World Health Organization

Annex 5

WHO biosafety risk assessment and guidelines for the production and quality control of human influenza pandemic vaccines

This document provides guidance to National Regulatory Authorities (NRAs) and vaccine manufacturers on the safe production and quality control of human influenza vaccines produced in response to a pandemic threat. The document outlines, in detail, international biosafety expectations for both pilot scale and large-scale vaccine production and control and thus is relevant to both development and production activities. It should be read in conjunction with the WHO Laboratory Biosafety Manual (WHO, 2004) and replaces the earlier WHO guidance 'Production of pilot lots of inactivated influenza vaccines from reassortants derived from avian influenza viruses *Interim biosafety risk assessment*' (WHO 2003) . Tests required to evaluate the safety of candidate influenza vaccine reference viruses by WHO Reference Laboratories prior to release to vaccine manufacturers are also specified in this document.

The following text is written in the form of guidelines instead of recommendations. Guidelines allow greater flexibility than Recommendations with respect to expected future developments in the field. These guidelines specify steps to minimize the risk of introducing influenza virus strains with pandemic potential from a vaccine manufacturing facility into the community. If a national regulatory authority so desires, these guidelines may be adopted as definitive national requirements, or modifications may be justified and made by a national regulatory authority. It is recommended that modifications to the principles and technical specifications of these guidelines be made only on condition that the modifications ensure that the risks of introducing influenza virus to the community are no greater than as outlined in the guidelines set out below.

Adopted by the 56th meeting of the WHO Expert Committee on Biological Standardization, 24-28 October 2005. A definitive version of this document, which will differ from this version in editorial but not scientific detail, will be published in the WHO Technical Report Series.

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Summary

International biosafety expectations for both pilot-scale and large-scale vaccine production of human influenza vaccines produced in response to a pandemic threat, and the quality control of these vaccines, are described in detail in these WHO Guidelines. Tests required to evaluate the safety of candidate influenza vaccine reference viruses prior to release to vaccine manufacturers are also specified in this document which is thus relevant to both development and production activities, and also to vaccine and biosafety regulators. A detailed risk assessment is presented. This concludes that the likelihood of direct harm to human health if non-reassortant wild-type H5 or H7 viruses with multiple basic amino acids at the HA cleavage site and high *in vivo* pathogenicity are used for vaccine production, they potentially would be high and such viruses pose a high risk to animal public health too. Stringent vaccine biosafety control measures, defined as BSL3 enhanced (pandemic influenza vaccine) are defined to manage the risk from vaccine production and quality control using such viruses in the pre-pandemic period. For all other vaccine strains, for example reassortants derived from H5 or H7 strains in which the multiple basic amino acid HA0 cleavage site has been removed, the direct risk to human health is very remote. Nevertheless, there is an indirect risk to human health since there is a theoretical possibility of secondary reassortment with normal influenza human viruses and that such reassortant viruses may be replication-competent in humans, whilst having avian-like coat proteins. Although very unlikely, the secondary reassortant could become adapted to human infection and transmission which, if vaccine production was taking place in the pre-pandemic period, would have serious public health consequences. The biosafety control measures that are proposed, defined as BSL2 enhanced (pandemic influenza vaccine), take this, and also potential risks to animal health, into account. Facility and personal protection specifications are provided for both BSL2 enhanced and BSL3 enhanced bioafety levels and guidance is provided on biosafety management and implementation within a vaccine production facility. Tests to

be performed on candidate vaccine reference strains prior to release to vaccine developers depend on the type of virus but include, at a minimum, ferrets (or other susceptible mammals) and also, where necessary, chickens, egg embryos, plaque assays and sequencing.

1. Introduction

The earlier WHO guidance 'Production of pilot lots of inactivated influenza vaccines from reassortants derived from avian influenza viruses *Interim biosafety risk assessment*' (2003) was prepared in response to the pandemic threat posed by highly pathogenic H5N1 avian influenza viruses and the need to begin development of experimental vaccines. This threat persists to the present day so that several countries are now planning large scale H5N1 vaccine production. The risk assessment that informed the WHO biosafety guidance for pilot lot vaccine production (WHO 2003) has therefore been reassessed in light of the intended greater scale of vaccine production where production facilities are likely to be different from those used in developing small pilot lots, and also in light of the experience gained from developing and testing vaccine reference viruses derived by reverse genetics from highly pathogenic avian influenza viruses.

This document follows the risk assessment scheme used in the above WHO risk assessment for pilot lot vaccine production. The risks associated with large scale production are likely to be different from pilot lots, e.g. the "open" aspect of some production processes and quantity of virus-containing waste. However, the development of pilot lot vaccine provides experience that assists in re-evaluating the hazards presented by the vaccine strain.

Furthermore, the range of vaccine development options is broader than originally considered in the WHO risk assessment for pilot lot production and the current document has been expanded to encompass current vaccine development pathways.

2. Scope of the Risk Assessment

In recent times, much effort has gone into H5N1 vaccine development and manufacture and so the guidance presented herein is influenced considerably by the experience gained with this strain and our knowledge of H5 strains in general. It is, nevertheless, intended that the guidance will also be applicable to future pandemic threats from other potential pandemic strains, such as H2 or highly pathogenic H7.

There is a range of possible pathogenicities in the candidate vaccine production viruses not only for humans but also for other mammals and avian species. On the one hand, H5 viruses that can be highly pathogenic for both humans and chickens have been used to produce reassortant viruses genetically modified to be of low pathogenicity for chickens and mammals. On the other hand for strains inherently less pathogenic for humans, wild-type virus might be used directly for vaccine production. Thus reassortants

derived by reverse genetics, empirically-derived reassortants, which may or may not be genetically modified, and native wild strains are within the scope of the guidelines.

Eggs have traditionally been used for influenza vaccine production, but cell culture techniques have been recently introduced and international expectations for production and quality control specifications defined (WHO, 2005a). For pandemic vaccine development, either method may be used; thus both egg and cell culture production methodologies are included within the scope of the document.

Most effort to date with candidate pandemic vaccine development has been targeted towards inactivated vaccines. In one country however two live attenuated virus vaccines are under development as potential pandemic vaccines. This may raise important issues beyond the risks to humans, namely the potential for excreted viruses to infect and replicate in livestock. As H5 and H7 are notifiable strains to the OIE, this could have very important economic and trade ramifications. Developers and regulators will need to assess both the human and the agricultural risk of live pandemic strain vaccines under development should shedding and replication be possible. Both vaccine types (inactivated and live) are therefore covered in the scope of these guidelines.

Furthermore it is intended that the risk assessment and the guidelines on containment measures apply to all facilities and laboratories that have a need to handle live vaccine virus. This includes not only the vaccine manufacturing facility but also to the quality control laboratories of the manufacturer and, if appropriate, to National Control Laboratories. The transport of live virus materials within and between sites should comply with international specifications (WHO, 2005c).

Finally it should be noted that the risks assessment for vaccine manufacture will vary according to whether production is occurring in a interpandemic period, in a pandemic alert period (as for example early in 2004 when H5N1 was threatening to circulate extensively in South East Asia) or in a pandemic period. The document is intended to minimize risks from vaccine manufacture especially during the interpandemic period. However, the document also indicates where modifications may be made during other pandemic periods.

3. Hazard Identification

Hazards associated with pandemic vaccine manufacturing and laboratory testing are dependent on the type of pandemic vaccine strain (reassortant versus wild type), method of production (egg-based versus cell-based) and intended virus viability in the product (inactivated versus live attenuated). The type of vaccine strain, the proposed testing schedule and containment are illustrated in table 1.

3.1 Hazards associated with the type of pandemic vaccine viruses

3.1.1 Hazards associated with the recipient virus in a reassortant strain

Pandemic vaccine reassortants have been produced on the human strain A/ PR/8/34 (PR8) as recipient virus. PR8 has had over 100 passages in each of mice, ferrets and embryonated chicken eggs. The result of such a passage history is complete attenuation of the virus and its inability to replicate in man (Beare et al, 1975).

