specimens in culture (also termed "virus isolation") to see if influenza viruses grow. In the past, virus isolation was usually done using embryonated eggs. More recently, this is done using MDCK cells although NICs are encouraged to continue using embryonated eggs when possible (see step 2a below). Once a virus has been isolated, the NIC then conducts preliminary analyses of the virus isolate using updated reagents to determine the virus type (Influenza A or B). If it is an influenza A virus, the virus is then subtyped to identify the HA and NA proteins of the virus. For example, influenza A (H3N2) is one subtype while influenza A (H1N1) is another subtype. The reagents to conduct this identification must be updated frequently and are produced by the WHO CCs and are provided free of charge to NICs through GISN. Following this step, NICs select samples of viruses to send forward to the WHO CCs. Viruses are selected either because they are considered "representative" of other influenza viruses in the country, based on temporal and geographical considerations, or because the viruses appear antigenically different (which are often called "low reactors"). These samples of viruses are sent then to one of the four WHO CCs located in Atlanta, London, Melbourne or Tokyo. Any NIC can send the viruses to any WHO CC of its choosing. The NICs send these viruses to a WHO CC so the viruses can be characterized more extensively (by antigenic and genetic methods) and so the testing results of viruses collected from around the world can be compared to identify important changes. Since 2005, WHO has paid the cost of shipping influenza viruses from NICs to WHO CCs when requested.

For H5N1 and other influenza A subtype viruses, such as H9N2, which are potential pandemic viruses, some of these procedures are different. When an avian influenza infection such as H5N1 is suspected in a human, the NIC or other national influenza reference laboratory conducts preliminary diagnostic testing, most often using polymerase chain reaction (PCR). This is a molecular technique that does not require a high-level of laboratory biocontainment to perform. Depending on the techniques used and quality of clinical specimens, PCR testing can take from a few hours to several days to complete. Some laboratories with appropriate laboratory high-level biosafety facilities (BSL3 and above) also isolate the H5N1 viruses in MDCK cells or eggs, and conduct partial or whole genome sequencing.

If genetic sequencing is done, the data usually are forwarded by the NIC to a WHO CC and/or a database for either general (public) or restricted access. If the sequencing is done by the WHO CC, an agreement is made with the NIC as to whether the sequencing information will be placed in a public or restricted database and if the latter, the criteria for accessing the data.

Step 2a. Virus isolation in eggs (for seasonal influenza vaccines) ~ 1-3 weeks
- Conducted by some NICs, WHO CCs in Atlanta, London, Melbourne and Tokyo, and TGA

For seasonal influenza vaccines, the vaccine virus must be initially grown in eggs because of national regulatory agency regulations. However, H3N2 viruses in particular have recently been found to grow increasingly poorly in eggs with an isolation rate generally below 3%. Partly as a
result, more NICs have been using MDCK cells instead of eggs to isolate influenza viruses, which has resulted in a decrease in the number of egg isolates available for seasonal vaccine development. In response to this problem, and to ensure that a sufficient number of viruses are available to support vaccine development and production, special projects for producing egg isolates have been established in the WHO CC in Atlanta and at the TGA in Canberra. These projects to increase egg produced isolates have been supported by industry through formal agreements known as CRADAs. In addition, WHO has suggested that NICs should store original clinical specimens for at least 18 months so, if needed to make vaccine, they can be used to isolate viruses using embryonated eggs as required by regulations.

**Step 3. Ferret antisera production ~ 3-5 weeks**  
- Conducted by WHO CCs in Atlanta, London, Melbourne and Tokyo (for seasonal influenza)  
- Conducted by WHO CCs in Atlanta, London, Melbourne, Memphis and Tokyo (for H5N1)

For both seasonal and H5N1 influenza viruses, the convalescent antiserum obtained from ferrets that were purposefully infected under laboratory conditions with a particular influenza virus strain is the most sensitive reagent for detecting antigenic variation among influenza viruses. In other words, ferrets can produce antibodies that are very useful for determining if an H5N1 virus is antigenically close or distant from other H5N1 viruses. This "antigenic distance" is an important factor in choosing viruses for vaccines and for determining when important changes are occurring among groups of viruses. However the process of producing of ferret antisera is difficult, partly because ferrets are naturally highly susceptible to circulating influenza viruses and therefore stringent precautions are required to keep ferrets uninfected until they can be infected by the particular virus of interest. This process requires facilities that have the appropriate laboratory biocontainment level. More stringent biocontainment measures (BSL3 and above) are required when ferrets are used to prepare antisera against H5N1 or other highly pathogenic influenza viruses.

