A review of production technologies for influenza virus vaccines, and their suitability for deployment in developing countries for influenza pandemic preparedness

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Preamble

Immunization against influenza is considered an essential public health intervention to control both seasonal epidemic and pandemic influenza. Thus, influenza vaccine development and deployment is a critical part of pandemic influenza preparedness. Most resource-constrained countries do not have the means for accessing seasonal influenza vaccines and may face the same challenge during an influenza pandemic. There is therefore a need for these countries to consider establishing influenza vaccine production capacity.

Several methods of production and also types of influenza vaccine exist, each with pros and cons for use, but also vastly different requirements in terms of capital investment, technology transfer, and production response time. Manufacturers in developing countries who are interested in establishing influenza vaccine production capacity need to consider which type of vaccine, and which type of production technology is appropriate for them, and can be sustained.

This document outlines the technologies currently available for the production of vaccines against influenza virus, and considers their suitability for influenza vaccine production in developing countries. The key advantages and disadvantages associated with each approach are identified, as well as the main hurdles that need to be overcome in the adoption of each of the technologies. Investment and time required for implementing production capacity are also discussed. The production technologies considered in the document are egg- and tissue-culture-based propagation of influenza virus, for the preparation of live attenuated or inactivated vaccines.

The analysis shows that egg-based production of live attenuated vaccine requires the least capital investment to establish and maintain, and therefore appears attractive to manufacturers in resource-poor settings. Nevertheless, parameters such as the regulatory pathway to licensure and Intellectual Property Right (IPR) issues also need to be considered. Egg-based production of inactivated virus is currently the most widely used technology., However large scale production of inactivated vaccine requires significant capital investment which may be difficult to justify in areas where there is only limited market for seasonal vaccine. Tissue-culture based production of live attenuated or inactivated vaccines requires greater financial investment, and access to proprietary technologies, but these technologies may have advantages in terms of logistics of vaccine production in event of a pandemic, and may also have long-term advantages for the manufacturer.

These pros and cons of each option are discussed in detail in the document, and graphically presented to assist manufacturers in selecting which technology is the most appropriate for them and for their country's pandemic preparedness plans.

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1 Acronyms and abbreviations

BPL .... betapropriolactone
BSL .... biosafety level
ca .... cold adapted
CEF .... chicken embryo fibroblast
CTL .... cytotoxic T lymphocyte
CAIV .... cold-adapted influenza vaccine
CPMP .... Committee for Proprietary Medicinal Products
EMEA .... European Agency for the Evaluation of Medicinal Products
EPI .... Expanded Programme on Immunization
FDA .... Food and Drug Administration (USA)
GMP .... good manufacturing practice
HA .... haemagglutinin (of influenza virus)
HAI .... haemagglutinin-inhibiting antibody
IIV .... inactivated influenza vaccine
IN .... intra nasal
IP .... intellectual property
LAIV .... live attenuated influenza vaccine
MDCK .... Madin Darby canine kidney cells
moi .... multiplicity of infection
MVA .... modified vaccinia Ankara
NA .... neuraminidase (of influenza virus)
NIBSC .... National Institute for Biological Standards and Control (UK)
OPV .... oral polio vaccine
pfu .... plaque-forming units
QA .... quality assurance
QC .... quality control
SPF .... specific pathogen free
TC .... tissue culture
ts .... temperature sensitive
WHO .... World Health Organization

2 Introduction

The purpose of this document is to provide an overview of the technologies currently available for the production of vaccines against influenza (flu) virus, and to consider their suitability for use for influenza vaccine production in developing countries. The key advantages and disadvantages associated with each approach are identified, as well as the main hurdles that need to be overcome in the adoption of each of the technologies and the investment and time required for implementing production capacity.

The technologies discussed in this document are egg-based and tissue-culture based virus propagation, and the use of inactivated virus (whole, split or subunit, with and without adjuvant) or live attenuated virus as the vaccine. Upstream technologies, including the production of influenza antigens in plants or insect cells, or vaccines based on pure recombinant antigens, peptides or DNA are only briefly discussed. While these technologies may have significant potential for influenza control in the future, there are still many unknowns regarding their development, manufacturing and regulatory approval pathway.

Because of the emergence and circulation of H5N1 influenza strains and the need to prepare for a possible influenza pandemic, it is important to consider methods for the production of seasonal influenza vaccine in the context of their relevance and impact on production of pandemic influenza vaccine and overall pandemic preparedness. Manufacture of seasonal and pandemic influenza vaccines are closely interrelated and emergency production of a pandemic vaccine may be dependent on manufacturing capacity for seasonal influenza vaccine. The sustainability of pandemic vaccine production preparedness will be dependent on maintaining a facility with trained operators, and this without incurring financial losses. Therefore, in selecting a technology for influenza vaccine manufacture, a country needs to consider the domestic market for seasonal influenza vaccine and also pandemic preparedness, as well as the financial and human investment required to establish and maintain the production capacity.

Other factors which manufacturers embarking on the process of establishing pandemic influenza manufacturing capacity need to consider include: the regulatory pathway to licensure of the vaccine; whether the facilities required for pandemic influenza vaccine production could serve for production of other vaccines, which could minimize the financial burden of maintaining preparedness; supply of critical components of the production process such as fertilized eggs, tissue culture medium and the fact that these supplies may be jeopardized in emergency situations.

3 Influenza vaccines: background

3.1 Seasonal influenza vaccine

3.1.1 Annual cycle of influenza vaccine production

Surveillance

Surveillance is undertaken year-round to monitor influenza strains in the population, and at the start of the influenza season WHO announces which dominant circulating strains should be included in the vaccine (Gerdil, 2003).

Seed strains

WHO Collaborating Centres generate and analyse the seed strains for vaccine production. Since the 1970s, this has been done by genetic re-assortment. Embryonated eggs are co-infected with the field strain selected for the vaccine and an A/PR8/34 (or similar) master strain that is known to give good yields on eggs. High-growth progeny virus is analysed to confirm the presence of surface glycoproteins from the field strain. (Gerdil, 2003).

Vaccine seed strains are distributed to the vaccine producers in order to evaluate their suitability for vaccine production. Factors such as yield when grown in eggs, antigenic stability, inactivation and purification process are evaluated. The results from all the producers are evaluated in meetings with the EMEA and FDA, typically held one month after the initial WHO decision on formulation (Gerdil, 2003).

It should be noted that currently the seed strains that are provided are intended for production of inactivated vaccines. The production of seed virus for live attenuated vaccines requires further re-assortment.

Vaccine manufacture

At the moment of preparing this document all marketed influenza vaccines are grown in embryonated eggs, however it is anticipated that tissue-culture derived vaccines will shortly receive marketing authorization. The basic procedures for viral propagation and options for downstream processing using both egg-based as well as tissue-culture based propagation are outlined in section 5. The expected number of doses to be manufactured needs to be estimated well in advance so that supply of the required number of eggs can be co-ordinated. Planning for egg supply typically commences six months before the start of vaccine production.

Vaccine characterization

Quantification of the haemagglutinin (HA) antigen in the vaccine is achieved by a single radial immuno-diffusion (SRID) assay. The reagents need to be specific for the strains in the vaccine, and take approximately three months to be produced and distributed. This can delay the release of the vaccine. Stability studies are also required for each new formulation and presentation.

Clinical studies

Clinical trials demonstrating the safety and immunogenicity of each new influenza vaccine formulation are required in Western Europe, but not in the United States or the Southern hemisphere (Gerdil, 2003).

Overall, the bulk production campaign for one season takes six months. Two such campaigns take place each year; one for production of vaccine for the Northern hemisphere, one for production of vaccine for the Southern hemisphere.

3.1.2 Inactivated influenza vaccines; composition

Seasonal influenza vaccines are usually trivalent, each dose containing 15 µg of each of two influenza A subtypes (e.g. H1N1 and H3N2) and 15 µg of one influenza B strain.

There are currently three predominant types of inactivated influenza vaccine: whole virus, split and subunit vaccines. All are currently produced in embryonated eggs. As discussed later, while egg-based production of virus is technically relatively well-understood, the need for a reliable supply of fertilized eggs is one factor that may make tissue-culture based production eventually more attractive. The fact that allantoic fluid does not need to be removed from the virus harvest can simplify downstream processing of tissue-culture (TC) derived virus.

Whole virus

For preparation of whole virus vaccine, in most cases only one purification step (gradient sucrose centrifugation) takes place before inactivation. Because there is a single inactivation step, a higher concentration of inactivating agent is required than for split vaccines. This may affect the integrity of the antigen. Nonetheless, whole-virus influenza vaccines are reported to be more immunogenic in immunologically naïve populations (Nicholson, 2004). However, they have been reported to be associated with a higher frequency of adverse events compared with other types of influenza vaccine, particularly in children, and have been little used so far (Stephenson et al., 2004). It is estimated that less than one third of all influenza vaccine production is whole-virus vaccine.

Split

The majority of influenza vaccines are 'split' vaccines, which are produced by detergent-treating purified influenza virus. The splitting process breaks the virus allowing the relevant antigens to be partially purified. Removal of some of the viral components results in a less reactogenic vaccine. The inactivation procedure can be less stringent than for the whole virus vaccine since the splitting procedure also serves to inactivate the virus.

