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

(Proposed revision of WHO TRS No. 941, Annex 5; H1N1 specific update 2009; and H7N9
update 2013)

NOTE:

This document has been prepared for the purpose of inviting comments and suggestions on the
proposals contained therein, which will then be considered by the Expert Committee on
Biological Standardization (ECBS). Publication of this early draft is to provide information
about the proposed WHO biosafety risk assessment and guidelines for the production and
quality control of novel human influenza candidate vaccine viruses and pandemic vaccines to a
broad audience and to improve transparency of the consultation process.

The text in its present form does not necessarily represent an agreed formulation of the
Expert Committee. Written comments proposing modifications to this text MUST be
received by 12 February 2018 in the Comment Form available separately and should be
addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention:
Department of Essential Medicines and Health Products (EMP). Comments may be submitted
electronically to the Responsible Officer: Dr TieQun Zhou at email: zhout@who.int.

The outcome of the deliberations of the Expert Committee will be published in the WHO
Technical Report Series. The final agreed formulation of the document will be edited to be in
conformity with the "WHO style guide" (WHO/IMD/PUB/04.1).

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1 Abbreviations

A/Len/17  Influenza A/Leningrad/134/17/57 virus
BSL  Biosafety Level
CVV  Candidate vaccine virus
EID  Egg infectious dose
GAP  WHO Global Action Plan for Influenza Vaccines
GISRS  WHO’s Global Influenza Surveillance and Response System
GMP  Good manufacturing practice
HA  Haemagglutinin
H&E  haematoxylin and eosin (staining)
HEPA  High-efficiency particulate air filtration
HP  High pathogenicity
HPAI  Highly pathogenic avian influenza virus
IVPI  (Chicken) Intravenous pathogenicity index. Any virus with an index greater than 1.2 is considered an HPAI
LP  Low Pathogenicity
LPV  Low-pathogenic avian influenza virus
MDCK  Madin-Darbey Canine Kidney (cells)
MVA  Modified Vaccine Ankara
NA  Neuraminidase
OIE  World Organization for Animal Health
PAPR  Powered air-purifying respirators
PFU  Plaque forming unit
PPE  Personal protection equipment
PR8  Influenza A/Puerto Rico/8/34 virus (A/PR8/34)
PTC  Pass through cabinets
RG  Reverse Genetics
TCID  Tissue culture infective dose
VCM  WHO Vaccine Composition meeting for Influenza Vaccines
VSV  Vesicular stomatitis virus
WHO CC  WHO Collaborating Centre for Influenza
WHO ERL  WHO Essential Regulatory Laboratory for Influenza
wt  Wild-type
Summary

International biosafety expectations for both the pilot-scale and large-scale production of human vaccines in response to a pandemic influenza virus, 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 viruses (CVVs) 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 that concludes that the likelihood of direct harm to human health would be high if reassorted H5 or H7 viruses or other subtypes that have high \textit{in vivo} pathogenicity are used for vaccine production; moreover, low pathogenic avian influenza viruses that are highly virulent for humans may also present a high risk and all such viruses could also pose a significant risk to animal health. Stringent vaccine biosafety control measures, defined as Biosafety Level (BSL)3 enhanced, are defined to manage the risk from vaccine production and quality control using such viruses in the pre-pandemic period. For all other vaccine viruses, for example reassortants containing a highly attenuated backbone (e.g., from PR8) or the handling of low pathogenic avian influenza viruses that are not highly virulent for avian influenza viruses, the direct risk to human health is considered to be low. Nevertheless, there is an indirect risk to human health due to a theoretical risk of secondary reassortment with circulating human influenza viruses, resulting in a novel virus capable of replicating in humans. Although extremely unlikely, such a secondary reassortant could become adapted to human infection and transmission and result in 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. Specifications for personal protection are provided for both BSL2 enhanced and BSL3 enhanced biosafety levels, and guidance is provided on biosafety management and implementation within a vaccine production facility. Tests to be performed on CVVs prior to release to vaccine manufacturers depend on the type of virus but include, at a minimum, \textit{in vivo} tests in ferrets and, where appropriate, chickens and embryonated eggs embryos, plaque assays and sequencing.
1. Introduction