**Step 4. Thorough antigenic and genetic analysis ~ 1-3 weeks**  
- Conducted by WHO CCs in Atlanta, London, Melbourne and Tokyo (for seasonal influenza)  
- Conducted by WHO CCs in Atlanta, London, Melbourne, Memphis and Tokyo (for H5N1)

Every year, the WHO CCs regularly conduct haemagglutination inhibition (HI) tests using ferret antisera on more than 5000 viruses and then further characterize a representative subset of these viruses by performing genetic sequencing. Thorough antigenic and genetic analysis is very time and resource consuming and all of the WHO CCs contribute. The work is done continuously so that at any given time, there is a comprehensive picture of the antigenic and genetic properties of contemporary influenza viruses causing human infections and the general evolution of those viruses. The relevance of these analyses for public health is tremendous but is dependent upon the quality, timeliness and the representativeness of the virus isolates/clinical specimens shared by NICs and other national laboratories with the WHO CCs.
Step 4a. Serological studies for seasonal influenza vaccine ~ 3-16 weeks
- Conducted by WHO CCs in Atlanta, Melbourne and Tokyo, and regulatory laboratories in FDA, NIBSC and TGA

In addition to the genetic and antigenic analyses of influenza viruses, the WHO CCs, in collaboration with the national regulatory laboratories in FDA, NIBSC and TGA, conduct collaborative studies of sera collected from immunized adults and sometimes children. These studies are conducted to determine whether the antibody levels produced by current vaccines are able to react sufficiently against contemporary influenza viruses. In other words, whether or not vaccines based on somewhat older viruses can still provide protection against more recent viruses. These studies are yet another way to determine if the antigenicity of recent viruses have changed significantly from older viruses.

Step 5. Review and selection of candidate viruses for vaccine use ~ 1-3 weeks
- Conducted by WHO CCs in Atlanta, London, Melbourne and Tokyo, and regulatory laboratories in FDA, NIBSC and TGA (for seasonal influenza)
- Conducted by WHO CCs in Atlanta, London, Melbourne, Memphis and Tokyo, and regulatory laboratories in FDA, NIBSC and TGA (for H5N1)

The data generated from antigenic characterization and gene sequence analysis are complementary to each other and both are used help "predict" what seasonal influenza strains will be circulating about 8 months later in the next influenza season. For H5N1, such data are needed to select H5N1 viruses for use in the development of new H5N1 vaccine viruses. For seasonal influenza viruses, WHO brings together representatives from the four WHO CCs in Atlanta, London, Melbourne and Tokyo, and the 3 national regulatory laboratories twice each year (in February and September) to carry out a comprehensive consultation of the cumulative data generated by individual laboratories. These consultations are preceded by multiple telephone conferences to conduct preliminary reviews of the available data. At the separate consultations for the northern and southern hemispheres, the representatives make recommendations for the viruses that should go into each hemisphere's seasonal influenza vaccines. Once the viruses for vaccine use have been selected, a public meeting is held right after the consultation and the WHO recommendations are communicated to all participants. Meanwhile, the recommendations are further communicated to healthcare professionals, influenza vaccine manufacturers and the general public through publication on the WHO website. Since 2004, with the widespread emergence of H5N1, this same group and a representative from the 5th WHOCC in Memphis use the opportunity of the consultation to also review the current data on H5N1 viruses and the status of H5N1 vaccine virus development. When and if warranted, the group selects new H5N1 viruses as potential vaccine virus candidates.
Steps 6a - 9a. Procedures for seasonal influenza vaccines

6a. Use of classical reassortment to produce high-growth viruses for H1N1 and H3N2 ~ 3-4 weeks
- Conducted by CSL, NIBSC and NYMC