Subunit or surface antigen vaccines

Sub-unit or surface-antigen vaccines are produced as for split virus, but more rigorous purification is carried out so that the vaccine consists almost exclusively of highly purified HA and NA. These purified antigens may be reconstituted in synthetic lipid bilayers, forming virosomes. Virosomal formulations of influenza vaccine are licensed in Europe.

3.1.3 Live attenuated influenza vaccines

Live attenuated influenza vaccines (LAIVs) were widely used in some parts of the world such as the Soviet Union, however their use declined as economical problems following the collapse of the USSR reduced demand for influenza vaccines. Recently they have been re-developed in the USA as an alternative to the standard inactivated influenza vaccine (IIVs). Potential advantages of LAIVs include:

- No down-stream processing required (harvested vaccine is simply packaged)
- High yield (as described later, 15 to 20-fold greater than inactivated vaccines)
- Needle-free delivery (administration is via an intra-nasal spray), which may facilitate administration in resource-poor settings.
- Induction of a broad immune response including mucosal, systemic and cell-mediated responses (Nichol, 2001). In contrast parenterally administered inactivated vaccines do not induce mucosal immunity.
- The potential to induce a protective immune response after a single administration in naïve individuals (yet to be confirmed). Two doses of vaccine are thought to be required for induction of protective immunity in naïve individuals with inactivated vaccine.

Live attenuated influenza vaccines are constructed by re-assorting NA and HA from circulating influenza viruses with six genes from master donor strains of two types:

- Temperature sensitive (*ts*) donor strains. The viruses contain mutations that limit growth at 37–39°C, restricting growth in the lower respiratory tract. Growth is still possible in the upper respiratory tract, so local and systemic immune responses can be stimulated.
- Cold-adapted (*ca*) donor strains, which are selected to grow well at a reduced temperature, so that growth is favoured in the upper respiratory tract.

Two live-attenuated, cold-adapted vaccines derived from the A/Leningrad/134/57 (H2N2) and B/USSR/60/69 virus have been licensed in Russia (Rudenko et al., 2001). A LAIV (CAIV, FluMist®, MedImmune Vaccines) with *ca*, *ts* and attenuated (*att*) phenotypes based on master donor strains A/Ann Arbor/6/60 and B/Ann Arbor/1/66 was licensed in the USA in 2003.

LAIVs are currently produced in embryonated eggs (section 7) however several groups are developing LAIVs in tissue culture.

3.1.4 Upstream influenza vaccine approaches

A number of approaches are being pursued to develop alternative forms of seasonal and pandemic influenza vaccines including:

- DNA vaccines expressing HA and NA, which are delivered to the epidermis by needle-free ballistic delivery of DNA-coated gold particles (PowderMed, Oxford, UK); Preliminary clinical data suggest that this approach is promising, and since DNA can be rapidly produced, this is attractive for pandemic influenza preparedness. The need however for a complex formulation and delivery system does remain a barrier to the widespread use of this technology.
- Recombinant HA produced by a baculovirus-expression system (Protein Sciences Corporation, CT, USA); or produced in transiently transfected plants (Microbix, USA). The high and rapid yield of antigen make these approaches attractive, however this must be weighed against the limited breadth of immunity that will be induced to a single antigen (compared to a split or live virus).
- Recombinant M2e, the extra-cellular domain of the ion-channel protein M2 (Acambis, Cambridge, USA). This is a highly conserved viral antigen and it is suggested that immunity to this antigen could theoretically protect against all A-strains of the virus and thus provide universal protection.

Because these programmes are at relatively early stages of clinical- or pre-clinical development or unlikely to be appropriate or available for use in developing countries in the near future, they are not discussed further in this report.

3.2 Pandemic influenza vaccine

3.2.1 Vaccine production capacity

A vaccine against a pandemic influenza strain would be monovalent i.e. containing antigens from the pandemic strain only. In theory only 15 µg of HA per dose would be required (rather than 45 µg); however, for several reasons, the production capacity for a pandemic strain could be less than for current seasonal vaccines.

Immunization regimen

The pandemic strain will be a new strain for which there is little or no immunological memory in the population. Therefore it is anticipated that two doses of inactivated vaccine will be required in order to induce protective immunity. If a LAIV is used, one dose may be adequate (still to be demonstrated). In order to avoid any possibility of reassortment of an LAIV strain carrying HA and NA from a potentially pandemic influenza strain and used for immunization with a circulating wild-type strain, which might theoretically generate a virulent pandemic strain, use in the general population of LAIV containing HA and NA from a highly virulent avian influenza strain is not recommended before the onset of pandemic.

Manufacturing
Current data suggest that egg-based production yields of HA expressed by H5N1 viruses generated by reverse genetics are only 30–40% of the average HA yield for seasonal influenza viruses (Stephenson et al., 2006), while virus yields are similar to those obtained usually. This could have serious implications for IIV production in the event of a pandemic. There are also concerns that growth of human influenza virus in eggs can lead to the selection of antigenic variants which may be less efficacious (Katz and Webster, 1989).

Immunogenicity
Data from phase 1 and 2 trials indicate that vaccines based on H5N1 strains may be less immunogenic than current seasonal vaccines. In a phase 1 trial of a non-adjuvanted sub-virion H5N1 vaccine (A/Vietman/1194/04), two doses of 90 µg were required to generate antibody responses that would be satisfactory for licensing (Treanor et al., 2006). In a further trial in which the sub-virion vaccine was formulated with aluminium hydroxide, two doses each of 30 µg were found to induce immune responses consistent with EMEA requirements for seasonal influenza vaccines. In contrast, two doses of 10 µg of an aluminium hydroxide-adjuvanted whole-virion vaccine induced seropositivity in 78% of vaccines (Lin et al., 2006). Therefore, for split or sub-unit H5N1 vaccines to induce protective levels of immunity, it appears that larger doses of HA (compared to standard influenza vaccines) and/or adjuvants will be required.

Thus, using current production technologies the antigen production yield is approximately three-fold lower, and six-fold more antigen is required (per strain), and two doses of vaccine (inactivated are required for protection). When establishing pandemic vaccine production capacity this significant reduction in functional yield compared to seasonal flu needs to be taken into account.

3.2.2 Adjuvants and delivery routes

Use of immunological adjuvants or alterative delivery routes may reduce the dose required to induce protective levels of immunity, thereby increasing overall global capacity.
Several adjuvants have demonstrated capacity to enable the induction of antibody levels that are considered to be protective, with lower inactivated antigen doses (generally evaluated with split, but also whole cell vaccines).

Aluminium-based adjuvants
Aluminium-based adjuvants including aluminium hydroxide, aluminium phosphate, and a combination of the two have been evaluated by numerous groups. While these adjuvants have been shown by some groups to permit induction of comparable antibody responses with up to five-fold less antigen from potential pandemic-strains, the results are not consistent and for some groups the effect is at best marginal or absent. A likely reason for this difference is the different antigen nature between the groups: for some the split antigen contains a relatively low ratio of lipid and other components (such as matrix protein M1) to the HA, and there is hence limited

competition for adsorption to the antigen to the alum by these other components. Other factors such as viral RNA in the vaccine (whole cell), contaminating endotoxins, and the presence of counter-ions which modify the charge on the gel may also affect the impact of aluminium adjuvants on the vaccine immunogenicity. This indicates that for any influenza vaccine antigen the potential contribution of alum to the immunogenicity and hence antigen dose needs to be evaluated. The use of alum-based adjuvants for pandemic vaccines appears attractive since there are only minimal intellectual property barriers (exception is D'Hondt et al, European Patent EP1618889), however the variable response, as well as formulation and characterization challenges suggests that other adjuvants should be considered.

Oil in water emulsions

The limited studies conducted to date, with oil-in-water emulsions have shown a greater dose-reduction potential than aluminium salts. MF59, an oil-in-water emulsion produced by Novartis (Chiron) comprising squalene, sorbitan rioleate and Tween 80 and which is a component of the company's seasonal influenza vaccine has been shown to permit the use of doses of pandemic-strain (H9N2) antigen as low as 3 µg. AS03, an oil-in-water emulsion produced by GlaxoSmithKline has been shown to permit significant dose sparing of H5N1 antigen, achieving what are considered protective levels of immunity with 3 µg per dose, whereas the unadjuvanted vaccine required 90 µg (Hannon and Stephenne patent application WO2006/100110). Both of these adjuvants are however proprietary and their use in vaccines produced by others would require a license. The original patent on MF59 emulsions **(**EP0399843) has been revoked in the European Patent Office (decision of 17 August 2000), however it may still be valid in other parts of the world. While the owners of the technology have publicly indicated that in the event of a pandemic they would permit their adjuvants to be used by others, pre-negotiated licenses and supply agreements would need to be established. This would reduce the independence of a company's pandemic response.

Other adjuvants

Several other adjuvants have been shown to be effective when used with influenza antigens in preclinical models. Most of these are proprietary, and as for the adjuvants above add to the complexity of manufacturing, formulation and characterization.

Intradermal delivery

Intradermal delivery has been shown to permit dose reduction for seasonal vaccine (Treanor 2004, Belshe 2004) however in the only clinical study to date with H5N1 antigen this route was not able to induce what are considered protective levels of immunity with a reduced antigen dose. This approach is attractive since reformulation of the vaccine is not required, however a number of parameters may affect the immunogenicity including the reliability of the intradermal injection, and further investigation of this approach is required.