Due to the inherent risks in handling pandemic influenza virus(es) for the production of vaccines where an uncontrolled release could have a significant public health impact, risk assessment, biosafety and biosecurity precautions are needed in laboratory and manufacturing environments. WHO developed a biosafety risk assessment and guidelines for the production and quality control of human influenza pandemic vaccines in 2005, published as Annex 5 to TRS 941, in response to the threat of a pandemic posed by the highly pathogenic avian influenza (HPAI) viruses A(H5N1) and the need to begin development of experimental vaccines (2). This threat still persists and several countries have produced and stockpiled H5N1 vaccine. Moreover, other threats have arisen since the appearance of H5N1, such as the H1N1pdm09 subtype virus causing the pandemic in 2009 and the emergence of low pathogenic avian influenza (LPAI) viruses A(H7N9) that were able to replicate in humans causing severe disease with a high fatality rate (mostly in adults and the elderly). The document was updated to include pandemic influenza A(H1N1) virus in 2009 and A(H7N9) in 2013 (3-5). However, it has been over a decade since the original publication of the WHO guideline and a revision was requested by industry, regulators and GISRS laboratories during several WHO informal consultations, such as the WHO Vaccine Composition Meetings (VCM), Global Action Plan for Influenza Vaccines (GAP) meetings and ‘Switch’ meetings (Meetings on Influenza vaccine response at the start of a pandemic), in order to review and reduce testing timelines for CVVs, which have been identified as one of the bottlenecks to rapid vaccine responses (6-10). Moreover, since the initial publication of TRS 941 Annex 5 guidance experience with viruses of pandemic potential and with pandemic viruses has increased globally and is reflected in this update. Further experience gained to date from the development and testing of CVVs derived by reverse genetics (RG) from HPAI viruses has also been incorporated. WHO convened a Working Group meeting during 9-10 May 2017 attended by experts and representatives of WHO Collaborating Centres (CC), Essential Regulatory Laboratories (ERL) and other national regulatory authorities for vaccine and biosafety regulation, manufacturers, and the World Organisation for Animal Health (OIE) to review the up-to-date experience, discuss the revision of TRS 941, Annex 5 and reach consensus on the outline and key issues for the revision (11). This document follows the risk-assessment scheme used in the WHO biosafety guidance for pilot-lot vaccine production, but is extended to include considerations relating to the greater production-scale needed to supply large quantities of vaccines (12, 13). 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. It also takes into account the considerable experience gained from handling HPAI viruses and those classed as low pathogenic in avian species but highly virulent in humans, as well as the hazards associated with such viruses.

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

2. Scope

Efforts have continued into the development and manufacture of H5N1 vaccines and the guidance presented in this document reflects experience gained with this virus and our greater knowledge of H5 subtype viruses in general. Moreover, a great deal has also been learned from our experience with the A/H1N1pdm09 viruses, and from the production of vaccines to this pandemic virus. It is, nevertheless, intended that the guidance will also be applicable to threats from other potential pandemic viruses (e.g., H2, H9, H7 subtype viruses, etc.), as well as low pathogenic avian and mammalian influenza viruses of various subtypes, which are potentially virulent in humans. Manufacturers and laboratories handling HPAI should consult their national regulatory authorities to determine whether additional biosecurity measures are required to handle these viruses.

Transmission and pathogenicity of influenza viruses are multi-factorial traits that are currently not completely understood (14). There is therefore a significant range of diversity in the pathogenicity of viruses used to make CVVs which are then used in vaccine production, not only for humans but also for other mammals and avian species. The haemagglutinin (HA) protein has been identified as a major determinant for virulence of avian influenza viruses (15). For example, H5N1 HPAI viruses that can also cause fatal disease in humans have been used to produce reassortant viruses containing an HA that has been genetically modified to generate viruses of low pathogenicity for chickens. In case of viruses that are inherently less pathogenic for humans, wild-type (wt) virus might be used directly for vaccine production (16). Thus, reassortants derived by classical means, RG, or synthetic means, which may or may not be genetically modified, as well as wt viruses are within the scope of these guidelines.