PR8 grows to high titre in embryonated chicken eggs and since the late 1960s, it has been used to produce 'high growth reassortants' in combination with the prevailing influenza A vaccine strain. Use of such reassortants as vaccine strains has increased vaccine yield many-fold. The reassortants are produced by a mixed infection of eggs with PR8 and the nominated vaccine strain, combined with a selection system based on anti-PR8 antibody and growth at high dilution.

Live attenuated influenza vaccines are licensed in some countries. The parental strains used in those live attenuated vaccines e.g. A/Ann Arbor/6/60, are also potential recipient strains for development of pandemic reassortant vaccines. These parental strains possess phenotypic markers of vaccine safety, such as temperature sensitivity, cold-adaptation and attenuation in ferrets or rodents and moreover have a demonstrated attenuated phenotype in man.

3.1.2 Hazards arising from the inserted gene product in a reassortant vaccine strain

The products of the inserted genes will be, at minimum, the haemagglutinin (HA) and neuraminidase (NA) of the pandemic strain virus. For reassortants derived from highly pathogenic H5 or H7 strains by reverse genetics, the HA will have been modified so that the multiple basic amino acids at the HA cleavage site, which are associated with high pathogenicity, will be reduced to a single basic amino acid. Any protein derived from the wild type strain, on its own will be neither inherently infectious nor harmful.

3.1.3 Hazards arising from reassortant viruses

3.1.3.1 Direct hazards

Without treatment, reassortant viruses may be expected to survive for at least a short time (hours) on surfaces or in a laboratory environment and thus provide a potential means of infection for laboratory workers. Although the surface antigens of reassortants, particularly the HA, can contribute to pathogenicity (Beare et al 1975, Kilbourne 1980) published information indicates that a reassortant between PR8 and a wild-type human influenza virus is likely to be avirulent in man (Beare and Hull, 1971; Beare et al, 1975 ; Florent, 1980; Oxford et al, 1978). Although such information is difficult to interpret because the genetic composition of the reassortants was not clear, it is known that the degree of attenuation increases as reassortants include more PR8 genes (Florent et al,1997; M Tashiro, unpublished data). The reassortants created by reverse genetics as H5N1 pandemic reference strains contain six out of eight viral genes from PR8 and the NA and modified HA genes of the H5N1 virus. Furthermore, the H5 HA retains a preference for α2,3 linked residues (see below), so the ability of the H5N1 reassortants to bind to and replicate in human cells should be minimal. It is therefore envisaged that an

H5N1 reassortant derived by reverse genetics according to WHO guidance (WHO 2005b) would be attenuated for humans compared to the H5 wild type. Furthermore, it is clear that such reassortants are expected to be of low chicken and animal pathogenicity compared to the highly pathogenic parental wild strains, and this has been borne out by experience to date. Nevertheless, as the factors affecting pathogenicity (see below) are not fully understood, genetic manipulation to remove the polybasic sites theoretically could have unpredicted effects on both transmissibility and pathogenicity.

For reassortants derived by traditional co-cultivation methods, the gene constellation is less predictable. There is a theoretical possibility of developing reassortants with more than two wild-type parental genes or even of selection of a mutant (non-reassortant) wild-type virus with improved growth characteristics. If vaccine production is occurring in the interpandemic phase there would be a need to determine the gene constellation of reassortants derived by traditional co-cultivation methods to facilitate the risk assessment.

Reassortants with a 6:2 gene constellation based on live attenuated recipient strains such as A/Ann Arbor/6/60, or other strains used as live attenuated vaccines, may also be used for pandemic influenza vaccine production. The attenuated A/Ann Arbor/6/60 strain has been used as a backbone in 6:2 reassortant live attenuated vaccines in clinical studies for more than 30 years using approximately 30 different vaccine strains, and the data demonstrate that the Ann Arbor/6/60 virus produces reassortant vaccine strains that are attenuated for humans (CDC, 2005). Live vaccines derived from the Ann Arbor strain have been licensed in one country. An adequate/appropriate level of attenuation should be expected for modified H5 reassortant strains. For each candidate pandemic strain, this should be verified by testing as described below (section 3.6.1).

Reassortants may be also be derived from non-H5 or non-H7 viruses (eg H9N2, H2N2) and may use either PR8 or an attenuated vaccine strain. The hazards associated with such reassortants depend on HA receptor specificity. If a reassortant has a preference for avian cell receptors (α2,3 linked sialic acid eg avian H2N2 viruses), the hazards are considered to be no different from those associated with the above-mentioned 6:2 reassortants derived from de-pathogenised H5 or H7 viruses (see section 3.4.3). However if a reassortant has a preference for mammalian cell receptors (α2,6 linkages eg human H2N2 pandemic virus from 1957), or possesses both avian and mammalian receptor specificities (eg H9N2), there is a greater hazard for human infection (see table 1).

3.1.3.2 Indirect hazards

Although it is considered that, for example, an H5N1/PR8 reassortant will be either attenuated or possibly non-infectious for man, an indirect hazard may exist through secondary reassortment with a human or animal influenza virus as influenza viruses are known to exchange genes by the process of reassortment. In order for secondary reassortants to be generated several events need to occur; firstly infection of

the production staff with the reassortant strain; secondly, for an infected worker to have a mixed infection with a wild type influenza virus, and thirdly for a reassortment event to take place. In practice, manufacturers have 30 years of experience with large scale production of vaccines based on PR8 reassortants and no reported cases of human illness (see section 3.5) although it should be noted that data are not available on whether human infections may have occurred . Also vaccine production workers can be vaccinated with current seasonal influenza vaccines to reduce the chances of infection with wild-type virus.

In practice, the lack of success in producing H5N1 reassortant vaccine strains in 1997 (UK: avian and swine viruses; Australia and USA: avian and PR8 viruses) suggests the probability of producing H5 reassortants between mammalian and avian viruses in human cells is slight. It should also be considered that poultry and pig farmers are continually exposed to animal influenza viruses and there have been few documented cases of human infection in this population with a reassortant between an avian or porcine and a human influenza virus. Based on these considerations the probability that a PR8 reassortant strain will replicate and combine with another influenza virus(es) in human cells is considered to be minimal. The risk of such secondary reassortments for animal species will be considered in the environmental risk assessment section.

3.1.4 Hazards arising from the use of wild type viruses for pandemic strain vaccine production

Wild-type strains may be considered for production purposes and different categories may be potential vaccine candidates. For example, they could be (i) an avian strain with no record of human infection (surrogate virus), (ii) an avian strain with documented human infection (potential pandemic virus), (iii) an actual human pandemic virus, or (iv) a past H2N2 pandemic virus. The hazards from wild type vaccine strains will differ according to the category of wild-type virus used but in all cases are compounded during vaccine manufacturing and associated vaccine product testing, due to the high volumes and/or high titres encountered. With the exception of surrogate viruses, the use of wild type pandemic-like influenza viruses to develop pandemic vaccine strains presents considerable biosafety risks to personnel in vaccine manufacturing facilities and testing laboratories, and also to the general community especially if manufacture is taking place for clinical studies and stockpiling of vaccines during an interpandemic period.

3.2 Hazards arising from the type of production

Vaccine manufacture takes place under Good Manufacturing Practices for Biologicals (WHO, 1992). GMP requires protection of the product from the operator and the environment and thus amelioration of certain hazards associated with production will require a suitable balance between GMP and biosafety requirements to be established.

3.2.1 Production in eggs

Influenza vaccine has been produced in embryonated hens eggs at large scale since the early 1950s. Much experience has been gained and some facilities are capable of handling large numbers of eggs on a daily basis with the aid of mechanised egg handling, inoculation and harvesting machines.