Experience over many years has shown that most original influenza wild type A viruses recommended for inclusion in vaccines do not grow efficiently in eggs despite the fact that this production method is still the one used by most vaccine manufacturers. To overcome this problem, Dr Edwin D Kilbourne of New York Medical College (NYMC) pioneered genetic reassortment techniques. Since 1971, his technique (termed classical reassortment) has been available for use. The technique is not patented and is used widely by public health and commercial laboratories for vaccine development and production work involving seasonal influenza viruses. The technique allows the genes from different viruses to be mixed together (i.e. reassorted) to produce a hybrid virus. In practice, six genes (the "backbone" genes) from the A/Puerto Rico/8/34 virus (often called PR8) and two genes encoding the haemagglutinin (HA) and neuraminidase (NA) proteins from the recommended virus are incorporated to form a new virus. This new hybrid virus will induce immunity against the virus from which the HA and NA were derived, and because of the genes from the PR8 virus, grow better in eggs. These hybrid viruses are called "high-growth reassortants" and are often used by vaccine manufacturers so that they can produce the required quantities of vaccine on time. Since these hybrid viruses are essential to vaccine manufacturers, they provide funding to CSL, NIBSC and NYMC to develop high-growth reassortants from selected egg isolates of seasonal influenza H1N1 and H3N2 viruses provided by the WHO CCs in Atlanta, London, Melbourne and Tokyo. These high growth reassortants are made available free of charge to all influenza vaccine manufacturers. At present, influenza B reassortant viruses do not have growth advantages and so influenza vaccines incorporate wild type viruses.

High-growth reassortants for live attenuated influenza vaccines are manufactured using the same techniques but the manufacturer uses its own attenuated parent strain as the donor of the backbone genes.

Step 7a. Antigenic and genetic characterization of reassortants ~ 4 weeks
- Conducted by WHO CCs in Atlanta, London, Melbourne and Tokyo

Before these high growth reassortants are used in vaccine production processes, additional steps are taken. The reassortant viruses are put into ferrets at one or more of the four WHO CCs in Atlanta, London, Melbourne and Tokyo to produce ferret antisera. The antisera then is used to ensure that the high-growth reassortants that have been created have the same antigenicity as the wild type virus from which the HA and NA genes were obtained. In addition, the WHO CCs also sequence the HA and NA genes of the reassortants to determine if any changes have occurred during laboratory manipulation, and if so, whether these changes are in amino acids that are critical to antigenicity.
Step 8. Evaluation of growth property ~ 3 weeks
- Conducted by influenza vaccine manufacturers

Before the high-growth reassortant viruses are selected by national regulatory authorities as a vaccine virus, the growth properties of the influenza A reassortants and the B viruses are evaluated by influenza vaccine manufacturers. This growth evaluation is done by several vaccine manufacturers in parallel due to the tight time constraints of making influenza vaccine imposed by the timing of influenza seasons.

If the results of antigenic or genetic characterization (Arrow A) or growth property evaluation (Arrow B) of the reassortants are not satisfactory, then the process starts over with the review and selection of candidate viruses. Depending on the situation, new reassortment viruses might need to be generated.

Step 9a. Development of standardizing reagents for inactivated vaccines ~ 6 weeks
- Conducted by influenza vaccine manufacturers, the WHO CC in Tokyo, and regulatory laboratories in FDA, NIBSC and TGA

WHO makes global recommendations on which virus strains should be used in influenza vaccines. However, the actual virus used in the vaccine is decided by national regulatory agencies. In practice, based on review of antigenic and genetic characterization data and virus growth properties conducted by some national authorities, national regulatory agencies then formally decide which exact viruses will be included in the influenza vaccines that are approved for marketing in their respective countries for the coming season. This can result in another virus that is antigenically similar ("like") to the recommended strain being put in the vaccine.

Before vaccines are released for sale to the public, they are tested to ensure they have the approved level of haemagglutinin antigen content (this is termed "potency"). The reagents for potency testing of inactivated seasonal human influenza vaccines are developed by the WHO CC in Tokyo and national regulatory laboratories (FDA, NIBSC and TGA) with the help of vaccine manufacturers. These laboratories then standardize the reagents and provide them to all manufacturers worldwide on request.

