

Reference viruses for seasonal and pandemic influenza vaccine preparation

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The production of seasonal and pandemic influenza vaccines depends on the timely availability of suitable reference viruses. Seasonal vaccines are traditionally produced from high-growth reassortant viruses, which have been derived empirically using well-established techniques. However, it is not possible to use such approaches in deriving vaccine reference viruses from highly

pathogenic H5N1 viruses and alternative techniques such as reverse genetics must be employed.

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Introduction

Most inactivated influenza vaccines available worldwide are produced in embryonated hens' eggs. Vaccine production is based on old technology,¹ which has nevertheless stood the test of time in delivering safe and effective vaccines against seasonal influenza. One of the key aspects in the vaccine production programme is the development and provision of a vaccine reference virus. If the reference virus is antigenically inappropriate, poor levels of immunity against epidemic influenza may result. Furthermore, if the reference virus grows poorly in eggs, there will be delays in vaccine production with resulting vaccine shortages, and inevitably a public health outcry. In the case of pandemic influenza vaccines, there are additional concerns over the safety of vaccine reference viruses, particularly, if the viruses are derived from highly pathogenic H5 or H7 avian subtypes.

Seasonal influenza vaccine viruses

The circulation and evolution of influenza viruses are monitored by the World Health Organization (WHO) global influenza surveillance network, which comprises 112 laboratories whose activities are coordinated by four WHO Collaborating Centres based in Australia, Japan, the UK and the USA. Twice each year, the directors of the four centres review data on epidemiology, antigenic and genetic characteristics of new influenza viruses and vaccine clinical trial serology,² before making recommendations on influenza

vaccine composition. One recommendation is made for northern hemisphere vaccines and another for southern hemisphere vaccines. However, the WHO recommendations are not made on the basis of growth properties of influenza viruses and a newly recommended virus may not always be suitable for use as a vaccine reference virus. If this is the case, alternative strategies must be adopted.

In 1969, Kilbourne³ described a method to improve the growth properties of influenza A viruses in eggs by the process of reassortment. This process utilizes an H1N1 virus called A/PR/8/34 (PR8), which was originally a human isolate and grows extremely well in hens' eggs. High-growth reassortant (HGR) viruses are selected to contain the haemagglutinin (HA) and neuraminidase (NA) from a WHO-recommended virus and the remaining six 'backbone' genome segments from PR8, and these can achieve up to eightfold increases in virus yield.

In the EU, an additional decision is taken about 1 month after the WHO recommendation, in order to approve the precise vaccine viruses (which may be an HGR) for vaccine development in the EU. Although the HGR technique has been in regular use to improve influenza vaccine yields for over 35 years, there is still an element of uncertainty about the outcome and there are occasional problems, as was seen in spring 2006.

In February 2006, the WHO recommended a new H3N2 vaccine strain A/Wisconsin/67/2005.⁴ As is usual, attempts were made to improve the growth potential of A/Wisconsin/67/2005-like viruses by producing HGRs, and in March two HGRs were generated: NYMCX-161 derived from

A/Wisconsin/67/2005 and IVR-142 derived from an A/Wisconsin/67/2005-like virus, A/Hiroshima/52/2005. Both HGRs were evaluated by vaccine manufacturers and it was observed that neither gave the high yields normally expected, nor was there a consensus about which HGR was more suitable overall. In the EU, a decision was taken to allow either HGR to be used, but it was not a satisfactory situation as reagents for standardization had to be prepared for each HGR. In late April, a third HGR became available: NYMCX-161B derived from A/Wisconsin/67/2005. This HGR had superior growth properties and it was accepted for use in EU vaccines; but this decision had been taken 6 weeks later than normal, well into the vaccine manufacturing timetable. Because of the initial uncertainties and poor vaccine yields, it is almost inevitable at the time of writing that there will be delays in the 2006 vaccine supplies in the EU.⁵

Could these delays have been prevented? The answer is probably 'yes', but there is no quick and easy solution. One solution would be to have more laboratories producing HGR vaccine viruses, thus giving a wider choice and hopefully more security about the availability of high-yielding HGRs. This would reduce, but not eliminate, the risks. A more certain approach would be to use the reverse genetics technology to generate seasonal reference viruses.