Eggs have traditionally been used for the production of influenza vaccines from defined influenza virus reassortants, but cell culture techniques and recombinant protein technologies have also been established and licensed for seasonal influenza vaccine production with
international expectations for production and quality control specifications defined (17). The principles outlined in this guidance should be applicable to these and other technology platforms for pandemic vaccine development.

Most effort to date with candidate pandemic vaccine development has been targeted towards inactivated vaccines; however, in both North America and Europe live attenuated pandemic virus vaccines have been produced and a ‘mock dossier’ approach has been accepted by the European Medicines Agency (EMA). This raises important issues beyond the risks to humans, namely the potential for excreted viruses or their derivatives to infect and replicate in non-human species particularly in those raised for commercial purposes, which could have a significant economic impact as well as ramifications for international trade. Developers and regulators will need to assess both the human and the agricultural risk of live pandemic virus vaccines for their ability to shed and to replicate in different hosts. Both inactivated and live vaccines are therefore covered in the scope of these guidelines.

Technologies not covered by this guideline, although the general principles could be applied, include new generation technology platforms that do not use live influenza vaccine viruses for production such as expressed recombinant proteins, virus-like particles, DNA- and RNA-based vaccines and vectored vaccines.

Furthermore, it is intended that the guidelines on containment measures should apply to all facilities and laboratories that handle live vaccine virus. This includes not only the vaccine manufacturing facility but also applies to the quality control laboratories of the manufacturer as well as National Control Laboratories and specialist laboratories as appropriate. The transport of live virus materials within and between sites should comply with international and national specifications (18).

Finally, it should be noted that the risk assessments for vaccine manufacture will vary according by whether production is occurring in an 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. These guidelines are intended to describe steps to minimize risks involving vaccine manufacture with emphasis on the interpandemic period, while indicating modifications that may be found appropriate during other periods.

3. Terminology

The definitions given below apply to the terms used in these guidelines. They may have different meanings in other contexts.
Aerosol: A dispersion of solid or liquid particles of microscopic size in a gaseous medium.

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 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.

Biosafety Level 2, enhanced or BSL3: 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.

Biosecurity: Laboratory biosecurity describes the protection, control and accountability for valuable biological materials within laboratories, in order to prevent their unauthorized access, loss, theft, misuse, diversion or intentional release.

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

EID 50: Egg Infectious Dose. A potency unit for measuring infectious activity of a biologic product or infectious agent equal to a base-10 logarithm of amount of product or agent preparation that causes infection in the 50% of embryos.

FFP3: A Mask that protect health professional against airborne particles and microorganisms in pharmaceutical work.

Fumigation: The process whereby gaseous chemical is applied to an enclosed space for the purpose of sterilizing the area.
**Good manufacturing practice (GMP):** 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 airborne particles with a mean aerodynamic diameter of 0.3 micrometres.

**Highly pathogenic avian influenza (HPAI) virus:** Avian influenza viruses of subtypes H5 or H7 containing a cleavage site in HA with multiple inserted amino acids (also referred to in the literature as ‘multibasic’ cleavage site) and causing a systemic infection in poultry associated with mortality of up to 100% (fowl plague). The designation “highly pathogenic” does not refer to the virulence of these viruses in mammalian and human hosts.

**Inactivation:** To render an organism inert by application of heat, chemicals (e.g. formalin, beta-propiolactone), UV irradiation or other means.

**Low-pathogenic avian influenza (LPAI) virus:** Avian influenza viruses containing an HA with a single basic amino acid preceding the site of proteolytic cleavage (also referred to as ‘monobasic’ cleavage site) causing localized infections in poultry leading to mild or moderate disease. The designation “low-pathogenic” does not refer to the virulence of these viruses in mammalian and human hosts.

**N95:** A surgical mask, also known as a procedure mask, designed to catch the microorganisms shed in droplets and aerosols from the wearer's mouth and nose with 95% efficiency at removing particles 0.3 micron and larger when correctly fitted to the user.

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

**Risk assessment:** A formalized documented process for analysing risks involving a systematic process of evaluating the potential risks that may be involved in a projected activity or undertaking.
**TCID 50:** median tissue culture infective dose 50%. The quantity of a virus suspension that will infect 50% of tissue culture inoculated with the suspension, expressed as TCID_{50}/ml.