Total Approximate Time Steps 6a-9a: 16 - 17 weeks

Total Approximate Time to Produce a Seasonal Influenza Vaccine
Steps 2-6 and 6a-9a: 25 ~ 50 weeks

Steps 6b - 9b. Procedures for human H5N1 vaccines

Step 6b. Reassortment of high-growth viruses using reverse genetics ~ 6 weeks
- Conducted by WHOCC in Atlanta and Memphis, and regulatory laboratories in FDA and NIBSC

The H5N1 viruses currently affecting many countries are highly pathogenic for poultry and eggs. Consequently, the development of high-growth reassortants from highly pathogenic avian...
influenza H5N1 viruses for human vaccine development and production is more complicated than for seasonal influenza viruses and requires a different process. First, the H5N1 virus must be made less pathogenic so that it will grow in eggs and be safe to use in vaccine manufacturing plants. This is accomplished using a patented technology called "reverse genetics" which allows the virus to be attenuated through the removal of certain amino acids at the cleavage site of the HA. Reverse genetics is also used to reassort the attenuated HA and the NA genes from the H5N1 virus with the backbone genes from the PR8 backbone virus. In this way, an H5N1 reassortant that grows well in eggs and does not require special containment facilities is developed. Once an attenuated H5N1 reassortant has been developed, additional strict safety testing procedures are required by WHOiii, including virus gene sequencing, plaquing, and safety testing in eggs, chickens and ferrets, before the reassortants can be released to research institutes and vaccine manufacturers. Currently the H5N1 reassortants for human vaccines are developed in two WHO CCs only, in Atlanta and Memphis, as well as in the regulatory laboratories at FDA and NIBSC.

The reassortants developed by the WHO system are available to all research institutes and vaccine manufacturers worldwide on request. Due to the use of reverse genetics, which is a patented technology, a Material Transfer Agreement (MTA) has to be signed by any recipient before the reassortant may be released.

**Step 7b. Antigenic and genetic characterization of reassortants ~ 4 weeks**
- Conducted by WHO CCs in Atlanta, London, Melbourne, Memphis and Tokyo

Similar to seasonal influenza reassortants, the H5N1 reassortants are put into ferrets at one or more of the 5 WHO CCs in Atlanta, London, Melbourne, Memphis and Tokyo to produce ferret antisera to ensure that the reassortants have the same antigenicity as the wild type virus from which the HA and NA were obtained. The 5 WHO CCs and NIBSC also sequence the HA and NA genes of the reassortants to ensure that any changes resulting from laboratory manipulation have not affected the amino acids critical to antigenicity or pathogenicity. If the antigenic and genetic characterization data are not satisfactory, the process must start over with the review and selection of candidate viruses and/or the development of new reassortants. (Arrow C)

**Step 8. Evaluation of growth property ~ 3 weeks**
- Conducted by influenza vaccine manufacturers

As with seasonal influenza reassortants, the growth properties of H5N1 reassortants are also tested by manufacturers. Typically, the H5N1 reverse genetics modified candidate vaccine viruses grow less well than seasonal influenza A candidate vaccine viruses. This may be another challenge for influenza vaccine manufacturers if large quantities of vaccine must be prepared in a short period of time.

**Step 9b. Development of standardizing reagents for inactivated vaccines ~ 6 weeks**
- Conducted by influenza vaccine manufacturers and regulatory laboratories in FDA, NIBSC and TGA
As with seasonal influenza reassortants, potency testing reagents for H5N1 inactivated vaccines are developed by the WHO CC in Tokyo and the national regulatory laboratories (FDA, NIBSC and TGA) with the financial assistance of vaccine manufacturers. The reagents then are standardized by the national regulatory laboratories (FDA, NIBSC and TGA). Reagents and are made available to all manufacturers worldwide on request.

Total Approximate Time Steps 6b-9b: 19 weeks

Total Approximate Time to Produce an H5N1 Human Vaccine
Steps 2-5 and 6b-9b: 28 - 52 weeks

---

3 http://www.who.int/biologicals/publications/ECBS%202005%20Annex%205%20Influenza.pdf