Reverse genetics is a laboratory technique by which an infectious influenza (or other) virus can be reconstituted from a cloned DNA copy of the genome. For influenza, this is achieved by using plasmids which express the individual genome segments of the virus under the control of a cellular RNA Pol I promoter and terminator.⁶⁻⁸ Cells transfected with these plasmids will generate exact copies of the viral genome. However, the viral RNA by itself is not infectious and the process also requires the co-expression of viral helper factors, in this case the three viral polymerases (Pa, Pb1 and Pb2) and the viral nucleoprotein. This is achieved from plasmid expression clones using Pol II promoters, either individually or combined with the Pol I rescue plasmids.⁶⁻⁸ The great value of this process is the ability to engineer the viral genome when it is in the form of a DNA plasmid.

The objective of producing an HGR for vaccine production is to derive a virus referred to as a 6:2 reassortant, i.e. one which has the six backbone genome segments from PR8 and the remaining two, encoding the HA and NA, from the wild-type strain. As the process of reassorting is serendipitous, a 6:2 reassortant is not always obtained, and a derived vaccine strain may be 5:3 or 4:4. Ultimately this might not matter, as long as the reassortant has good growth properties.

Reverse genetics can be used quite readily as an alternative and rational means of developing a high-growth reference virus. In this case, six plasmids encoding the internal

proteins of PR8 and two plasmids encoding the HA and NA of the wild-type virus are used alongside the helper plasmids to rescue a 6:2 reassortant directly. No other reassortant will be generated and although the formation of a 6:2 reassortant does not guarantee high growth properties, studies to date have shown that all reverse genetics-derived reference viruses grow as efficiently as traditionally derived HGRs.^{9,10}

The process of rescuing a vaccine strain in this way is relatively robust and generally takes no more than 3 weeks from start to finish. Thus, the application of reverse genetics to derive a vaccine strain for an A/Wisconsin/67/2005-like virus would have avoided the problems encountered in 2006 with the northern hemisphere decision. It is now known that the high-yielding NYMCX-161B virus is a 6:2 reassortant whereas the lower-yielding NYMCX-161 virus is a 1:7 reassortant, with only a single gene from PR8 and the remainder from the wild-type virus (D. Bucher, New York Medical College, New York, USA, personal communication).

The rescue process requires the use of a mammalian cell line for virus rescue, and this imposes additional quality issues not applicable to traditional egg reassorting. The quality of a reverse genetics-derived vaccine virus can be assured by using a cell line validated for vaccine production and by applying appropriate quality control and quality assurance procedures. However, such viruses are not currently being provided on a routine basis to industry for vaccine production because of the reluctance to use them due to intellectual property issues associated with this technology.

Pandemic influenza vaccine viruses

It is generally accepted that vaccines will provide our best intervention strategy against pandemic influenza. In recent years, the episodes of avian influenza virus infection of humans suggest that the next pandemic virus may be a highly pathogenic avian strain and we are faced with the problem of generating a vaccine from a potentially lethal virus. The virus would thus be hazardous for vaccine production staff, and both wild and domestic animals in the vicinity of the production facility would be at risk of infection. We can try to solve this problem in two very different ways: we can search for a suitable non-pathogenic virus, which resembles the pandemic virus (a surrogate vaccine virus) or we can try to attenuate the highly pathogenic virus.

When H5N1 viruses first started causing human infections in Hong Kong in December 1997, the first of these options was available. It was quickly realized that a non-pathogenic avian H5N3 virus, A/Duck/Singapore/97, with an antigenically similar HA to the highly pathogenic Hong

Kong virus, could be used as a surrogate H5 vaccine virus. The drawback to this approach, however, was the poor growth of this virus in hens' eggs. Attempts made to produce HGRs in eggs met with little success. This virus would certainly not have been suitable for large-scale vaccine production. However, some experimental lots of A/Duck/Sing/97 vaccine were made and protection against the lethal H5N1 infection in mice was demonstrated,¹¹ whilst good immune responses with the MF-59 adjuvanted subunit vaccine were obtained in a phase I clinical trial.¹² When human infections with H5N1 viruses reappeared in late 2003 and in 2004, there were no suitable surrogate non-pathogenic H5 viruses, so the second course of action was needed.