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

### 4. Hazard identification

Hazards associated with pandemic vaccine manufacturing and laboratory testing are dependent on the type of pandemic vaccine virus (reassortant or wild-type), method of production (egg-based or cell-based or other), whether it is an inactivated, live (attenuated) virus or a recombinant virus-vectored vaccine, and whether or not there are any deliberate modifications of the virus for attenuation or for enhanced immunogenicity and/or increased yield. For recombinant virus-vectored vaccines, (e.g. MVA, adenovirus, or VSV) which use replicating recombinant constructs based on viruses other than influenza, virus homogeneity, the nature of the transgene, shedding and the potential for recombination are important factors to evaluate (19, 20).

#### 4.1 Candidate vaccine viruses (CVV)

CVVs for live and inactivated influenza vaccines are generally produced via reassortment with well-defined backbones from established parent viruses, such as human virus A/Puerto Rico/8/34 (PR8), A/Ann Arbor/6/60, or A/Leningrad/134/17/57 (A/Len/17); wt viruses may also be used for vaccine production. Further, new donor strains for reassortment are being developed and evaluated to enhance vaccine yields. It is likely that a high growth reassortant will provide the basis for pandemic vaccine development, although it is conceivable that a wt virus could be used.

#### 4.1.1. Reassortants

The genome of influenza A viruses is composed of eight individual single-stranded RNA segments of negative polarity. Segments 4 and 6 encode the two surface glycoproteins, HA and neuraminidase (NA), respectively. The HA is the major surface antigen of the virus and antibodies directed against HA can protect from infection. Antibodies to NA are not neutralizing but can inhibit virus release from infected cells thereby reducing severity and duration of disease and limiting viral shedding. The remaining six RNA segments (“internal genes”) encode internal and non-structural viral proteins. The segmented structure of the
genome allows for the exchange of the individual RNA segments between influenza viruses, a process defined as reassortment, upon coinfection of a single cell with two or more viruses.

The classical or conventional method for reassortment involves preparing CVVs by co-inoculation of a WHO recommended wt virus and a backbone donor virus with a high growth phenotype in embryonated chicken eggs. Concomitant infection allows for reassortment of genetic segments between the two viruses. Antiserum is used for negative selection against the donor virus surface glycoproteins; amplification in eggs results in positive selection for growth. The resulting high growth reassortant CVV must contain the HA and NA genes of the wt target virus. This system takes time and is likely to take several weeks for the production of a high growth reassortant.

An alternative approach is through the use of RG methodology to produce a reassortant vaccine virus. (21). This process is based on incorporating the six RNA segments encoding the internal genes of the (high growth) donor virus and the two segments encoding the HA and NA from the circulating wt virus into plasmids. The plasmids are subsequently transfected into cells to rescue the reassortant to be used for vaccine manufacturing. This system allows for the direct genetic manipulation of the influenza gene segments and is generally faster and more accurate than the use of classical reassortment. Moreover, if an HPAI virus is used in the RG process, the HA gene can be easily modified to remove a specific motif of amino acids at the HA cleavage site that is known to convey high pathogenicity in poultry (22). The reassortant can thus be specifically designed to serve as a low pathogenic CVV. The RG system has been reported to be able to produce a CVV within 9 to 12 days (23).

The distribution and receipt of the circulating wt virus as a source of RNA for construction of RG HA and NA plasmids adds extra time to the reassortant process and can be bypassed if the reassorting laboratories can produce plasmids by site-directed mutagenesis of the template DNA of an existing related virus, or by the use of synthetic DNA (24, 25).

### 4.1.1.1 Backbone donor viruses

PR8 is a common donor virus for generating reassortant vaccine viruses as it reaches a high titre in embryonated chicken eggs. It was originally used in the late 1960s to produce “high growth reassortants” and the use of such reassortants as vaccine viruses increases vaccine yield many-fold. Moreover, 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 rendering it incapable of replication in humans (26).
Improved donor viruses are under development in an effort to enhance vaccine yields from CVVs used to manufacture inactivated influenza virus vaccines. Such donor viruses may be new derivations of PR8 but also may have genes from viruses other than PR8, be synthetically generated, and be optimized for specific HA and NA subtypes (23). Demonstration of adequate attenuation for new/improved donor viruses will be needed.