4 Review of influenza vaccine production technologies

4.1 Scale

For the purposes of this review, each manufacturing technology is considered at two scales of operation: the laboratory and pilot scale manufacture, and industrial scale manufacture. This separation has been included to permit interested vaccine manufacturers to determine the investment required to proof-of-concept at the pilot level, and then the challenge and investment of scaling up to full production. As indicated below, some technologies are relatively easy to establish at pilot-plant level, but require massive investment to scale up, whereas others are scaled up relatively easily.

4.1.1 Laboratory and pilot-scale manufacture

In this case, the requirements to establish and run a process that would be capable of producing a few thousand doses of vaccine under current Good Manufacturing Practice (cGMP) are considered. The material would be suitable and sufficient for phase I and phase II clinical trials to demonstrate immunogenicity and safety of the product.

4.1.2 Industrial-scale manufacture

In this case, the investment and infrastructure needed to establish a process capable of producing millions, or tens of millions, of doses per year are considered. The capacity should be sufficient for meeting local and/or regional demand for seasonal influenza vaccine. It should also be sufficient, or at least readily scaleable, to meet the local and/or regional demand for a pandemic vaccine. For the purpose of comparing production costs and time to establishment between technologies we have arbitrarily chosen to fix the production at 20 million doses per year.

4.2 Assumptions

In estimating the resources required to establish influenza manufacturing, it has been assumed that the process and facility will be established by an existing developing-country manufacturer of human vaccines. It has also been assumed that influenza-vaccine production will be installed on an existing ‘campus’ with access to trained staff familiar with GMP requirements, as well as all the necessary Quality Control (QC), Quality Assurance (QA) and other supporting functions. Access to vaccine-filling lines and associated labelling and packaging infrastructure has also been assumed to be available. Where these are not available, the implementation of pandemic influenza vaccine production will be far more costly and take far longer than indicated in the following paragraphs. Finally, cost estimated are based on figures that would apply in the USA or Europe; they may be different for developing country or resource poor settings.

5 Inactivated influenza vaccines (IIV); egg-based manufacture

5.1 Introduction

Producing influenza vaccine in embryonated hens’ eggs has been carried out for more than 60 years, and has not yet been replaced as the standard industrial process, despite the recent development of alternative methods.

5.2 Laboratory scale

5.2.1 Manufacturing process

At the laboratory scale, it is possible to produce hundreds to thousands of doses of IIV manually, without the need for automation and using only standard laboratory equipment.

Embryonated eggs from a certified source are obtained and used 9–12 days after fertilization. The eggs are candled to locate the air sac. The egg is pierced under aseptic conditions, and the seed-virus is inoculated into the air-space with a syringe. The hole is then sealed with wax. The procedure can be carried out on a laminar flow bench. The inoculated egg is incubated for two to three days in a humidified atmosphere. At the end of this period, it is transferred to 4°C, which kills the embryo

and aids clarification of the allantoic fluid. The top of the egg is cut off, the membrane pierced with a pipette and clear allantoic fluid is removed. This is then clarified by centrifugation to remove cell debris. Harvests from the eggs are pooled and sterility tested for three to four days.

The degree and method of purification used depends on the type of vaccine being produced.

Whole virus.
The pooled harvest is concentrated and purified by ultracentrifugation on a sucrose gradient. After harvesting from the gradient, the virus is diluted, and inactivated either by formaldehyde or betapropriolactone (BPL). The concentration of the inactivating agent needs to be determined and validated for each strain. A filtration step may be included to remove egg debris. Because relatively little purification is involved, the yield of whole-virus vaccine per egg is higher (roughly 2-fold) than with the other types of IIV.

Split virus.
The procedure above is followed to the point of harvesting from the sucrose gradient. At that point, the virus is diluted and then 'split' by the addition of detergent such as Triton X-100, sodium lauryl sulphate or Tween 80 to extract proteins from the lipid membrane. The optimal conditions for each strain need to be determined but typically this step can take two to three days. The preparation is then sucrose-gradient purified once more and the HA-rich fraction is harvested. Alternatively, the detergent may be removed by diafiltration. The product is inactivated and sterile filtered.

Compared to the whole-virus preparation, split vaccines are better characterized, contain less ovalbumin and are claimed to be less reactogenic. However the yield of HA can vary significantly from strain to strain and year to year, and from one manufacturer to the next (between 0.7 to 3 eggs required per dose of trivalent vaccine).

Subunit vaccine.
This is prepared in a similar manner to split virus; however, different, more extensive purification steps are used in place of the second sucrose-gradient purification. This results in the isolation of relatively pure HA with minimal contaminating N, matrix protein, nucleoprotein and lipid.

Whether whole, split or subunit vaccine is being produced, a final dilution is made in the formulation buffer. Typically, no adjuvant or stabilisers are used, although preservatives such as thiomersal are frequently added (Nicholson, 2004). Use of adjuvants would require well-characterized material to ensure consistent formulation. Furthermore, adsorption of alum adjuvants is influenced by the lipid to protein ratio, which varies between the different types of IIV.

It should be noted that the methods described above are suitable for production of vaccine candidates for pre-clinical testing and phase I clinical trials. They can provide a useful guide for the development of industrial process, but the final methods used for industrial production would be dependent on, and a result of the know-how of the company developing the process.

5.2.2 Resource required

The infrastructure requirements for the process are straightforward. Inoculation and harvesting of eggs can be performed manually. Minimum requirements would be:

- a relatively simple laboratory with one or two laminar flow benches;
- a simple incubator;
- and a standard ultracentrifuge.

Assuming access to a manual or small-scale filling-line is available and full QC and QA functions are in place, a facility could be established with investment in the order of US$100,000s dollars. Establishing such a facility, developing the process, producing and releasing a batch of vaccine suitable for use in a phase I clinical trial would take approximately 12-18 months (Figure 1), depending on the specific expertise and prior experience of the manufacturer.

5.3 Industrial scale

5.3.1 Manufacturing process

In order to produce sufficient inactivated vaccine for routine seasonal immunization against influenza or pandemic preparedness, the process requires automation and scale-up at all stages. A realistic goal would be a facility capable of producing 20 million doses of trivalent IIV in a four to five month production cycle.

5.3.2 Resource required

Egg supply.

In optimized conditions, each egg can produce approximately between 45 and 90 ug of a classical seasonal antigen, which corresponds to one to two doses when formulated as a trivalent vaccine. As noted above, the yield for H5N1 strains may be significantly less. Manufacture of 20 million doses of trivalent seasonal influenza vaccine requires 15 million to 20 million fertilized eggs over the production cycle. Supplying eggs in these numbers requires careful planning and a long lead-time (6-12 months) in order to reach the numbers required. Furthermore, the supply has to be carefully controlled and timed because the eggs have to be inoculated at a fixed point after fertilization. The egg supply needs to be secure in terms of quantity and quality of the eggs.

Automation.

Automatic inoculators, incubators and harvester are required to speed up the process and increase capacity. Automatic inoculators can operate at approximately 10,000 eggs per hour, enabling a plant to process up to 100,000 eggs per day. However, this also means that less care is taken with the process because there is no longer the opportunity to inspect each egg for contamination. Careful cleaning and pooling procedures are needed to prevent the contamination of one egg being spread by a harvester or inoculator to many other eggs, resulting in the loss of a whole bulk harvest.

Infrastructure and equipment.

A large plant will be required to house the necessary equipment, including equipment for the initial upstream process steps:

- inoculation machines
- incubators
- automatic harvesters
- collection vessel (>1000 litres capacity) for the harvest
- cold rooms

Additional specialized equipment will be needed for downstream processing. The exact nature of this will be dependent on the specific process employed, but is likely to include:

- high-volume, low-speed centrifugation
- ultracentrifugation, and possibly diafiltration, which will require thousands of litres of buffer for counter-flow.
- waste disposal. Approximately 80% of the mass of the 100,000 eggs received per day (equivalent to approximately 4,000 kg) will be solid, infectious waste, which will need to be inactivated and disposed of.

It is estimated that the capital investment required to establish a plant for large-scale production of influenza vaccine in eggs on an existing vaccine manufacturing campus is approximately US$1 per dose of capacity, i.e. US$20 million investment for a plant capable of producing 20 million doses of trivalent vaccine per year. Building and validating the large-scale facility would require at least two years (Figure 1).

5.4 Inactivated influenza vaccine production in eggs: summary

5.4.1 General considerations

Laboratory-scale production of IIV in eggs should be readily achievable. The equipment needed is relatively small, standard and inexpensive and the skills required for the process are straightforward. It is estimated that a plant and process could be established and produce a clinical lot in less than 18 months, for an investment in the order of US$100,000s.

Scaling up to the industrial process requires significant investment. In addition to the financial cost, one of the major hurdles will be accessing the necessary expertise. Egg-based production of influenza vaccine using automated procedures is a unique process not used in other vaccine manufacturing plants, and the expertise required are not readily transferable from other vaccine-manufacturing plants, even other egg-based processes such as yellow-fever vaccine production where the volumes used are orders of magnitude less. Since much of the equipment is specific to production of influenza vaccine, the adoption of the technology will require training of personnel in facilities already equipped and performing automated inoculation and harvesting.