The major molecular basis for the virulence of H5N1 viruses is linked to the cleavability of the H5N1 HA spike protein, in addition to important features of the internal viral proteins, and the enhanced cleavability is due to an extra four to six basic amino acids in the HA structure at the cleavage site. It is in generating a pandemic vaccine strain against the H5N1 virus that the power of reverse genetics really becomes apparent. Using simple genetic engineering techniques, these extra basic amino acids can be excised from a cloned copy of the HA segment. At the same time, by rescuing the NA segment from the potential pandemic strain along with the backbone of six PR8 segments, a virus can be generated which has the outer coat proteins of the pandemic strain, has the ability to grow well in eggs and is non-pathogenic, both for the eggs in which it will be grown and for the staff working in vaccine production. The use of PR8 as a genetic backbone for pandemic reference viruses has been approved by the WHO and the rationale is as follows.

PR8 was originally a human isolate and has been propagated so extensively in eggs and in other substrates that it has become wholly attenuated for man.¹³ A reassortant virus with the HA and NA genes from a human H3N2 virus and the remainder of its genes from PR8, can grow in humans but retains attenuated characteristics compared with the circulating human H3N2 strain.^{13,14} It is thus anticipated that a pandemic reference virus based on PR8 will also be attenuated in man. Additionally, the PR8 virus contributes a high growth phenotype in eggs to the rescued virus, thus improving the potential yield of vaccine.

In early 2004, the WHO requested their reference laboratories to develop a pandemic vaccine strain from the avian H5N1 viruses causing the epizootic in poultry and serious infections in humans. It was considered that a pandemic of influenza based upon the highly virulent H5N1 virus was a high probability. Although more than 2 years later no pandemic has occurred, the epizootic and occasional human infections continue and the concern remains high that eventually the virus will mutate to become highly transmis-

sible amongst humans. Two laboratories, St Jude Children's Research Hospital, USA¹⁵ and we at NIBSC in the UK,¹⁰ rapidly developed H5N1 vaccine viruses using reverse genetics. The traditional approach of reassorting was out of the question because of the major pathogenic trait mentioned above that resides within the HA gene and which would carry over into any resulting reassortant virus. In addition, the lethality of the virus for embryonated eggs and the need to work at high containment laboratories would have made traditional reassorting problematic. In deriving the vaccine viruses by reverse genetics, the extra basic amino acids within the HA were excised and the resulting viruses containing the altered HA along with the NA were rescued from a human Vietnamese viral isolate with the remaining six segments from PR8.

The NIBSC H5N1 vaccine strain (NIBRG-14) was rescued in validated Vero cells in a high containment laboratory, following procedures that assured the quality of the reference virus for use in the development of a human vaccine.¹⁰ However, there was a further major difference between the rescue of an attenuated H5N1 6:2 reassortant and the generation of a reassortant from a seasonal H3N2 or H1N1 virus, and that was the need to perform safety tests. Although deletion of the polybasic amino acids from the H5 HA should in theory provide a high level of attenuation, it was incumbent on those producing these strains to prove that they were indeed safe to handle by vaccine manufacturers. First, sequencing was used to demonstrate that the deletion within the HA gene had not re-inserted itself. Indeed, during the engineering of the DNA clone, further base substitutions were introduced to reduce the likelihood of the deleted section being re-introduced by viral polymerase stuttering, the most likely method by which the coding region for these extra amino acids is introduced in nature. A further *in vitro* assay was to assess the ability of the rescued H5N1 virus to plaque in cell culture in the absence of added trypsin. Most influenza viruses require added trypsin to cleave the HA and hence to form plaques in cell culture; highly pathogenic strains do not. This test can be used to assess the pathogenic potential of an influenza virus and the test indeed demonstrated that NIBRG-14 was not highly pathogenic.