Hazards occur only for the production stages and quality control laboratory activities prior to virus inactivation. The most hazardous production stage is egg harvesting when the eggs have to be opened to harvest the allantoic fluid. The volume and titre of virus is higher than at any other stage. The open nature of the operations provides a greater exposure to aerosols and spills. In contrast during egg inoculation, the virus used is dilute and a relatively small volume. The allantoic fluid that is harvested from the eggs is invariably manipulated thereafter in closed vessels and so hazards arising from live virus during downstream processing and during the virus inactivation process, if used, are less than during virus harvest. Egg waste collection and disposal is a potential major environmental hazard. For egg-grown vaccines safe disposal of the waste, both within the plant and beyond, is therefore critical

3.2.2 Production in cell cultures

Biosafety risks associated with production and testing of pandemic cell-based influenza vaccines are associated primarily with the nature of the cell culture system utilized for virus production. Closed systems, such as bioreactors, normally present little to no exposure opportunities to live virus during normal operation, but additional measures must be taken during procedures where samples are introduced into or taken from the bioreactor, and during procedures to deal with accidental spills. Roller bottles and cell culture flasks used for virus production may generate exposure to live virus through aerosols, spills, and other operations during virus production and become contaminated solid materials following virus infection.

The possibility exists that genetic mutations may be selected in pandemic vaccine viruses during passage in mammalian cells that render them more adapted to humans. Sequence analysis of the region of the HA gene encoding the receptor binding site may be useful. However, it should be noted that little is known about the relation between cell substrate and virus reversion or adaption. Beare et al., (1975) tried to de-attenuate PR8 by multiple passage on organ cultures of human tissue but failed, whilst studies with MDCK cells (Robertson et al, 1995) demonstrated that human viruses that retained their α2,6 receptor specificity (human-like) were likely to mutate to an α2,3 specificity (avian-like) as this provided a replicative advantage on MDCK cells, rather than the reverse. Overall, hazards arising from the inherent properties of a reassortant or wild type virus are likely to be far greater than the probability of adaptation of the virus to a more human-like phenotype.

3.3 Factors affecting pathogenicity for humans

3.3.1 HA receptor specificity

The influenza HA is responsible for attachment of virus to the target cell and has specificity for sialic acid receptors on cell surface molecules. The HAs present on human influenza A viruses preferentially bind to receptors containing α2,6 linked sialic acid residues, whereas avian influenza viruses preferentially bind to α2,3 linked sialic acid (Rogers and D'Souza, 1989). Human tracheal cells have mainly α2,6 linked residues (Nelson et al, 1993), so the acquisition of an avian HA by PR8 virus is expected to minimize potential binding to human respiratory epithelial cells. Although the α2,3 receptor specificity of avian viruses will reduce the efficacy of such binding, it may not completely prevent infection in man. Moreover, the presence of avian-like receptors has been demonstrated in human respiratory tract epithelium (Matrosovich et al, 2004). Beare and Webster (1991) found that extremely large quantities of avian viruses (between $10^{6.8}$ and $10^{9.2}$ egg infectious doses) were needed for replication in man and, since replication was poor, that it was not possible to induce person-to-person transmission, ; .

There have been many human infections with avian H5N1 viruses since 1997 in SE Asia. It is possible that exposure to high titre H5N1 virus in contaminated chicken or duck carcasses or animal products, may have overcome the avian specificity of HA receptor binding. Virus replication in such human cases was much better than in the earlier experimental studies of avian influenza viruses in humans (Beare and Webster, 1991); however, the extensive replication of H5N1 viruses in these persons is inexplicable on the basis of current knowledge of receptor specificity since the viruses isolated from them retained the α2,3 avian specificity.

3.3.2 HA cleavability

The HA of influenza virus must be cleaved into HA1 and HA2 by host cell proteases for a productive infection. Pathogenicity of H5 and H7 influenza A viruses for chickens is largely determined by the nature of the amino acids at the HA cleavage site. H5 and H7 viruses with multiple basic amino acid sequences are highly pathogenic and their HA can be effectively cleaved by ubiquitous furin-like proteases, which are expressed in most organs of birds and humans. In contrast, the HA of H5 and H7 viruses of low pathogenicity for birds and certain laboratory animals contain a single basic residue at the cleavage site, a feature common to all other subtypes of influenza HA, and which can only be cleaved by trypsin-like proteases, which are restricted to certain cell types, e.g. epithelial cells lining the respiratory tract of man and the gut of birds. Thus, HA cleavability influences tissue specificity and is a major determinant of pathogenicity for H5 and H7 viruses in chickens and certain laboratory animals. Multiple basic amino acids at the cleavage site have not been observed for any other HA subtype.

Direct evidence has been obtained that both HA cleavage and HA receptor specificity have an effect on tissue tropism of an avian H7N1 virus, A/Fowl Plague/Rostock/34 in chicken embryos (Feldmann et al, 2000). Similarly, the available evidence from the 1997 H5N1 infections, demonstrates that the high degree of pathogenicity in chickens, mice and ferrets is directly influenced by the presence of the multiple basic amino acids. Webby et al (2004) demonstrated that removal of the basic amino acids changed H5N1 infections from a fatal systemic infection to a localized non-pathogenic infection in chickens (i.e low pathogenicity for chickens), mice and ferrets.

Hatta et al (2001) and Lipatov et al (2005) have also shown by reverse genetics that high cleavability of H5N1 HA due to the presence of multiple basic amino acids was an essential requirement for a lethal mouse infection. It is not appropriate to examine pathogenicity of influenza virus infection in man, but an examination of H5N1 viruses by Gao et al (1999) provided evidence that pathogenicity in mice can resemble that in man. The occurrence of multiple organ failure after human H5N1 infections is suggestive of an unusual tissue tropism. Although evidence for viral replication outside the lung has been described for at least one human case (Uiprasertkul et al 2005 *)*, such evidence remains difficult to document (To et al, 2001).

The available evidence suggests that virulence of the 1997 and later H5N1 viruses for man is related to the presence of the HA multiple basic amino acids. It is therefore considered imperative to remove them, if present, in the HA of any H5N1 virus being developed as a vaccine strain in order to reduce the potential for harm to humans. This procedure will also increase the safety of the reassortants for avian species (see later under environmental risk assessment) as cleavage site modifications have resulted in reduced killing of avian embryos (Nicolson et al., 2005). It should be noted that during production of reassortants by reverse genetics, base substitutions are introduced in order to stabilize the removal of multiple basic amino acids during passage of reassortants.

3.3.3 Other factors affecting pathogenicity

Whilst it is clear from experience in SE Asia from 1997 to the present that H5N1 influenza viruses that display α2,3 sialic acid specificity could replicate in humans, it must be noted that influenza virus pathogenicity does not depend solely on HA, but is a polygenic trait. The 1997 H5N1 virus had unusual PB2 and NS1 genes that influenced pathogenicity whilst the 2004 H5N1 viruses possess complex combinations of changes in different gene segments that affect pathogenicity in ferrets (Govorkova et al, 2005). Changes in the PB2 gene of the 1997 H5N1 viruses were sufficient to attenuate them for mice (Hatta et al, 2001) and changes in the NS1 protein rendered these viruses resistant to the effects of interferons and other cytokines produced as part of the innate immune response (Seo et al, 2002). The NS1 changes conferred a highly virulent phenotype which allowed replication to proceed unchecked *in vivo*. In this case even a virus with a poor affinity for its receptor was able to replicate (although not to transmit). In contrast, viruses with a PR8 internal protein gene constellation were clearly sensitive to the innate immune mechanisms which prevent the establishment of infection by an avian virus in humans. This may well explain why in H5 avian influenza outbreaks before 1997, no evidence of transmission from birds to man has been noted. Further, prior to the 2003 outbreak in the Netherlands, transmission from birds to man of H7 viruses was documented in just two cases (Campbell et al, 1970, Kurz et al, 1996). Also during many years of laboratory handling of high titre avian viruses (of which one H7 strain [A/FPV/Dobson] is known to contain a gene which adapts it for replication in mammalian cells [Almond, 1977]), there is only one report in the literature of a worker being affected by these viruses. This was a laboratory worker in Australia who developed conjunctivitis after accidental exposure directly in the eye with a H7N7 virus [Taylor and Turner, 1977]. The PR8/H5N1 6:2 reassortants and the A/Ann Arbor/6/60 live attenuated

6:2 reassortants created by reverse genetics for H5N1 vaccine production do not contain the gene constellation considered necessary for pathogenicity in chickens, mice and ferrets and in contrast have internal genes that confer sensitivity to the innate immune response.