Finally, the cycle of seasonal influenza vaccine manufacture is such that manufacture for a single market takes place in a four to five month period. Therefore a manufacturing plant may remain unused for six months of the year; alternatively additional markets in a different hemisphere need to be identified.

5.4.2 Advantages of egg-based IIV production

- **Experience.** Virus grown on embryonated chicken eggs and then inactivated has been widely used for influenza vaccine production for more than 60 years and is the standard method for flu-vaccine production. Billions of doses of IIV have been produced to date using this technology. Reassortant virus vaccine strains have been developed that are optimized for growth in eggs. The regulatory pathway to licensure, as well as the necessary analytical assays are well established with the result that few unknowns remain in this process. .

5.4.3 Disadvantages of egg-based IIV production

- **Cost**. Establishing manufacture at the industrial scale is expensive, and many of the skills and equipment required are not readily transferable to the production of other vaccines.
- **Egg supply.** The supply of eggs from a certified source is critical. A lead-time of several months is required to establish a reliable supply of fertilized eggs of suitable quality to meet the requirements of seasonal flu-vaccine production. In the event of a pandemic, scale-up of egg production would take several months. If the eggs are not produced locally, in the event of a pandemic the limited supply of eggs may be used by the manufacturer in the country of origin of the eggs. Having its own chicken farm and chicken flocks can be a strategic advantage for a manufacturer, both for seasonal and pandemic vaccine

production. It is technically possible and necessary to secure the flocks against avian influenza, to ensure that egg-based production is possible, even in the worst-case scenario that the pandemic virus infects both mammals and birds.
- **Waste disposal.** Up to 80% of the mass of eggs used (potentially 4,000 kg per day) will be solid infectious waste, which will require inactivation and disposal.
- **Selection of variants** Growth of human influenza virus in eggs can lead to the selection of variants that differ antigenically from the original seed virus (Katz and Webster, 1989). It is therefore necessary to adapt the strains to egg-growth.
- **Yield in eggs.** Many years of experience producing reassortant viruses as vaccine strains have made this technology robust and reproducible. However, individual virus isolates may exhibit different growth characteristics in eggs, thereby affecting the yield of the manufacturing process. This might particularly be the case with an avian-derived pandemic strain. Experience to date with H5N1 viruses generated by reverse genetics has shown that these reassortant viruses produce only 30-40% of the yield of HA of seasonal vaccine strains, despite growing to normal titres in eggs.

5.4.4 Outstanding questions and issues

Due to the decades of experience with this process, many issues have been resolved. However, the process for producing influenza vaccine in eggs is high specialized and to some extent, is specific to each vaccine strain. The procedure can still be complicated by the need to introduce changes in the process steps that arise when novel strains are incorporated into the vaccine. When considering establishing a new facility, three of the key outstanding issues are:

- **Egg availability.** How many eggs would be required to support the process? What grade of eggs is needed? How secure, dependable and rapid would the supply be in the face of pandemic influenza?
- **Economic viability.** Can the process be made economically viable for seasonal vaccine production for a single region? Will this require manufacturing for both Northern and Southern hemispheres, and can suitable markets be identified?
- **Specific experience.** Due to the highly-specialized nature of manufacture of IIV at large scale, it would be necessary for a new manufacturer to seek advice on the process in order to avoid the time and cost of acquiring the expertise. This expertise currently resides within the large vaccine multinational manufacturers.

6 Inactivated influenza vaccines: cell-culture-based manufacture

6.1 Introduction

Growing influenza virus in tissue culture is being developed in order to overcome some of the limitations of egg-based production. The potential advantages of this approach compared with egg-based manufacture include:

- No requirement for a supply of large numbers of eggs
- A more-rapidly scaleable process (using technologies that are used in other vaccine production plants)
- Improved aseptic handling during manufacture
- Avoidance of the risk of embryonated eggs containing avian retroviruses (Ghendon et al., 2005).
- Elimination of egg components in the vaccine and the resultant risk of potential allergic reactions.

6.1.1 Cell lines

Three cell lines are currently in late-stage development as substrates for the growth of influenza virus. Some manufacturers have filed for the approval of their influenza vaccines produced in cell lines and authorization is expected in 2007.

Vero cells
Vero cells are a well-characterized and widely used African green monkey kidney-cell line. They have been used for the production of polio vaccine for more than 20 years. They are an adherent cell line and some strains can be cultured in serum-free medium in stationary culture or on microcarriers.

MDCK
Madin Darby canine kidney cells were originally isolated in 1958 and have been widely used for the production of veterinary vaccines. They can produce high quantities of influenza virus (Tree et al., 2001). Like Vero cells, they are adherent and can be cultured in serum-free medium.

PER.C6
PER.C6 is a relatively new, immortalised human retinal-cell line. It can be cultured in serum-free medium, but only grows in suspension cultures.

Others
Several other cell lines that may be particularly suitable for production of influenza virus are under investigation, including a chicken-derived cell line from Vivalis (Nantes, France). These cell lines have not yet been fully characterized and several years of research and characterization will be required before these can be approved for the production of human vaccines. An potential alternative is the use of chicken embryo fibroblasts (see section 9) which are used in the production of measles vaccine. How well the yield of growth of influenza virus on CEF compares to immortalised cell lines needs to be established.

6.2 Laboratory-scale

6.2.1 Manufacturing process

A laboratory-scale cell-culture-based process should be capable of producing a few thousand doses using relatively straightforward equipment such as roller bottles or cell factories and standard incubators. Roller-bottle cultures of MDCK cells can produce up to $1x10^9$ pfu/ml of influenza virus (Tree et al., 2001).

The cells need to be expanded to the desired quantity from working cell banks. They are then infected with influenza virus and incubated for three to five days. Parameters such as multiplicity of infection (moi), incubation time and temperature need to be optimized for each cell line and each strain of virus. After the incubation period, the virus is harvested by removing the tissue-culture supernatant.

The harvested supernatant is concentrated using diafiltration or centrifugation. Further downstream processing is then required to remove contaminating host-cell DNA. Typically, this involves ion-exchange chromatography to reduce the level of DNA, combined with the addition of benzonase (or a similar reagent) to reduce the size of the DNA fragments that are still present.

Procedures for splitting and inactivating the virus are similar to those as described for egg-based manufacture (section 5.2.1). Inactivated whole-virion vaccines are currently not produced by cell culture.

6.2.2 Resource required

The infrastructure requirements for this process are straightforward. Standard tissue-culture facilities can be used: class II microbiological safety cabinets, incubators and centrifuges. In addition, an efficient cell-culturing system such as one using roller bottles, hollow fibres or cell factories will be necessary.

At the laboratory scale, the technology required and overall complexity of the cell-culture process are similar to the egg-based process. Arguably, the cell-culture methodology is slightly more complex and would take longer to set up; in particular additional QC assays will be required to measure levels of host-cell DNA. However, the level of investment required at this scale is likely to be similar for both systems and is of the order of magnitude of US$100,000s.

6.3 Industrial scale

6.3.1 Manufacturing process

Scaling up the laboratory process will require establishing a fermenter-based cell culture; either using suspension cells or a micro-carrier-based culture. It is likely that 1,000 - 2,000 litre sized fermenters would be required to produce 20 million doses per season. It has been estimated that an optimized 1,000 litre bioreactor using solid microcarriers and MDCK cells would be comparable with approximately 31,000 eggs (Tree et al., 2001).

Another consideration in the process is the time required to expand cells from the working cell bank through a series of passages to produce the necessary numbers required for seeding the final fermentation culture.

6.3.2 Resource required

Considerable capital investment in plant and equipment would be needed for the scale-up of a cell-culture based process; several 1,000 – 2,000 litre fermenters supported by several small fermenters for progressive building-up of cellular biomass would be needed to produce tens of millions of doses. Cell culture at this scale requires large amounts of consumables and media; therefore, secure dependable supplies are needed. One 2,000 litre fermenter might require more than 5,000 litres of cell-culture medium in a single production run. Supporting processes for the production of large volumes of buffers for diafiltration and purification will also be needed. Housing all the necessary equipment will require a relatively large facility. Overall, it is estimated that the cost of establishing such a facility would cost five to ten-fold more than an equivalent facility for egg-based production, i.e. more than US$100 million dollars for a facility sized to produce 20 million doses per year.

6.4 Inactivated influenza vaccine production in cell culture: summary

6.4.1 General considerations

At the laboratory scale, the cell-culture based process is as manageable as egg-based manufacture. More complex QC assays are required for the cell culture process, such as those to detect contaminating host-cell DNA. These techniques are, however, standard. In view of the additional complexity, producing the first clinical lot from a laboratory scale cell culture process is likely to require slightly longer than for an egg-based process (Figure 1).

The costs associated with scale-up to the industrial scale are considerable, and estimated to be in the order of US$100 millions. In theory, large-scale cell-culture fermentation technology could be used for the production of other vaccines in between influenza vaccine manufacturing campaigns, which could mitigate the investment.