Live attenuated seasonal influenza vaccines are licensed in some countries using reassortants with a 6:2 gene constellation based on live attenuated donor viruses such as the A/Ann Arbor/6/60 and A/Len/17 backbones. The attenuated A/Ann Arbor/6/60 virus has been used as a backbone in 6:2 reassortant live attenuated vaccines in clinical studies for over 40 years using approximately 30 different vaccine viruses, and the data demonstrate that the Ann Arbor/6/60 based reassortant vaccine viruses are attenuated for humans (27). To date, live attenuated seasonal influenza vaccines derived from the A/Ann Arbor virus have been licensed in North America and Europe while the A/Len/17 backbone has been licenced in Russia, China, Thailand and India (28). These donors may also be used for the development of pandemic influenza vaccine and an adequate level of attenuation has been shown for modified reassortant viruses of various subtypes (29). For each candidate pandemic vaccine virus, this should be verified by testing as described in section 5.1. H5N1-specific LAIV versions (LPAI) made from A/Ann Arbor/6/60 reassortants have been licensed as pandemic preparedness vaccines in several countries.

4.1.1.2 Gene segments from wild type viruses

Reassortants with a 6:2 gene constellation are considered to be ideal but are not always possible to isolate by traditional co-cultivation methods and for seasonal vaccines some CVVs with different gene constellations, such as 5:3, have been used. As the resultant gene constellation is less predictable using classical methods there is a theoretical possibility of developing reassortants with more than two wt parental genes or even of selection of a mutant (non-reassortant) wt virus with improved growth characteristics. The gene constellation of reassortants derived by traditional co-cultivation methods should be determined.

4.1.1.3 Virulence factors associated with HA of wild type viruses

The CVV gene products derived from the wt virus will be, at a minimum, the HA and NA. For reassortants derived from highly pathogenic H5 and H7 avian viruses by RG, the HA should be modified so that the inserted amino acids at the HA cleavage site are reduced to a single basic amino acid; for some H5 viruses additional nucleotide substitutions can be introduced in the
vicinity of the cleavage site in order to increase the genetic stability of the created monobasic
motif during virus amplification for large-scale manufacture. Also, the modification of the
cleavage site alone is not a guarantee of low pathogenicity as there are LPAI that are virulent in
humans due to the presence of other virulence factors (30). It should also be noted that this
procedure will increase the safety of the reassortants for avian species as cleavage site
modifications have consistently resulted in a reduction of their pathogenicity in avian embryos
and in poultry (31). Reassortants for vaccine production are expected to be of low
pathogenicity in poultry compared to the highly pathogenic wt parental viruses (32).

The hazards associated with reassortants depend in part on HA receptor specificity. If a
reassortant has a preference for avian cell receptors (i.e. \( \alpha_2,3 \)-linked terminal sialic acid) the
hazard is considered to be minimal to humans; however, if a reassortant has a preference for
mammalian cell receptors (\( \alpha_2,6 \)-linkages, e.g. human H2N2 pandemic virus from 1957), or
possesses both avian and mammalian receptor specificities (e.g. H9N2), there is a greater risk
of human infection. For H5 reassortants, the HA retains a preference for \( \alpha_2,3 \)-linked terminal
sialic acid residues, so the ability of the H5N1 reassortants to bind to and replicate in human
cells should be reduced. It is envisaged that an H5N1 reassortant derived by RG according to
WHO guidance (33) would be attenuated for humans compared to the wt H5 virus.
Nevertheless, it should also be noted that the human lower respiratory tract contains \( \alpha_2,3 \)-
linked sialic acid receptors and exposure to high doses of H5N1 viruses represents a risk of
infection. Moreover, humans are immunologically naive to H5 and many other avian subtypes,
which is also an important risk factor.