3.4 Hazards arising from the vaccine

Inactivated pandemic influenza vaccines present no biosafety risks provided that results from the inactivation steps show complete virus inactivation, as the viral vaccine is rendered incapable of replication.

In an interpandemic or pandemic alert period, pilot scale live attenuated pandemic influenza vaccines may be developed for clinical evaluation. As there is some uncertainty with regard to biosafety risks arising from release into the environment, potentially with each dose administered to a human vaccinee, clinical trials in the interpandemic or pandemic alert phase should be conducted under appropriate clinical isolation conditions for the subjects participating in the clinical trial. If not, indirect hazards for man will arise as considered in section 3.1.3.2. Furthermore, for pandemic human influenza vaccine strains that express H5 or H7 avian influenza genes, there will be potential consequences for agricultural systems. This is because all viruses of H5 or H7 subtype are notifiable to OIE. An accidental exposure of livestock could have serious economic and trade implications for national agriculture systems if the viruses were transmissible.

If a human pandemic has started the hazards from live attenuated vaccines elaborated above will not be relevant.

3.5 Prior large scale experience with reassortants

Reassortants derived from PR8 have been used routinely for production of inactivated influenza vaccines for the past 30 years. This work involves production of many thousands of litres of infectious egg allantoic fluids, which will create substantial aerosols of reassortant virus within manufacturing plants. Most of the reassortants were made from wild type human strains that had not yet been in widespread circulation. Thus, although the manufacturing staff would have some susceptibility to infection with the wild type virus, there have been no anecdotal or documented cases of work-related human illness resulting from occupational exposure to the reassortants although, as noted previously, data are lacking concerning the frequency of silent infections . Nevertheless, this is further testimony to the attenuation of PR8 reassortants.

Reassortants derived from the attenuated A/Ann Arbor/6/60 strain have been used for production of live attenuated vaccine for at least three years. There have been no anecdotal or documented cases of work-related human illness resulting from exposure to the reassortants although, as noted previously, data are lacking concerning the frequency of silent infections . Nevertheless,this is further testimony to the attenuation of Ann Arbor reassortants.

However, unlike the situation with annual vaccine production, staff manufacturing an H5N1 vaccine would have no previous immunological experience of the avian virus, so staff would be expected to be susceptible, although the risk of work-related human illness and of transmission outside of the facility is expected to be slight and lower than for non-reassortant strains.

3.6 Testing of reference viruses being considered for vaccine production

Vaccine reference viruses will be developed by a WHO laboratory or by one approved by a national regulatory authority (hereafter, for ease of reference, referred to as a WHO laboratory). The following tests and specifications have been developed based on experience gained in the evaluation of 6:2 reassortant H5N1 viruses produced on the PR8 and A/Ann Arbor/6/60 backbones. The principles outlined should be applicable during the interpandemic period to other reassortant strains, but exceptions may be made if appropriately justified. Tests on wild-type viruses being considered for vaccine production will need to be determined on a case-by-case basis. The tests below are usually conducted by the WHO laboratory developing the reference strain.
In a pandemic alert period, or in a pandemic period, the need for the conduct or the completion of some or all of these tests prior to the distribution of a candidate reference strain may not be required based on the risk assessment. For example, in a pandemic alert period , a candidate reference strain which on the basis of molecular analyses is expected to have a low risk of human infection and transmission could be distributed to vaccine manufacturers in order for them to begin preparation of their seed stocks prior to the completion of time consuming tests such as the chicken and the ferret pathogenicity tests. If a pandemic has begun, and the pandemic virus has become adapted to human infection, there may be no need to perform all the pathogenicity tests indicated below.. A risk assessment should be performed for each candidate reference strain and the outcome will depend on the nature of the strain and the pandemic period declared by WHO.

3.6.1 In vivo tests to evaluate pathogenicity of H5 and H7 viruses:

For optimal interpretation of tests, the pathogenic properties of the candidate reference virus, should be compared with the parental backbone strain and the wild-type strain.

These tests should be performed under appropriate high laboratory containment conditions (see section 4.3) Tests to be performed on the candidate vaccine reference strain (see Table 1) by the WHO reference laboratory that develops the reassortant strain include:

- the ability to plaque in the presence/absence of added trypsin. Viruses of high pathogenicity can replicate in mammalian cell culture in the absence of added trypsin whereas low pathogenicity viruses generally do not.
- the ability to cause chicken embryo death. Highly pathogenic viruses cause rapid chicken embryo death upon inoculation into eggs whereas removal of the multiple

basic amino acids from a highly pathogenic strain results in embryo survival (Nicolson et al., 2005).

- the viruses must be found to be of low pathogenicity in the statutory chicken intravenous pathogenicity test (IVP index of 1.2 or less) (OIE, 2005). This is an important statutory test required by veterinary authorities before the reassortants can be removed from high containment. Development of specifications to indicate that the test articles have been correctly administered in the IVP test would be beneficial.
- the viruses should be shown to be attenuated in ferrets or in other suitable animal models, provided they have equivalent virus sensitivity to ferrets and a similar ability to discriminate between highly pathogenic and non pathogenic influenza viruses. These tests are done in comparison with the wild-type virus. Detailed test procedures are described in Appendix 1. In the case of H5N1 reassortants the criteria used to evaluate this test were that virus replication and clinical symptoms should be comparable to those induced by the attenuated PR8 parent virus and should be milder than the wild type human H5N1 virus infection.

Ferrets were chosen because they have been used extensively as a good indicator of influenza virus virulence for man (reviewed by Smith and Sweet, 1988). Typically, human influenza viruses cause lethargy, nasal discharge and occasionally fever in ferrets, and virus replication is usually limited to the respiratory system. PR8 virus has been assessed in ferrets and it causes little or no clinical signs and virus replication is limited to the upper respiratory tract. However, the 1997 and 2004 wild type human H5N1 viruses replicated in ferrets throughout the body, caused fever, weight loss and occasionally death (Zitzow et al, 2002; Govorkova et al, 2005). Thus, in terms of predicting a highly pathogenic human infection or an infection which is attenuated for man, in the absence of human data, the ferret is the best available model.

It would be useful to measure transmissibility as well as pathogenicity of strains, but currently there is a lack of a well-characterized methodology to do so. Intranasal administration of chickens may be one such method, and has been shown to be possible, but to date the test is not standardized. Uninoculated birds in close contact with infected birds in the intravenous pathogenicity test may provide some information on transmissibility. Transmission studies in ferrets are also available after oral and ocular inoculation, but also need to be standardized.

Tests for safety in mice may provide useful information if the parent strain is virulent in mice. Detailed test procedures are described in Appendix 1.

Only after appropriate compliance with the above tests has been shown should a reassortant virus be used for vaccine manufacture. For H5 and H7 strains, the nucleotide sequence corresponding to the HA cleavage site should be determined by the WHO laboratory to demonstrate the absence of multiple basic amino acids in the vaccine candidate. After WHO has declared a pandemic manufacturers may receive candidate reference strains that have not been assessed fully for pathogenicity. In this case they

should handle the viruses appropriately depending on the nature of the virus and the pandemic situation.

3.6.2 Genetic stability of H5 and H7 viruses

Genetic stability is an important issue as it is known that in poultry, wild type low pathogenicity H5 and H7 avian viruses can become highly pathogenic by mutation (insertion of basic amino acids at the HA cleavage site) and this is the origin of highly pathogenic H5 and H7 strains. Although the derivation of low pathogenicity candidate reference viruses by reverse genetics involved the introduction of silent mutations in the region of the HA cleavage site that should minimize the re-insertion of multiple basic amino acids, during vaccine production such viruses may be passaged several times and so it is important to evaluate their genetic stability at the cleavage site. Several attenuated reassortants have now been produced between PR8 virus and highly pathogenic H5N1, H5N3 and H7N1 viruses by reverse genetics (Liu et al, 2003; Nicolson et al 2005; Subbarao et al, 2003; Webby et al, 2004, FLUPAN website) and following extended passage in eggs (up to 10), they have each retained their attenuated phenotype.