6.4.2 Advantages of cell-culture-based IIV manufacture

In comparison with producing inactivated influenza vaccine in eggs, cell-culture technology offers a number of advantages:

- **Security of supply.** It is possible to stock-pile reagents and cells so that a batch of cells can be thawed and expanded whenever it is needed.
- **Faster scale-up of production capacity.** Eventually, the long lead times required for co-ordinating an increased supply of eggs would be avoided, although as described in section 5.4.3 other actions that can also be taken to improve egg-supply.
- **A cleaner, better characterized vaccine** can be produced, without the presence of the egg-derived contaminants that can lead to allergic reactions in some individuals.
- **Selection of variants.** Adaptation of virus to growth in eggs is not required, and therefore the theoretical risk of selecting variants during the growth of the vaccine strain is reduced.

6.4.3 Disadvantages of cell-culture-based IIV manufacture

- **Cost.** Producing influenza vaccine in cell culture is generally regarded as being more expensive than egg-based production for quantities less than approximately 25 million doses per year.
- **Biomass/cell substrate.** For egg-based methods, the biomass needed for growth of the virus is provided in the form of eggs, whereas in cell-culture-based vaccine production there is the additional the time and expense of expanding cells up from a master bank to the numbers required for production.
- **Regulatory perspective.** At the time of writing, no cell-culture-produced influenza vaccines have regulatory approval. in 2002, Influject®, a whole-virion vaccine produced in Vero cells (Baxter Vaccines, Vienna, Austria) was approved in The Netherlands. However, subsequent phase II/III trials of this vaccine were suspended due to a higher than expected rate of mild fever and associated symptoms in the trial participants. influenza vaccines grown in each of the cell-types described in section 6.1.1 are in late-stage development, and several are anticipated to be licensed in 2007.
- **Intellectual property.** Use of proprietary cells for producing influenza vaccine is usually covered by intellectual property that would have to be licensed.
- **Characterization and approval of cell lines**. Full characterization and documentation of the manufacturers' own master and working cell banks of an already approved cell line would be required. Alternatively, a manufacturer could isolate and establish its own cell line and carry out the necessary characterization work to secure approval. However, this process could take years, with no guarantee of success.

6.4.4 Outstanding issues and questions

- It is possible, or even likely, that manufacturers will have a proprietary position on the use of their approved cell line for flu-vaccine manufacture.
- It will be important to establish whether a cell-culture flu-vaccine facility could realistically be used for production of other vaccines to maximize return on investment.

7 Live attenuated influenza vaccines; egg-based manufacture

7.1 Introduction

Live attenuated influenza vaccines have been undergoing development for more than 30 years. Two LAIV vaccines have also been licensed in Russia (Rudenko et al., 2000). In 2003, one cold-adapted LAIV, FluMist® was licensed in the USA (Glezen, W. 2004). They are delivered intranasally and so are expected to elicit a similar immune response to natural infection with influenza. Clinical studies have reported the induction of specific nasal IgA, serum antibodies, and cell-mediated responses by LAIV (reviewed by Belshe, 2004). All licensed LAIVs are currently produced in embryonated specific pathogen-free (SPF) eggs. A cell culture (MDCK) cold-adapted LAIV is also being developed (Ghendon et al., 2005).

7.2 Laboratory scale

7.2.1 Manufacturing process

The process for producing LAIV at the laboratory scale is relatively straightforward, once the vaccine master strain has been constructed, and is similar to the process described in section 5.2.1 for the production of inactivated vaccine. Specific pathogen-free (SPF) eggs are inoculated, incubated and the allantoic fluid harvested and clarified by centrifugation to remove cellular debris. Other than sterile filtration and dilution to the appropriate dose, no additional downstream processing is required. Thus, the number of doses produced per egg is significantly higher and can be in the range of 20 to 50 doses of monovalent vaccine per egg. In addition, by avoiding the splitting and inactivation steps, several days are saved in the production of each batch of vaccine (Figures 2 and 4).

7.2.2 Resource required

The resource required for laboratory scale production of LAIV is less than that needed for production of IIV. The requirements for sterile working areas and incubators and human resources are the same for the two processes. However the absence of concentration and purification steps in LAIV manufacture removes the need for expensive ultracentrifuges. As for IIV, it should be possible to establish a facility for US$100,000s. Due to the simpler process, production of the first clinical lot may be slightly faster than with IIV, and could be achieved in less than one year (Figure 1).

7.3 Industrial scale

7.3.1 Manufacturing process

Because the LAIV manufacturing process has a high yield of vaccine per egg, scale-up should be possible without introducing full-scale automation or complex, expensive equipment. Considerable manufacturing capacity should be achievable simply by increasing the available space and human resources. Consequently, scale-up would be very inexpensive and fast compared with the other technologies (Figure 1).

7.3.2 Resource required

At full capacity, a non- or semi-automated plant could process an estimated 100,000 eggs per week, which could produce more than 10 million doses of monovalent vaccine

per week. Because of the simplicity of the scale-up process, it is estimated that a plant with this capacity could be established for a US$1 million to US$2 million investment.

7.4 LAIV production in eggs: summary

7.4.1 General considerations

Laboratory-scale production of LAIV should be readily achievable, as is the case for IIV. However, the LAIV process is expected to produce at least one order of magnitude more vaccine than the IIV process for the same scale of operation.

Expanding to large-scale LAIV production does not involve a step-change to automation or large-scale equipment. It is generally accepted that the manual process is ‘cleaner’ than automated processes because there is more monitoring of the process. Consequently, there is less chance of losing a large bulk harvest to contamination.

One issue that remains to be addressed is containment. Although the vaccine itself will be non pathogenic, there is concern that co-infection of an individual carrying a wild-type influenza strain could lead to the production of reassortant viruses that lack the *ca* phenotype. Therefore, the entire manufacturing process may need to be carried out under conditions that meet or exceed the requirements for biosafety level 2 (BSL-2).

7.4.2 Advantages

- **Yield.** This should be higher for LAIV compared to IIV (30–50 times more doses per egg). Therefore, the rate of vaccine production in a pandemic will be significantly faster (Figure 4).
- **Dosing regimen.** Two doses of IIV are anticipated to be necessary to protect an individual against a novel pandemic influenza strain. It is possible, that due to the nature of the induced immune response only a single dose of LAIV will be required. However, current guidance for LAIVs recommends two doses for children the first time that they are immunized (Belshe, 2004).
- **Cross-protection.** A live replicating vaccine might be expected to induce a broader immune response than IIV. Several clinical studies have shown that immunization with LAIV can protect against antigenically drifted influenza strains (Belshe, 2004).
- **QC release assays.** The potency assay and sterility test will be the principal assays for vaccine release. There is no need to quantify HA in a vaccine lot, thus vaccine release should not be delayed by waiting for HA-specific reagents. Therefore, release of the initial batches of a pandemic vaccine could be several weeks ahead of the first batches of IIV (Figure 2).
- **Needle-free delivery.** LAIV vaccines are administered nasally, either with a spray device or dropper. The ability to administer without needle-and-syringe may facilitate mass immunization in the event of a pandemic. The absence of needles will also make the administration safer since the risks of iatrogenic infection from unsafe injection practices is reduced.

7.4.3 Disadvantages

- **Supply of Specific Pathogen Free (SPF) eggs** may be more problematic and vulnerable than standard embryonated eggs. SPF eggs will also be more expensive.
- **Pre-pandemic priming.** LAIVs are licensed for seasonal influenza vaccination, and are expected to be suitable for pandemic vaccination once a pandemic is

underway. However, LAIV carrying HA and NA from highly pathogenic avian influenza strains are currently not considered to be suitable for inter-pandemic use, such as the priming of the population with H5N1 vaccine ahead of pandemic; this is due to the theoretical risk of reassortment. The use of LAIV may therefore be limited to pandemic alert stages 4&5. However, it should be noted that the value or need for pre-pandemic priming with an H5N1 vaccine strain has not been established.

- **Genetic instability / selection of variants.** The potential exists for the accrual of mutations that affect vaccine safety or efficacy during growth in eggs. However, data from the evaluation of manufacturing process of nine CAIVs indicated that no mutations occurred (Buonagurio et al., 2006).
- **Containment facilities** may be required for the manufacture and storage of a pandemic strain LAIV to prevent accidental release of virus.
- **Contraindications.** Since the vaccine is cold-adapted there is no risk of viraemia in immunocompromised persons. Nonetheless FluMist® is currently not recommended for use in immunodeficient or immunosuppressed subjects, although small numbers of HIV-positive subject have been vaccinated with no untoward events (Belshe, 2004). FluMist® is also contraindicated in children taking aspirin, due to the risk of Reye's syndrome. LAIVs may also contain more egg-derived components than split or subunit vaccines or cell-culture-produced vaccines.
- **Intellectual property.** Use of current LAIV strains is protected by intellectual property.