4.1.1.4 Other factors associated with influenza virus virulence

Although it is clear from experience in south-east Asia from 1997 to the present that H5N1
influenza viruses that display preference for \( \alpha_2,3 \)-linked sialic acids can still 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 whereas the 2004 H5N1 viruses possessed complex combinations of changes in
different gene segments that affected pathogenicity in ferrets (34, 35). In these cases even a
virus with a poor affinity for the mammalian receptors was able to replicate in humans
(although it was not transmissible). Further, prior to the 2003 outbreak in The Netherlands,
only two cases of transmission of H7 viruses from birds to humans were documented (36). Also
during the many years of laboratory handling of high-titre avian viruses (of which
A/FPV/Dobson/27 is known to contain a gene which adapts it for replication in mammalian cells (37, 38), there has only been one report in the literature of a laboratory worker being affected by these viruses. This was a laboratory worker in Australia who developed conjunctivitis after accidentally being exposed to an H7N7 virus directly in the eye (39). The PR8/H5N1 6:2 reassortants and the A/Ann Arbor/6/60 live attenuated 6:2 reassortants created by RG for the production of H5N1 vaccine do not contain the gene constellation considered necessary for pathogenicity in chickens, mice and ferrets and in contrast have internal genes that are likely to confer sensitivity to the innate immune response.

Compared to HA, the NA protein of influenza viruses has a less prominent record as a virulence factor. It is known that a balance of the counteracting activities of HA (receptor binding) and NA (receptor destruction and virus release) is required for efficient viral replication (40, 41). Further, specific adaptations in NA have been identified upon transmission from wild aquatic birds to poultry. However, specific determinants for the adaptation to and virulence in humans have so far not been detected in the NA protein, although there is some evidence that the NA can mediate HA cleavage in rare H1N1 viruses (42, 43). Of note, resistance to the viral inhibitors oseltamivir and zanamivir is caused by specific mutations in either NA or HA, the latter reducing the affinity of HA to the receptor determinant.

4.2. Manufacture

Vaccine manufacture requires the establishment of both WHO Good manufacturing practices (GMP) and appropriate biosafety requirements for biological products, as well as related national regulations, technical standards, and guidelines (44, 45). GMP requires the protection of the product from the operator and protection of the operator and the environment from the infectious agent, thus minimizing the risk of any hazards associated with production. It should be noted that reassortants derived from PR8 have been used routinely for the production of inactivated influenza vaccines for the past 40 years. This work involves the production of many thousands of litres of infectious egg allantoic fluids, which creates substantial aerosols of reassortant virus within manufacturing plants. Most of the reassortants were made from wt human influenza viruses. Although staff in the manufacturing facility may have some susceptibility to infection with the wt virus, there have been no anecdotal or documented cases of work-related human illness resulting from occupational exposure to the reassortants.

Similarly, reassortants derived from the A/Ann Arbor/6/60 virus have been used for the production of LAIV for many years and no anecdotal or documented cases of work-related human illness have been reported. While no conclusive study yet has been conducted to detect
silent infections for either the PR8 or live-attenuated viruses, the attenuation status of these CVVs continues to be supported by their excellent safety record to date. Furthermore, for pandemic human CVVs that express avian influenza genes there may be potential consequences for agricultural systems. If influenza A viruses of H5 or H7 subtype or any influenza A virus with an IVPI greater than 1.2 are introduced into poultry (46), the presence of infection would be notifiable to OIE and should lead to implementation of biosecurity measures that aim at preventing the spread of disease (46, 47). Infection with influenza A viruses of high pathogenicity in birds other than poultry, including wild birds, should be reported to OIE; however, a Member Country should not impose bans on the trade in poultry and poultry commodities in response to such a notification, or other information on the presence of any influenza A virus in birds other than poultry, including wild birds (46, 47).