Nevertheless, manufacturers should assess any H5 and H7 seed viruses and vaccine virus harvests by sequence analysis of the HA cleavage site. The need for studies of genetic stability for seed viruses prepared from candidate reference strains derived by other methods should be assessed on a case-by-case basis. At least one *in vivo* test (section 3.6.1) should be applied, for example the egg embryo lethality test.

3.6.3 Evaluation of wild type non-pathogenic H5 or H7 viruses or reassortants derived from them.

In view of the propensity for non-pathogenic H5 and H7 viruses to acquire mutations leading to increased pathogenicity, it is advisable to conduct the full spectrum of pathogenicity tests (ferret, chicken, chicken embryo) , as indicated in section 3.6.1.
3.6.4 In vivo evaluation of non-H5, H7 viruses or reassortants derived from them.
Ferret tests are required for non-H5, non-H7 candidate vaccine strains prior to manufacture. The tests should be conducted under biocontainment levels equivalent to that required for the production of the reference strain. The other tests specified in 3.6.1 and 3.6.2 are not required since they are specific for reassortants derived from highly pathogenic H5 and H7 viruses.

4. RISK ASSESSMENT

4.1 Health protection

4.1.1 Likelihood of harm to human health

By virtue of PR8 attenuation, avian receptor specificity, loss of multiple basic amino acids at the HA cleavage site and the absence of other H5N1 genes associated with pathogenicity in humans (i.e. NS1 or PB2 genes), it is envisaged that an H5N1 x PR8 2:6

reassortant, while possibly infectious for man and ferrets, will have only a low probability of causing harm to human health. On the basis of these arguments, reassortants derived from H5 or H7 strains in which the multiple basic amino acid HA0 cleavage site has been removed, using either PR8 or strains attenuated for humans e.g the A/Ann Arbor/6/60 as the recipient virus, likely would be similarly attenuated. Reassortants derived from all other subtypes or from low pathogenicity H5 and H7 subtypes, in which the multiple basic amino acids were not present, should also be attenuated by virtue of the receptor specificity of the avian HA and the attenuating effect of the 6 PR8 genome segments (absence of any other avian genes). The same arguments are also valid for reassortants prepared from live attenuated virus strains such as A/Ann Arbor/6/60.

If staff at a vaccine production plant are exposed to aerosols containing high titre reassortant virus, there is a possibility that sub-clinical infections could result. If this happened, it is very unlikely that a reassortant virus would transmit to human contacts as it is likely that replication will be attenuated and virus shedding, if it occurs, may well be below the titres considered to be needed for human infection.

However, although there is no precedent, as described above there is a theoretical possibility of secondary reassortment with normal influenza human viruses and that such reassortant viruses may be replication-competent in humans, whilst having avian-like coat proteins. Although very unlikely the, the secondary reassortant could become adapted to human infection and transmission in which case the public health consequences would be serious . Vaccine biosafety control measures should be considered to take this into account.

If non-reassortant wild-type viruses with multiple basic amino acids at the HA cleavage site and high *in vivo* pathogenicity, are used for vaccine production, they potentially would be highly pathogenic and transmissible in humans. Stringent vaccine biosafety control measures should be considered to manage the risk from vaccine production using such viruses. Non-reassortant wild-type viruses , without multiple basic amino acids at the HA cleavage site with low *in vivo* pathogenicity and avian receptor specificity are likely to be less pathogenic and less transmissible in humans (Beare and Webster, 1991) than the wild type viruses described above. However risks remain of secondary reassortment with normal human viruses and that such reassortant viruses may be able to replicate in humans. Appropriate vaccine biosafety control measures should be considered to manage the risk from vaccine production using such viruses. Non-reassortant wild-type viruses, without multiple basic amino acids at the HA cleavage site, with low *in vivo* pathogenicity and mammalian receptor specificity (eg human H2N2 and H9N2) are also likely to be less pathogenic than the wild type viruses described above, but their ability to transmit to humans is unknown. Consequently because of the risks of secondary reassortments, appropriate biosafety control measures should be considered.

4.2 Environmental protection

4.2.1 Nature of the work

Egg-based vaccine production represents a relatively open system with several operations likely to generate virus aerosols: seed virus preparation, egg inoculation, harvest of infected egg fluids, use of laminar outward air flows, segregation of contaminated eggs, cleaning (that may include high powered spraying) and decontamination of contaminated egg trays, and disposal of waste products.

Cell culture vaccine production involves handling, prior to virus inactivation, large volumes of high-titre preparations of live influenza virus. The majority of operations are carried out in closed systems. Nevertheless, leaks can occur from valves or during procedures where material is introduced into or taken out of the system, such as taking samples for testing purposes. Furthermore, if roller bottles and/or cell culture flasks are used for virus production, operations with these culture vessels may result in aerosols, spills, and other accidents involving release during virus production and become contaminated solid materials following virus infection.

4.2.2 Environmental considerations

Influenza A viruses are endemic throughout the world in some agricultural animals (swine and horses) and some populations of wild birds, specifically birds in the families *Anseriformes* (ducks, geese and swans) and *Charadriiformes* (shorebirds) (Swayne & Halvorson, 2003). Of the influenza A viruses, a number can cause disease in domestic poultry, such as H5, H7, and H9. H5 and H7 are thought to be highly pathogenic in poultry, whereas H9 is typically less so. In addition, sporadic infections by influenza A viruses have been reported in farmed mink, wild whales and seals, dogs and captive populations of big cats (tigers and leopards) (Swayne & Halvorson, 2003; Keawcharoen *et al.*, 2004). In dogs, the influenza A infections were caused by H3N8 viruses closely related to endemic equine viruses, and in the big cats, the infections followed consumption of dead chickens infected with H5N1 viruses.

In the case of an H5N1 reassortant, the virus will have avian receptor specificity, and thus birds would be the species theoretically most susceptible. The contribution of the six PR8 internal genes to replication and virulence in birds is unknown. However, Hatta et al (2002) has recently demonstrated by the use of reverse genetics that acquisition of only one PR8 gene by an avian influenza virus can abolish virus replication in ducks. Experimental evidence has demonstrated that PR8 virus is attenuated in not only humans (see above) but also chickens (Subbarao et al, 2003). Furthermore, a reassortant between PR8 (internal protein genes) and the 1997 Hong Kong H5N1 virus (NA and HA with a single basic amino acid) was barely able to replicate in chickens and was not lethal. Similar studies have been performed with the 2003 Hong Kong H5N1 virus at the WHO Collaborating Centre, Memphis, USA (R Webster, unpublished data), where the 6:2 PR8 reassortant did not replicate or cause disease signs in chickens. The removal of the multiple basic amino acids from the H5 x PR8 reassortants in both studies probably played a major role in reducing the risk for chickens. Although replication occurs in chicken embryos, for reasons that are unknown, the risk of environmental transmission via such replication in nature is remote.

Pigs are uniquely susceptible to infection by all strains of influenza A virus because they have both alpha 2,3 and alpha 2,6 receptors in abundance. Although pigs are not susceptible to infection with PR8, a reassortant containing a single gene (HA) from an A/New/Jersey/76 (H1N1) isolate infected pigs and the animals excreted virus (Kilbourne 1980 check Webster if further references). It is thus conceivable that pigs are susceptible to infection by an H5N1 reassortant, as viruses with avian receptor specificity are known to replicate in this species. It is also possible that these species would be susceptible to secondary reassortments between the H5N1 reassortant and a pig virus. There is in fact evidence that triple reassortants between avian, pig and human influenza viruses can circulate in pigs in the USA (Webby et al, 2000).