7.4.4 Outstanding issues/questions

- **Formulation:** A formulation of influenza vaccine that is stable at 2–8°C would be preferable. Such formulation has been developed for FluMist® (CAIV-T) and is undergoing late-stage clinical testing. A stable formulation was used in Russia for many years.
- **Administration:** LAIVs are delivered intra-nasally. FluMist® is supplied pre-filled in its special administrator and is delivered as an intra-nasal spray. Administration of drops intra-nasally might also be an appropriate means of administration. However, a delivery method will need to be developed and filling lines will have to be adapted for the final container. Oral polio vaccine (OPV) might represent a relevant precedent; this vaccine is produced in several developing countries and is delivered with a dropper.
- **SPF eggs.** It will be important to establish whether fully SPF eggs will be essential for LAIV production, or whether other accredited sources might be acceptable.
- **Intellectual property.** The proprietary position of companies such as MedImmune (Gaithersburg, MD, USA) and Nobilon (Boxmeer, NL) regarding LAIV strains needs to be defined. There may also be relevant intellectual property covering the use of reverse genetics to construct the vaccine strain from the master donor strains (Krattiger et al 2006). It is possible that the latter could be achieved via standard reassortant methodology.
- **Correlates of protection.** Pre-clinical and clinical data suggest that protection afforded by LAIV is likely to be at least as good as that induced by IIVs. However, because the immune mechanisms involved are different, LAIV is unlikely to meet the standard CPMP criteria for licensing annual influenza vaccines. Correlates of protection for IIVs are based on titres of serum haemagglutinin inhibiting (HAI) antibody. Clinical challenge studies have elucidated some correlates of protection for FluMist®, including nasal-wash anti-HA IgA antibodies (reviewed by Belshe, 2004). Further challenge studies will be needed to establish the immune correlates of protection.

8 Live-attenuated influenza vaccines: cell-culture-based manufacture.

8.1 Introduction

Producing LAIVs using cell-culture-based methods could potentially combine the advantages of LAIV in terms of immunogenicity, ease of administration and simplicity of the purification process with some of the advantages of cell-culture-based production, namely avoidance of egg-derived contaminants in the final product and no dependence on an external supply of eggs.

A cell-culture (MDCK cells) live cold-adapted attenuated vaccine based on the A/Leningrad/134/47/57 (H2N2) cold-adapted donor strain has been proposed (Ghendon et al., 2005). Development and pre-clinical evaluation of an H5N1 LAIV produced in MDCK cells is now underway.

8.2 LAIV production in cell-culture: summary

Production of LAIV in fermenters should not require the complex purification or concentration steps involved in IIV production (Ghendon et al., 2005). However, some of the key advantages of using LAIV in eggs, namely: high yield, ease of scale-up, simplicity and speed of the process, are lost when LAIV are produced in cell culture, while some drawbacks remain:

- **Complexity and cost.** The high capital investment required to establish an industrial-scale facility is likely to be the same as for IIV.
- **Regulatory pathway**. The uncertainties mentioned in section 6.4.3 regarding which cell lines will be approved for flu-vaccine manufacture and when this will happen, will also apply to LAIV production.
- **Intellectual property.** This could present a significant barrier. Licences might be needed both for access to LAIV master donor strains and for a suitable cell line.

However, as for tissue-culture production of inactivated vaccines there are some advantages that may make this route attractive for manufacturers entering the field:

- If the facilities that are to be used for TC-production of LIAV could also be used for the manufacture of other tissue-culture derived vaccines, the large capital investment could be recouped through other vaccines. This could ensure sustainability of the production plant and expertise.
- If the same cell line was being used this would also ensure a more rapid availability of tissue mass for pandemic LAIV production should the need arise.
- The yields of LAIV (number of doses per ml of tissue culture) are likely to be far greater than for inactivated vaccine produced from tissue culture. Little public data is currently available however extrapolated from egg-based production one could expect 15-30 fold greater yield. A smaller facility would therefore be able to produce vaccine for a larger population, and would be cheaper to establish than a production plant of the same capacity based on inactivated vaccine.

At the moment of writing this report there are no manufacturers in late-stage development with tissue-culture based LAIV, and it is therefore difficult to establish reliable data for yield, cost, regulatory pathway etc. Manufacturers interested in this approach should discuss with the groups who have initiated development of this approach (e.g. Nobilon, NL or MedImmune, USA).

9 Production of influenza vaccines in chicken-embryo fibroblasts

In the preceding discussion on tissue-culture based production of influenza vaccines the tissue-culture under consideration was established cell lines such as MDCK, PerC6, Vero etc. One approach to production of influenza vaccines which has hitherto received little attention is the possibility of propagating the virus on chicken-embryo fibroblasts (CEFs).

9.1 Introduction

Primary cultures of chicken-embryo fibroblasts (CEFs) are currently used for the production of measles and mumps vaccine and modified vaccinia Ankara (MVA) smallpox vaccine. These processes are well established at vaccine manufacturers in some developing countries. Therefore, in some cases, the potential exists to adapt the existing process and facilities for manufacture of a pandemic influenza vaccine.

9.2 Laboratory scale

9.2.1 Manufacturing process and resource required

The process should be relatively straightforward to establish at laboratory scale, with similar requirements to the cell-culture-based processes described in section 6.2. However, consideration of CEF-based production of influenza vaccines is only likely to be worthwhile in settings where CEFs are already being used for large-scale production of other vaccines.

9.3 Industrial scale

9.3.1 Manufacturing process

Chicken embryos are removed from SPF eggs and the head and viscera are discarded. The remaining tissue is treated with trypsin and then seeded into roller bottles. CEFs would then be inoculated with influenza vaccine strain, incubated and the supernatant harvested. No additional downstream processing other than the addition of stabilizers, preservatives, dilution to the correct potency per dose and sterile filtration is likely to be required. CEF propagation of virus could also be used for the production of inactivated vaccine. The pros and cons of TC-derived IIV remain (as discussed in section 6.2) however the cost of establishing and marinating the facility are reduced.

9.3.2 Resource required

Transfer of an existing CEF-based process to production of a pandemic influenza vaccine should be relatively straightforward and inexpensive. The plant would already have a rolling capacity for supply and handling of eggs. Appropriate QC assays will need to be established and validated. However, it should be possible to achieve this in less than one year.

9.4 Influenza Vaccine production in CEFs; summary

9.4.1 General considerations

Production of LAIV or IIV in CEFs may represent the fastest route to establishing large-scale capacity for flu-vaccine manufacture. However, this approach may only be relevant to manufacturers already producing other vaccines by this method.

9.4.2 Advantages

- **Cost.** Relatively large-scale production should be achievable for a modest capital investment and potentially short time-frame.
- **Risk-spreading.** Production in CEFs would represent an alternative manufacturing technology for influenza vaccines, thereby increasing the number of possible approaches for producing a pandemic influenza vaccine.

9.4.3 Disadvantages

- **Reagent supply.** CEFs require calf serum for growth. This is used at low concentration (approximately 1%), but is expensive. There is also the risk that supply could become limiting during a pandemic.
- **Capacity** is likely to be limited to those manufacturers that already use CEF-based processes. Therefore, the contribution of this approach to pandemic preparedness might be limited.
- **Egg supply**. Production of CEFs requires SPF eggs. As discussed in section 7, this has drawbacks and limitations. It is also possible that the supply of SPF eggs would be limited even further during an avian influenza pandemic.

9.4.4 Outstanding issues and questions

There is little or no information available on the feasibility of this approach. Therefore, a number of key questions need to be addressed:

- **Yield.** The likely yields of vaccine that might be achievable from CEFs need to be determined.
- **Which vaccine should be produced?** CEFs could be used for production of LAIV or IIV. It will be important to establish if CEFs are equally suitable for LAIV and IIV production.
- **Process development**. Although it is envisaged that a CEF-based process will be straightforward, full process development will have to be carried out, and the degree of downstream processing involved will need to be determined.

10 Conclusions

For more than sixty years, influenza vaccines have been produced by inactivating virus grown in embryonated eggs. Although this is still the standard method used for the production of the vast majority of all globally produced seasonal influenza vaccine, a number of alternative methods are available or could be developed. This report reviews the available technologies for their suitability for transfer to vaccine manufacturers in developing countries to establish the capacity to produce seasonal influenza vaccines and thereby create a platform for manufacturing pandemic influenza vaccine. The key features for each of the technologies are listed in Table 2 and are summarized below.

Table 2: Comparison of three technologies for the production of influenza vaccines

	Inactivated flu vaccine **Egg-based process**	**Inactivated flu vaccine** **Cell-culture process**	**Live attenuated vaccine** **Egg-based process**	**Live attenuated vaccine** **Cell-culture process***
Technology transfer and investment				
Capital investment	**High**, >US$20m	**Very high**, >US$100m	**Low,** US$2-3m	**High**
Time to establish manufacturing facility	3 – 4 years	4 – 5 years	1 – 2 years	? > 5 years
Technology requirement	**High**	**Very high**	**Low**	**High**
Manufacturing process				
Cost per dose	**Moderate**	**Moderate – high**	**Low**	**Moderate**
Vaccine production time[1]	>8 weeks	>8 weeks	3 – 4 weeks	? 3-4 weeks
Pandemic vaccine lead time[2]	>12 weeks	>12 weeks	10 weeks	10 weeks
Dependence on external resources	**High**. Requires large numbers of eggs	**Moderate**. Requires large volumes of culture medium	**High**. Lower numbers of eggs required, may need to be SPF	**Low-moderate.** Requires less volume of culture medium than IIV
Barriers to entry				
Intellectual property	**Low**. No IP barrier on basic process	**Potentially high**. May need to license cell-line once it has approval	**Potentially high**. Will need to license CAIV	**Potentially high**. Will need to license cell line and CAIV
Regulatory pathway	**Low**. Standard method of production for flu vaccine	**High**. No cell lines are approved for flu vaccine production	**Potentially high**. Unlikely to meet current immunological correlates for protection	**Potentially high**. Unlikely to meet current correlates for protection. No cell line approved yet
Product				
Immunogenicity	**Good**	**Good**	**Potentially excellent**. Should stimulate mucosal and cell-mediated responses	**Potentially excellent**. Should stimulate mucosal and cell-mediated responses
Reactogenicity	**Low**	**Very low**	**Potentially low** due to intra-nasal delivery.	**Potentially low** due to intra-nasal delivery.