The rescued virus was also found to be non-lethal for embryonated eggs, even when inoculated neat, providing additional evidence of attenuation, whilst in the intravenous pathogenicity index test in chickens, an OIE requirement for assessing the pathogenicity of any H5 (and H7) avian strain, NIBRG-14 scored 0.0 (the requirement for low pathogenicity being below 1.2).¹⁶ The final safety test performed in ferrets is a new development, agreed by the experts of WHO group. Whilst the above tests assure the safety of a virus with respect to avian species, it is also desirable to assess the virus in a mammalian system. The mouse model is used extensively in

influenza virus research; however, the infectious nature of influenza strains for the mouse is highly variable and disease signs do not necessarily mimic those of humans. The ferret, in contrast, has long been known to mimic human infection in many ways, including disease signs and pathogenesis, and was instrumental in the discovery of human influenza virus in the 1930s.¹⁷ Thus, the WHO recommends comparing the virulence of vaccine viruses derived from highly pathogenic H5N1 strains with both the parental wild-type strain and PR8, with the requirement that the vaccine strain must be comparable to those induced by PR8 and less pathogenic than the wild type. The NIBRG-14 virus caused mild disease signs and pathology in the ferret comparable to PR8, and considerably less than wild-type H5N1 virus. Some of the above tests were also applied to virus that had undergone a further six passages so as to demonstrate the genetic stability of the virus (with respect to pathogenicity).

Altogether, NIBRG-14 was shown to be a highly attenuated H5N1 strain and thus suitable, from a safety point of view, for use in vaccine production. Nonetheless, the virus remains a live infectious virus and recent WHO guidance for the manufacture of vaccine based upon such strains recommends the application of an enhanced BSL2 level of biological containment during vaccine manufacture.¹⁸ The reasoning behind this recommendation is to avoid unnecessary exposure of staff and the environment to a virus that has the capability of recombining with a wild-type seasonal human strain and the formation of a new virus with an H5N1 coat and the internal genes from a human virus. NIBRG-14 is now being used worldwide for vaccine development.

Specialist laboratories within the WHO surveillance network have continued to analyse isolates both antigenically and genetically from the prevailing H5N1 epizootic and it is recognized that some antigenic drift has occurred. To maintain the availability of suitable H5N1 vaccine strains, further attenuated viruses have been developed by reverse genetics, by us and by other WHO reference laboratories. Recently, vaccine viruses have been derived from 2005 isolates from Indonesia, China, Mongolia and Turkey.^{19–21}

A number of clinical trials ongoing are investigating the optimal and most dose-sparing formulation for an H5N1 pandemic vaccine. Antigen-sparing approaches are essential to provide the maximum number of vaccine doses and non-proprietary aluminium-based adjuvants and other proprietary adjuvants are being assessed. However, the earliest results show H5N1 to be a poor immunogen and recently a plea has been made for an increased assessment of the immunogenicity of various formulations and an investigation into possible surrogate markers of protection in animal models.²²

It has also become apparent that WHO reference laboratories need to improve H5N1 vaccine viruses so that they

are more suitable for vaccine production. Manufacturers have reported that current H5N1 strains provide poor yields of HA antigen compared with seasonal vaccine strains (about one-third reduction), despite adequate growth of the virus. The problem appears to lie with a low content of HA antigen within the individual viral particles. This is being investigated with a view to understanding the genetic basis for antigen content and to ensure that reference vaccine strains have the maximum antigen content in addition to generating a maximal yield of virus particles.