4.3 Assignment of containment level

The production of influenza vaccine reassortant reference viruses, by WHO Collaborating Centres, from highly pathogenic H5 or H7 wild type viruses should take place at a high level of biocontainment (BSL 3 enhanced or BSL 4, as advised by WHO and national authorities) (WHO 2005b). The collaborating centres provide characterized reassortant reference viruses to vaccine manufacturers who may develop vaccine seeds and vaccines from these materials.

In consideration of hazards associated with egg and cell culture H5 and H7 vaccine production and quality control with reassortant viruses of demonstrated low pathogenicity in chickens and/or in ferrets (and mice if applicable), as specified in 3.6.1 and 3.6.2, the assigned containment level is BSL2 enhanced (pandemic influenza vaccine), as defined below (see Table 1). This applies to both pilot scale and large scale production during the interpandemic phase and pandemic alert period (WHO 2005d) when the site of vaccine production is geographically remote from the site of the emerging pandemic. Any subsequent relaxation of the levels of containment during the developing pandemic, should be done on a case by case basis after careful evaluation of the risks.

In consideration of hazards associated with egg and cell culture vaccine production and quality control with wild-type viruses (non-H5 and non-H7) of demonstrated low pathogenicity in ferrets, as specified in 3.6.3, the assigned containment level is BSL2 enhanced (pandemic influenza vaccine), as defined below. This applies to both pilot scale and large scale production during the interpandemic phase and pandemic alert period (WHO 2005d) when the site of vaccine production is geographically remote from the site of the emerging pandemic. Any subsequent relaxation of the levels of containment during the developing pandemic, should be done on a case by case basis after careful evaluation of the risks.

In consideration of hazards associated with cell culture vaccine production and quality control with highly pathogenic H5 or H7 wild-type viruses, the assigned containment level is BSL-3 enhanced (pandemic influenza vaccine) , as defined below. This applies to both pilot scale and large scale production during the interpandemic phase

and pandemic alert period (2005d) when the site of vaccine production is geographically remote from the site of the emerging pandemic. Any subsequent relaxation of the levels of containment during the developing pandemic, should be done on a case by case basis after careful evaluation of the risks.. In addition, the parts of the facility where such work is done (both production and quality control) should meet the OIE requirements for containment, which includes not only biosafety but also requirements for biosecurity. (OIE, 2005). In view of the open nature of large scale egg-based vaccine production, it is not possible to operate at BSL-3 enhanced (pandemic influenza vaccine).Therefore egg-based vaccine production from high pathogenicity H5 or H7 wild-type strains is not recommended.

For vaccine production and quality control using other types of vaccine virus (eg reassortants derived from non-H5 or H7 viruses; wild type low-pathogenic H5 or H7 viruses), the assigned containment level is BSL2 enhanced (pandemic influenza vaccine), as defined below. This applies to both pilot scale and large scale production during the interpandemic phase and pandemic alert period (2005d) when the site of vaccine production is geographically remote from the site of the emerging pandemic. Any subsequent relaxation of the levels of containment during the developing pandemic, should be done on a case by case basis after careful evaluation of the risks..

It should be noted that implementation of the containment conditions described in this section within a production and quality control testing facility must take into account the large quantities and high titres of live virus that are produced, the industrial scale of facilities, as well as the rules and regulations governing the manufacture and testing of medicinal products known as Good Manufacturing Practices (WHO, 1992). The facility requirements for a specific biosafety level within a manufacturing plant will differ from the facility requirements within a laboratory handling smaller quantities of infectious material such as a laboratory producing reassortant reference viruses or in a pilot scale facility. It should also be noted that these biosafety requirements apply to the production and quality control operations involving live viruses; virus lots shown to be inactivated by a validated process need not be handled under these conditions.

4.4 Environmental control measures

Each vaccine manufacturer must review their own control measures in light of the intended work,the nature of laboratory and production facilities and the need to maintain GMP. Influenza specific enhanced containment measures (defined in 4.4.1 and 4.4.2) should be in place for open manipulations with live virus, especially virus harvesting in egg production facilities. Quality control facilities need to meet production containment requirements, and in some regions, a second approval will be needed to meet other requirements such as GMO status.

Local safety regulations provide guidance for disposal of potentially infectious waste. Contaminated waste from current production facilities may reach high titres. Decontamination methods should be validated. If possible, decontamination of waste

should take place on site. However if this is not possible, there should be procedures in place to ensure that material is safely contained and transported prior to decontamination off site. Guidance on regulations for the transport of infectious substances is available from WHO (WHO 2005c). In all cases the procedures should be validated for the scale of manufacturing.

In view of the possible exposure to high titre pandemic strain virus and the need to reduce the chance of simultaneous infection with human influenza viruses, staff should be prophylactically vaccinated with seasonal influenza vaccines. It is anticipated that before large scale vaccine production is attempted, pilot lots of pandemic strain vaccine will have already been produced. Experimental vaccines inducing protective antibody levels are recommended for use by staff before large scale vaccine production commences if possible. Antiviral treatment must be available in case the situation warrants it.

Each manufacturer should also assess the risk of contamination of avian or porcine species based on the likelihood of birds or pigs being in the vicinity of the manufacturing plant and the manufacturing controls in use. Staff or other personnel entering the live virus area potentially exposed to live virus should avoid visiting pig, horse or bird facilities (e.g. farms, equestrian events, bird sanctuaries) for at least 14 days following occupational exposure. This period should be extended to 14 days after the symptoms resolve if conjunctivitis or respiratory signs indicating the potential development of influenza infection or disease develop during this 14 day period..

It is also known that mice can be experimentally infected with some influenza viruses and the PR8 strain is known to be lethal for mice. It is not known whether a reassortant based on PR8 will be able to replicate in mice, but steps should be taken to prevent exposure of wild mice and escape of laboratory mice and rodent control measures should be in place.

4.4.1 Specifications for "BSL2 Enhanced (pandemic influenza vaccine)"

Specifications for BSL2 enhanced (pandemic influenza vaccine) facilities include the following in addition to the principles for BLS2 facilities as specified in the WHO Laboratory Biosafety Manual (Third edition 2004, WHO Geneva).

4.4.1.1 Facility

The facility should be designed and operated according to the stage of the manufacturing process to meet the demands of protection of the recipient of the vaccine, the staff producing and testing the vaccine, and of the environment. It is noted that different solutions may be needed depending on the risks inherent in the operation(s) conducted in an area. Specialized engineering solutions will be required that may include:

Use of relative negative pressure biosafety cabinets when possible

- Use of HEPA filtration of air prior to exhaust into public areas or the environment
- Use of positive pressure with negative pressure in-line sinks prior to exhausting to the non-viral zone

In addition the following decontamination procedures should take place:

- Decontamination of all waste from BSL-2 enhanced (pandemic influenza vaccine) areas
- Decontamination of manufacturing and quality control areas at the end of a production campaign through cleaning and validated decontamination for example gaseous fumigation.

4.4.1.2 Personal protection

- Full-body protective laboratory clothing (for example Tyvek disposable overalls) worn only in controlled BSL-2 enhanced (pandemic influenza vaccine) area
- If activities cannot be contained by primary containment and open activities are being conducted, the use of respiratory protective equipment, such as N95, FFP3 (European Union 2001) or equivalent respirators is strongly recommended. Minimal specifications for the filtering/absorbing capacity of such equipment should be met, and masks, if used, must be fitted properly and the correctness of fit tested.
- Personnel should be instructed, in a written document to which they sign their agreement, not to have contacts with avian and porcine species, in particular farm animals fourteen (14) days post departure from the facility where vaccine has been produced.. Currently the risks involved with contacts with household dogs and cats are not considered to be significant, but the available scientific evidence is sparse.
- Staff should be prophylactically vaccinated with seasonal inactivated influenza vaccines.
- It is anticipated that before large scale vaccine production is attempted, pilot lots of pandemic strain vaccine will have already been produced. Experimental vaccines inducing protective antibody levels are recommended for use by staff before large scale vaccine production commences if possible.
- Antiviral treatment must be available in case the situation warrants it.