1 Once master vaccine strains are available
2 Minimum time required to produce first batch of vaccine from selection of pandemic vaccine strain
* Tissue-culture based production of live attenuated vaccine is currently being developed, however insufficient public data is available to permit a detailed comparison with the other technologies

10.1 Egg-based manufacture of IIV

Egg-based production of inactivated influenza vaccines is the best characterized, most reliable process, and offers the following advantages:

- **Experience.** The process has been widely used for decades
- **Vaccine strains.** Systems are in place for efficient, routine supply of vaccine seed strains to manufacturers
- **Pre-pandemic priming.** The value of pre-pandemic priming has not yet been established. However if this approach was recommended, inactivated vaccines could be used ahead of a pandemic to prime the population against suspected, likely pandemic strains.

The main drawbacks of egg-based IIV production are:

- **Investment.** The capital investment required to establish large-scale, egg-based production of IIV is estimated to be tens of millions of dollars and at least an order of magnitude greater than for LAIV.
- **Technology transfer.** At the industrial scale, the process is complex and requires process-specific automated equipment.
- **Time.** The time required to establish a large-scale plant is estimated to be approximately four years.
- **Egg supply**. Dose for dose, IIV production requires at least 20-fold more eggs than production of LAIV. However, IIV production does not currently have the strict requirement for SPF eggs that are needed for LAIV manufacture since there is an inactivation step.

10.2 Cell-culture-based manufacture of IIV

Cell-culture-based production of influenza vaccines is being pursued by a number of large vaccine manufacturers as a means to reduce dependence on the supply of eggs. However, from the developing-country perspective, there are still several obstacles associated with this technology.

- **Investment and time to establish**. The cost of establishing a plant is estimated to be an order of magnitude higher than for egg-based production, possibly hundreds of millions of dollars, and is likely to take a further two to three years.
- **Regulatory uncertainty**. To date, a cell-culture grown influenza vaccine has not been licensed or marketed. Therefore, there are still unknowns in the regulatory pathway, and the time required to obtain approval for a new vaccine and/or new cell substrate for vaccine growth is not clear.
- **Access to cell lines.** Once a cell-line has been approved, there may be IP barriers preventing use of that line. In addition, each manufacturer will have to fully characterize and obtain regulatory approval for their cell banks.

One possible means to circumvent the concerns surrounding cell-culture-based production of influenza vaccines would be the use of CEFs in instances in which a manufacturer already had processes in place for producing vaccines such as mumps or measles vaccine. In these situations, it may be relatively straightforward and inexpensive to transfer the process to flu-vaccine manufacture.

10.3 Egg-based manufacture of LAIV

Producing LAIV in eggs has several features that would make it a suitable technology for use in developing countries:

- **Cost of establishment.** The relatively high yield of the process means that a facility producing large quantities of vaccine can be established for a fraction of the capital investment required for plants producing IIV in eggs or cell culture.
- **Technology transfer.** The technology required is straightforward and simple, both at the laboratory and industrial scale. Complex equipment and procedures are not required.
- **Speed.** The time to establish and validate a plant capable of producing millions of doses is relatively short, less than two years. In addition, the time required for each vaccine-production cycle is shorter than with other methods. This fact, coupled with the high yield of the process means that the time taken to produce a vaccine following the declaration of a pandemic is less than with the other approaches.
- **Cost per dose.** The process has a 15–30 fold higher yield per egg than the production of IIV in eggs or cell-culture.
- **Efficacy.** Data suggest that a mucosally delivered live attenuated vaccine will induce more-relevant immune responses and, possibly, better protection than parenteral delivery of an inactivated vaccine.
- **Needle free delivery.** LAIV is administered intra-nasally, either by spray or droplet. Use of sharps and the concomitant hazards of accidental needle-stick injury or improper use of needles are avoided.

However, a number of key issues need to be resolved before this approach can be recommended for implementation.

- **Intellectual property and strain availability.** A license to existing LAIV master vaccine strains will be needed.
- **Regulatory pathway.** LAIVs are not likely to fulfil the standard CPMP immunological criteria for licensure. Therefore new immunological correlates may need to be established. Clarity on the regulatory requirements for approval is required.
- **Formulation and delivery.** A formulation stable at 2–8°C needs to be developed along with suitable final containers and administration method.
- **Egg supply.** LAIV production currently requires a supply of SPF eggs. This could be limiting, particularly during an avian-flu pandemic. Therefore, it needs to be established whether SPF eggs are a critical requirement. Cell culture production of LAIV would avoid this issue, but would require addressing the issues listed in section 10.2, namely IP, investment and regulatory uncertainty.

10.4 Action points and next steps

In order to support these activities and address the issues listed above, WHO intends to:

- Examine the IP landscape surrounding use of LAIV strains, and to ensure that vaccine strains are available.
- Consult with reference laboratories such as National Institute for Biological Standards (NIBSC) to determine whether LAIV vaccine seed strains can be produced on a rolling basis.
- Clarify the requirements for regulatory approval for new LAIV strains. In particular, to ascertain clinical-trial endpoints and requirements for the quality of eggs used for production.

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Figure 1: Timelines for establishing influenza vaccine production facilities

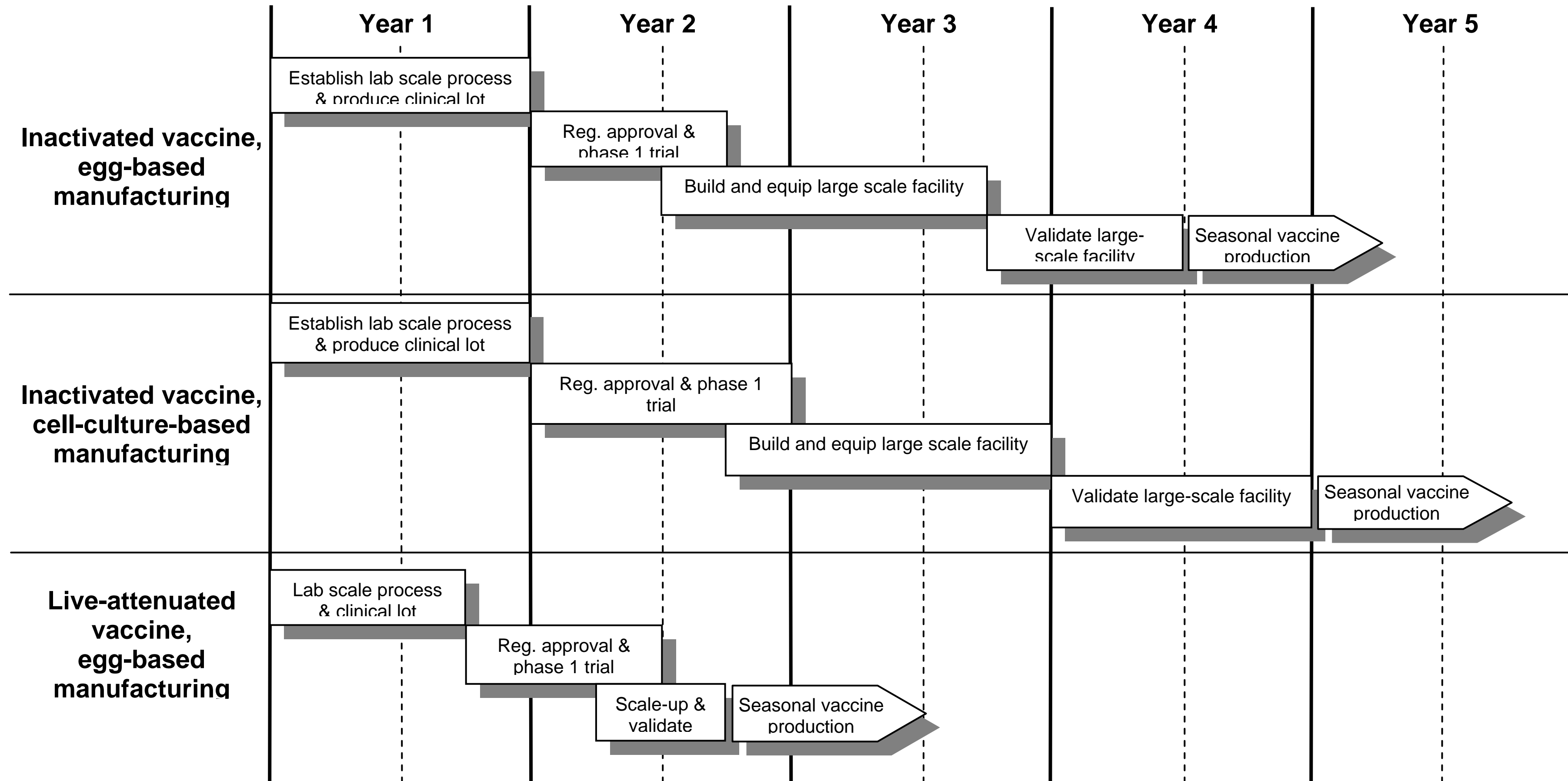


Figure 2. Comparative times for producing a batch of pandemic influenza vaccine

Week	1 - 6	7	8	9	10	11	12
IIV Eggs	Production of vaccine strain[1]	Inoculation and culture	Harvest, split & purify		Sterility testing[2]		Release[3]
IIV Cells		Inoculation and culture	Harvest, split & purify		Sterility testing[2]		Release[3]
LAIV Eggs		Inoculation culture and harvest	Sterility testing		Release		