In the event of a pandemic, it is absolutely vital to prepare vaccine reference viruses as quickly as possible. In 2004, when we were faced with the threat of human H5N1 infections in Vietnam and Thailand, it took 2.6 months to produce the fully tested NIBRG-14 virus at NIBSC. Since that time, we have tried to streamline as much of the process as possible, with the result that our current capability is 1.4 months. However, there is an opportunity to do even better. If we consider that after just 1 month it is possible to have a newly derived H5N1 reverse genetics virus which has been tested for absence of multiple basic amino acids at the HA cleavage site, for the inability to kill chicken embryos and the inability to form plaques in mammalian cells without the addition of trypsin, we would thus have a great deal of confidence, based on our experience and that of others, that the candidate H5N1 vaccine virus is safe. The remaining tests for safety in ferrets and chickens, which need a further 0.5 months, would not have been completed. According to current OIE regulations, an H5N1 virus which has not been tested for pathogenicity in chickens is considered to be highly pathogenic,¹⁶ and it would not be possible to distribute a partially tested H5N1 virus unless it is handled at either BSL3 or BSL4 according to locally implemented laws. A saving of 0.5 months would be vital in preparing a pandemic vaccine and discussions are now underway between WHO and OIE to resolve this issue. In the USA, a partially tested H5N1 virus is designated 'select agent' status by the USDA and urgent discussions are also needed to relax such laws under emergency situations.

If we consider that it will take 1 month to release a partially tested reference virus, a further 1 month to generate a seed virus by vaccine manufacturers and about 3 months to produce the first vaccine doses, an H5N1 vaccine produced *de novo* from a pandemic virus is not likely to be ready before 5 months. Therefore, such an H5N1 vaccine will probably not be available to combat the first wave of pandemic activity anywhere in the world. How can we improve on this? There are a number of options being considered by different countries, including vaccine stockpiling, pre-pandemic use of vaccine to antigenically prime populations and use of a library of potential pandemic vaccine viruses. Each option has its strengths and weaknesses and

as the first two options are outside the scope of this review, we will explore the option of using library viruses.

The WHO has identified three priorities for the influenza subtypes likely to cause pandemic activity:

- 1 H5N1 and H7 viruses;
- 2 H9N2 and H2N2 viruses;
- 3 H6 and H4 viruses.

The concept of a pandemic library is to prepare vaccine viruses in advance, using either reverse genetics or more conventional means, to represent the major antigenic variants within each of the priority subtypes. Of course if time and resources allow, the work would continue to cover the remaining avian influenza subtypes. Ideally, each of the library viruses would be distributed to vaccine manufacturers, so that working seeds can be prepared. This action could save nearly 2 months from vaccine production times discussed above, so that the first doses of a library vaccine may be ready within 3 months of a pandemic declaration. This would not be the only benefit as it would also be possible to produce vaccine potency testing reagents to match the library vaccine virus, so that no unnecessary delays would be encountered in quality control testing of pandemic vaccines. There are obviously some disadvantages in this approach, the chief of which is the likelihood that a library vaccine virus will not be an accurate antigenic match with a newly emerging pandemic virus. Does this 'mismatch' in fact matter, when arguably the main purpose of a pandemic vaccine is to save lives? There are some experimental data emerging in mice and in ferrets to show that inactivated H5N1 vaccines will in fact protect against a lethal H5N1 challenge virus which is antigenically different from the vaccine virus (J.M. Wood, unpublished data;²²). Should we now reconsider what is required from a pandemic vaccine as has been suggested recently by Stohr *et al.*?²³ We certainly need more immunogenicity and challenge data in animals to answer these questions.

Despite long-term experience of HGR virus development for seasonal vaccine production, various problems persist, with 2006 being a typical example as described earlier. Most of the problems lie with the time constraints in developing a new HGR and manufacturing vaccine in readiness for the following winter's influenza season. New reverse genetics technology allows for the rational design of suitable vaccine strains but has yet to be used routinely for seasonal strains and embraced by the manufacturing community. However, in preparation for a pandemic, these new tools have permitted WHO reference laboratories to generate rapidly, safe H5N1 vaccine strains and although vaccine derived from them is currently being assessed clinically, a lot remains to be achieved before we have a cost-effective, safe and highly dose-sparing pandemic vaccine.

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