4.4.1.3 Monitoring of decontamination

- Cleaning and decontamination methods need to be checked periodically as part of a master validation plan to demonstrate that the protocols, reagents, and equipment are effective in the inactivation of pandemic influenza virus on facility and equipment surfaces, personnel garments, and waste materials, and within cell growth and storage containers. Once decontamination procedures for influenza virus are fully described and validated, there is no need to repeat them for each new strain. Validation studies

using influenza viruses may be supplemented by studies with biological (for example bacterial) markers selected to be more difficult to inactivate than influenza.

4.4.2 Specifications for "BSL3 Enhanced (pandemic influenza vaccine)"

Specifications for BSL3 enhanced (pandemic influenza vaccine) facilities include the following requirements in addition to the principles for BLS3 facilities as specified in the WHO Laboratory Biosafety Manual (Third edition 2004, WHO Geneva), and are additional to the specifications given above in section 4.4.1.

4.4.2.1 Facility

- The facility should be designed and operated to meet the demands of protection of the recipient of the vaccine, the staff producing and testing the vaccine, and of the environment. This will require specialized engineering solutions that may include:
- Negative pressure secondary containment areas
- HEPA filtration on supply and exhaust air
- On-site liquid effluent decontamination
- Floor dams erected around bioreactors or other large scale equipment including storage tanks to contain spillage of virus from large virus-containing vessels

4.4.2.2 Personal protection

- Full clothing change into manufacturing facility specific garments
- Full body protective single use laboratory clothing (for example Tyvek disposable overalls) donned upon every entry
- When open activities are being conducted, eye protection and the use of respiratory protective equipment, such as N95, FFP3 (European Union 2001) or equivalent respirators such as positive pressure air purifying respirators is required. Minimal specifications for the filtering/absorbing capacity of such equipment should be met, and masks, if used, must be fitted properly and the correctness of fit tested.
- Shower out is recommended after normal operations at BSL-3 enhanced (pandemic influenza vaccine), but must be used when staff may have been exposed to vaccine virus.
- Personnel should be instructed, in a written document to which they sign their agreement, not to have contacts with animals, in particular farm animals fourteen (14) days post departure of the facility where vaccine has been produced. Currently the risks involved with contacts with household dogs and cats are not considered to be significant, but the available scientific evidence is sparse.
- Staff should be prophylactically vaccinated with seasonal influenza vaccines.
- It is anticipated that before large scale vaccine production is attempted, pilot lots of pandemic strain vaccine will have already been produced. Experimental vaccines inducing protective antibody levels are recommended for use by staff before large scale vaccine production commences if possible.
- Antiviral treatment must be available in case the situation warrants it.

4.5 Biosafety management and implementation within a vaccine production facility

4.5.1 Management structure

The implementation of the biosafety levels described in these guidelines requires that the institution employ a Biosafety Officer who is knowledgeable in large-scale viral production and containment, but is independent of production in his or her reporting structure. The Biosafety Officer is responsible for the independent oversight of the implementation of the biosafety practices, policies, and emergency procedures in place within the company or organization and should report directly to the highest management levels within the company. A Biosafety Officer is needed in addition to a Qualified Person who, in some countries, has overall responsibility for a medicinal product. There should also be a Biosafety Committee comprising representatives of viral production and quality control that is responsible for reviewing the biosafety status within the company and for coordinating preventative and corrective measures. It must include the institutional Biosafety Officer as a member. The chairperson of the Committee should be independent of both the production and quality control functions. The management and governing board of the manufacturing company should ensure that adequate priority and resources are made available to the Committee to implement required measures.

4.5.2 Medical surveillance

Occupational health departments at vaccine manufacturers of pandemic strain influenza vaccines should provide training of the clinical signs of influenza infection to company physicians, nurses, and vaccine manufacturing supervisors, who must make decisions on the health of personnel associated with pandemic strain influenza vaccine manufacturing and vaccine testing. Local medical personnel in the catchment area of the manufacturing site should receive special training in the diagnosis and management of pandemic influenza infection. Any manufacturer embarking on large-scale production should have documented procedures for dealing with influenza-like illness in the staff involved, or their family members, including diagnostic procedures and prescribed treatment protocols. Manufacturer's should ensure that staff understand they have an obligation to seek medical attention and to report any influenza-like illness to the occupational health department or equivalent. Manufacturers should hold supplies of one or more effective antiviral agent and have defined means of quarantining staff if necessary.

4.5.3 Implementation

A detailed and comprehensive risk analysis should be conducted to define possible contamination sources to personnel or the environment that may arise from the production or testing of live influenza virus within the establishment. For each procedure or system, this analysis should take into account the concentration and stability of the virus at the site, the potential for inhalation or injection that could result from accidents, and the potential consequences of a major or minor system failure. The procedural and technical measures to be taken to reduce the risk to workers and the environment should

be considered as part of this analysis. The results of this risk analysis should be documented.

A comprehensive Biosafety Manual must be created and implemented that fully describes the biosafety aspects of the production process and quality control activities and defines such items as emergency procedures, waste disposal, and the requirements for safety practices and procedures as identified in the risk analysis. The manual should be made available to all staff of the production and quality control units, with at least one copy present in the containment area(s). The manual should be reviewed and updated when changes occur and at least annually.

Comprehensive guidelines outlining the response to biosafety emergencies, spills, and accidents should be prepared and made available to key personnel for information and for coordination with emergency response units. Rehearsals of emergency response procedures are helpful. These guidelines should be reviewed and updated annually.

The implementation of the appropriate biosafety level status in the production and testing facilities should be verified through an independent assessment. National requirements concerning verification mechanisms should be in place and complied with.

Table 1 Comparison of properties and proposed containment for pandemic vaccine production using different vaccine reference viruses

Vaccine virus	HA receptor specificity	Tests needed on reference virus[1]	Proposed containment for vaccine production
H5, H7 reassortants, from HP viruses[2]	α2,3 residues	Ferret, Chicken, Sequence, Plaquing, Egg embryo	BSL 2 Enhanced (pandemic influenza vaccine)
H5, H7 reassortants, from NP viruses[2]	α2,3 residues	Ferret, Chicken, Sequence, Plaquing, Egg embryo	BSL 2 Enhanced (pandemic influenza vaccine)
Non-H5, H7 reassortant	α2,3 residues	Ferret	BSL 2 Enhanced (pandemic influenza vaccine)
Non-H5, H7 reassortant	α2,6 residues	Ferret	BSL 2 Enhanced (pandemic influenza vaccine)
H5, H7 HP viruses	α2,3 residues	Not applicable	BSL 3 Enhanced (pandemic influenza vaccine)
H5, H7 NP viruses	α2,3 residues	Ferret, Chicken, Sequence, Plaquing, Egg embryo	BSL 2 Enhanced (pandemic influenza vaccine)
Non-H5, H7 viruses	α2,3 residues	Ferret	BSL 2 Enhanced (pandemic influenza vaccine)
Non-H5, H7 viruses	α2,6 residues	Ferret	BSL 2 Enhanced (pandemic influenza vaccine)

1. Test performed by WHO reference laboratory

2. Highly Pathogenic and Non Pathogenic viruses

Glossary

Aerosol: A dispersion of solid or liquid particles of microscopic size in a gaseous medium.

Air balance: The necessity to keep air supply and exhaust systems in balance by means of measurements of static pressure, fan and motor performance, and air volumes.

Airlock: Areas found at entrances or exits of rooms that prevent air in one space from entering another space. These generally have two doors and a separate exhaust ventilation system. In some cases a multiple-chamber airlock consisting of two or more airlocks joined together is used for additional control.

Biosafety Committee: An institutional committee of individuals versed in the subject of containment and handling of infectious materials.

Biosafety Level 2(or 3) (enhanced pandemic influenza): A specification for the containment of pandemic influenza during vaccine manufacture and quality control testing with specialized air handling systems, waste effluent treatment, immunization of staff, specialized training, and validation and documentation of physical and operational requirements.