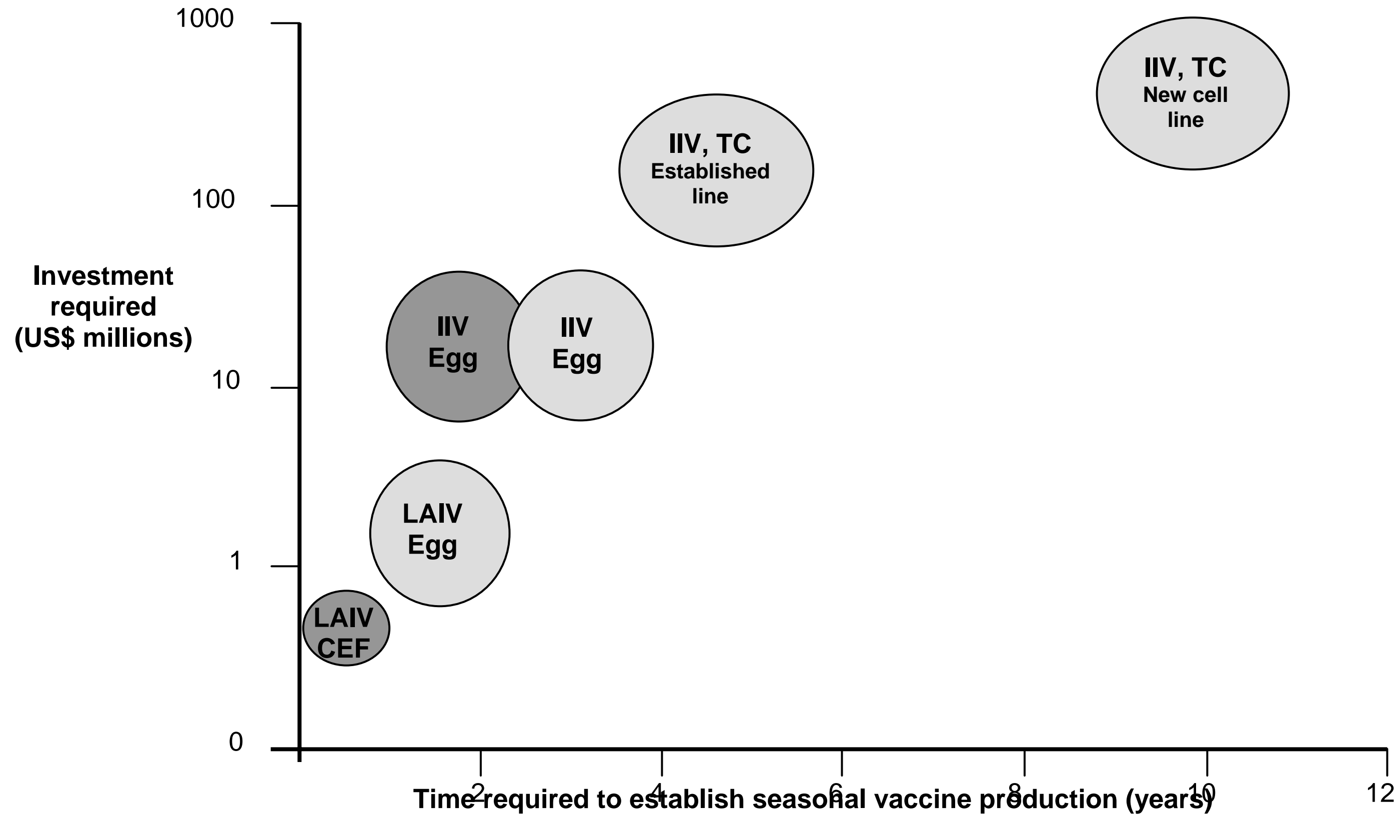
Notes and assumptions

1 – Production of vaccine seed on LAIV background strain is not routinely undertaken at present. This would have to be established at WHO collaborating centres

2 – Additional QC testing will be required in addition to sterility testing. However sterility testing would be the rate-limiting step determining release date (but see note 3).

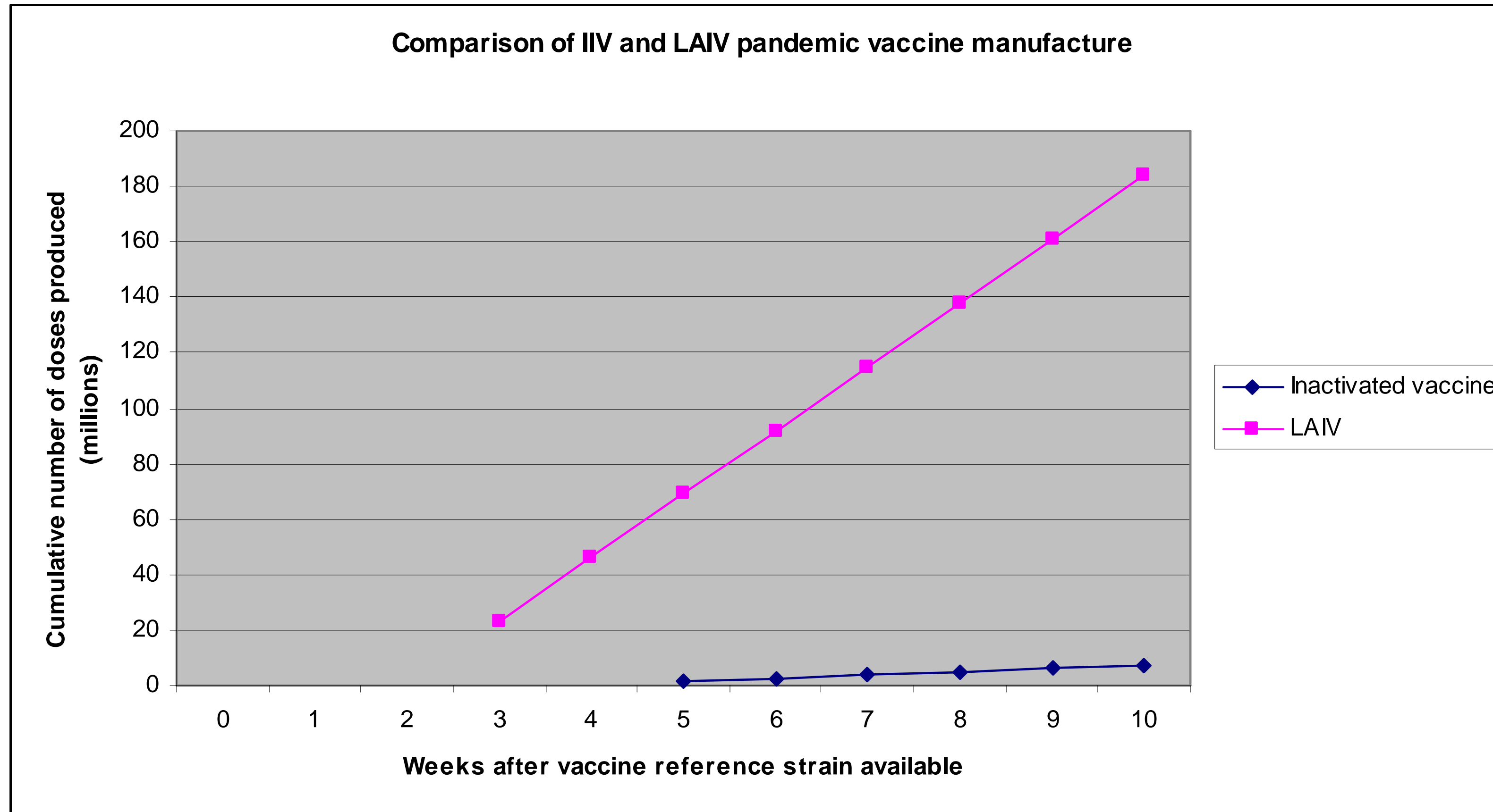
3 – Assuming release of IIV is possible on protein quantification data only. Reagents to quantify HA content may not be available until weeks 16 – 20.

Figure 3. Comparative timeline and cost required to establish large-scale seasonal influenza vaccine production capacity



Dark shading – adapt or scale-up the process in an existing facility using the same methodology
Light shading – set up process from scratch on an existing vaccine campus
Area of circle represents relative scale of production

Figure 4



For facilities of the same size and with the same number of eggs, the speed of production of LAIV is much greater than for IIV since the yield per egg is greater, there is only limited downstream processing for the LAIV, and the QC and release of LAIV is more rapid. This results in far greater cumulative doses produced in the window after a vaccine reference strain becomes available.