Biosafety Manual: A comprehensive document describing the physical and operational practices of the laboratory facility with particular reference to infectious materials.

Biosafety Officer: A staff member of an institution who has expertise in microbiology and infectious materials, and has the responsibility for ensuring the physical and operational practices of various biosafety levels are carried out in accordance with the standard procedures of the institution.

Biological indicators: The use of organisms to test the efficacy of sterilization processes.

Biological safety cabinet: Primary and partial containment work enclosure used for manipulation of materials that may cause infections or sensitization to workers. They are equipped with high efficiency particulate air (HEPA) filters and may or may not be open fronted.

Certification: Documentation that a system qualification, calibration, validation, or revalidation has been performed appropriately and the results are acceptable.

Decontamination: A process by which and object or material is freed of contaminating agents.

Floor dams: Purpose-built elevations to enclose liquid spills.

Fumigation: The process whereby gaseous chemical is applied to an enclosed space for the purpose of sterilizing the area.

Good Manufacturing Practices: That part of quality assurance which ensures that products are consistently produced as controlled to the quality standards appropriate to their intended use and as required by the marketing authorization.

HEPA filter: A filter capable of removing at least 99.97% of all particles with a mean aerodynamic diameter of 0.3 micrometers.

Inactivation: To render an organism inert by application of heat, or other means.

Seed lot: A culture of micro-organism distributed from a single bulk container in a single operation, in such a manner as to ensure uniformity and stability and to prevent contamination.

Positive pressure laminar flow hood: An enclosure with unidirectional outflowing air, generally used for product protection.

Primary containment: A system of containment, usually a biological safety cabinet or closed container, which prevents the escape of a biological agent into the immediate working environment.

Respirator: A respiratory protective device with an integral perimeter seal, valves and specialized filtration, used to protect the wearer from toxic fumes or particulates.

Risk analysis: A formalized documented process for analysing risks.

Secondary containment: A system of containment, usually involving specialized air handling, airlocks, and secure operating procedures, which prevents the escape of a biological agent into the external environment or into other working areas.

Sterilization: Sterility is the absence of viable micro-organisms. In general, an item is assumed to be sterile if the validation of the sterilization process applied to it indicates that only one item in one million items subjected to the process will contain a viable micro-organism.

Validation: The documented act of proving that any procedure, process, equipment, material activity, or system actually leads to the expected results.

Authors

The following series of background papers, commissioned by the WHO Secretariat, were prepared in the period April-July 2005.

1. A review of WHO biosafety guidelines for Manufacturing Avian Influenza Vaccines (Frey, Richmond, Robinson)
2. A risk assessment for large scale manufacture of inactivated influenza vaccines from reassortants derived from avian influenza viruses (Wood, Robertson, Logan)
3. Industry pandemic biosafety position paper (IFPMA influenza vaccine supply international task force)
4. Conceptual risks of reassortants for the environment (Swayne)

These documents were discussed in a teleconference on 27 July 2005 convened by WHO (D Wood, S Lambert, M Ali, B Kay) and the following participants:

Patrick Celis
Tony Colegate
Jackie Katz
Catherine Gerdil
Gary Grohman
Alan Hampson
Alan Hay
Roland Levandowski
Paul Logan
Jim Robertson
Robin Robinson
David Swayne
Jonathan Richmond

A first draft document was prepared by the WHO Secretariat (D Wood) based on the outcome of the teleconference and the commissioned papers. Comments on this first draft were received from Drs Alexander, Hampson, Hay, Logan, Robertson, Swayne and the IFPMA influenza vaccine supply international task force. Aversion of the document for public comment (WHO/BS/05.2026) was prepared by the WHO Secretariat (D Wood) taking into account the comments received and further review by Drs J Robertson and J Wood.

The current version of the document (WHO/BS/05.202612 October 2005) was prepared by the secretariat (D Wood, S Lambert) taking into account comments from participants at a WHO informal consultation on WHO/BS/05.2026, held in Geneva 19-20 September 2005 attended by the following persons: (*to be added*) (include Alan Hampson, Ingegerd Kallings, Paul Payette, Jim Robertson, John Wood, and the Influenza Vaccine Supply Task Force for providing additional written comments)

Appendix 1 Testing for attenuation of influenza vaccine strains in mammals

Titration of test virus

The 50% infectious dose of an egg- or mammalian cell-passaged stock of vaccine virus and the parental viruses will be determined by titration in eggs (EID_{50}) or cells ($TCID_{50}$) as appropriate. Titration of vaccine virus stock and parental virus stocks should be determined within the same laboratory and titres should be sufficiently high such that these viruses can be compared using equivalent high doses in mice or ferrets (10^7 to 10^6 EID_{50} or $TCID_{50}$).

Ferrets

Experimental procedure: Outbred ferrets 4-8 months of age are sedated either by intramuscular inoculation of a mixture of anaesthetics (e.g. ketamine [25 mg/kg], xyalazine [2 mg/kg] and atropine [0.05 mg/kg]) or by a suitable inhalant. A standard dose of 10^7 EID_{50}/$TCID_{50}$ (as appropriate) (10^6, if the higher dose is not possible) in 1 ml phosphate-buffered saline is slowly distilled onto the nares of the sedated animal, making sure that the virus is inhaled and not swallowed or expelled. A group of 4-6 ferrets should be infected. One group of ferrets (2-3 animals) will be euthanized on day 3 or 4 post-infection and the following tissues should be collected for estimation of virus replication: nasal turbinates and/or swabs, lung (tissue samples from each of 4 lobes and pooled), brain (tissues from anterior and posterior sections sampled and pooled), spleen or intestine. Additional lung tissue may be collected and processed for haematoxylin and eosin (H&E) staining for microscopic evaluation of histopathology. The remaining animals are observed for 14 days for signs of weight loss, lethargy (based on a previously published index [Reuman, 1989]), respiratory and neurological symptoms. Neurological involvement may be confirmed by collection of brain tissue on day 14 post-infection at the termination of the experiment and processing as above for histopathology.

Expected outcome: Viral titres of the vaccine strain in respiratory tissues should be no greater than either parental strain; a substantial decrease in lung virus replication is anticipated. Replication of the vaccine candidate should also be restricted to the respiratory tract and replication in the spleen or intestine is not expected. While isolation of the vaccine strain from the brain is not desirable, if high viral titres are found in the nasal turbinates there may be some detection of virus in the brain based on previous results with non-virulent human H3N2 viruses (Zitzow, 2002). The significance of such a finding may be confirmed by performing a histopathological analysis of brain tissue on day 14 p.i. Neurological lesions detected in H&E stained tissue sections should confirm virus replication in the brain and observation of neurological symptoms. Neurological symptoms and histopathology would indicate a lack of suitable attenuation of the vaccine candidate. Likewise clinical signs of disease such as weight loss and lethargy should indicate lack of attenuation in the vaccine strain, assuming that the wild-type avian virus also causes these symptoms.

Mice

Experimental procedure: The 50% lethal dose (LD_{50}) of the vaccine strain and parental virus strains is determined in 6-8 week old female BALB/c mice. Mice are lightly anesthetized with an inhalant and groups of mice (4-8 per group) are infected intranasally with 0.05 ml of serial 10-fold dilutions of virus (expected dose range 10^7 to 10^1 EID_{50}). Mice are observed daily for disease signs and the numbers of deaths at each virus dilution are recorded. The LD_{50} values are calculated by the method of Reed and Muench (1938). An additional 3 mice infected with a high dose of virus (e.g. 10^6) are sacrificed on day 3 or 4 post-infection and organs, including the lungs and brain, are harvested for estimation of virus replication.

Expected outcome: If the wild-type avian strain replicates in the brain and is highly lethal for mice, the vaccine candidate should exhibit at least a 1000-fold reduction in lethality (i.e. ≥3 log increase in the LD_{50} value). Lung and brain titres of the vaccine strain should be lower than those of either parental strain, consistent with an attenuation of replication in mouse tissues.

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