4.2.1 Production in eggs
Influenza vaccine has been produced in embryonated hens’ eggs since the early 1940s. Much experience has been gained and some facilities are capable of handling large numbers of eggs on a daily basis with the aid of mechanized egg handling, inoculation and harvesting machines. Hazards may occur during the production stages and quality control laboratory activities prior to virus inactivation. During egg inoculation the virus used is dilute and of a relatively small volume. The most hazardous production stage is egg harvesting when the eggs have to be opened to harvest the allantoic fluid, as the open nature of the operations may lead to a greater exposure to aerosols and spills. The allantoic fluid that is harvested from the eggs is invariably manipulated thereafter in closed vessels and hazards arising from live virus during downstream processing and during the virus inactivation process, if used, are therefore less than during virus harvest. Collection and disposal of egg waste is potentially a major environmental hazard. Safe disposal of the waste from egg-grown vaccines, both within the plant and outside, is therefore critical.

4.2.2 Production in cell cultures
For pandemic influenza vaccines produced on cell cultures, the biosafety risks associated with manufacturing will depend primarily on the nature of the cell culture system employed. Closed systems, such as bioreactors, normally present little to no opportunity for exposure to live virus during normal operation, but additional safety measures must be taken during procedures where samples are introduced into or removed from the bioreactor. Roller bottles and cell culture flasks used for virus production may allow exposure to live virus through aerosols, spills, and
other operations during virus production and, thereafter, additional risks are associated with the
inactivation and disposal of the large quantities of contaminated liquid and solid waste,
including cellular debris, generated by this method.

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 HA may be useful to detect such changes most likely occurring within or close to the
receptor-binding domain of the protein; however, it should be noted that little is known about
the relation between cell substrate and virus reversion or adaption. Beare et al. (48) tried to de-
attenuate PR8 by multiple passage in organ cultures of human tissue, but failed, whereas
studies with MDCK cells 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 (49). Overall, hazards
arising from the inherent properties of a reassortant or wild type (wt) virus are likely to be far
greater than the probability of adaptation of the virus to a more human-like phenotype.

4.3 Hazards from the vaccine

Inactivated pandemic influenza vaccines present no biosafety risks provided that the results of
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 concerning the
biosafety risks associated with shedding or other unintentional release into the environment
following vaccination, subjects participating in clinical trials in the interpandemic or pandemic
alert phase should be kept under appropriate clinical isolation conditions. If this is not done,
indirect hazards for humans could arise.

While it is unlikely that a reassortant will be harmful to humans, an indirect hazard may exist
through secondary reassortment with a human or animal influenza virus (50); however, recent
studies have shown that there is a clear time dependence of coinfections and the generation of
reassortants limiting this possibility (51). For secondary reassortants to be generated, several
events need to occur:

• Infection of production staff (or recipients of live attenuated vaccine in clinical trials)
  with the reassortant virus
• A worker to simultaneously contract infection with a wt influenza virus and the reassortant virus
• A reassortment event would have to take place.

Evidence to date indicates that the probability of generating secondary reassortants is low. For example, manufacturers have more than 30 years of experience with large-scale production of vaccines based on PR8 reassortants and there have been no reported cases of human illness. Moreover, containment procedures have significantly improved over the last 30 years and production staff can be vaccinated to reduce the chances of an infection with a circulating wt virus thus minimising the risk for secondary reassortment and appropriate personal protective equipment (PPE) can also be provided.

**5. Safety testing of candidate vaccine viruses (CVVs)**

CVVs can be developed by either a WHO laboratory, or by a laboratory approved by a national regulatory authority. The following tests and specifications have been developed based on experience gained with the evaluation of CVVs derived from viruses of various subtypes. The required safety testing of different CVVs and proposed containment levels are summarised in Table 1. The information summarised in this table should be considered as guidance and changes to these requirements may be determined on a case-by-case basis by WHO and/or national authorities. It is recommended that for CVVs developed from newly emerging viruses of pandemic potential, an appropriate WHO expert group review the associated data from safety testing and advise WHO; WHO will then provide further guidance as to the appropriate biocontainment requirements through its expert networks such as GISRS.

The requirement to conduct or complete some or all of these tests prior to the distribution of a CVV may be relaxed based on additional risk assessments; these assessments should take into account WHO pandemic phase, evolving virological, epidemiological and clinical data as well as national regulatory requirements applying to shipment and receipt of infectious substances.

**5.1 Tests to evaluate pathogenicity of CVVs**

The recommended tests for CVVs are dependent on the parental viruses from which they are derived (Table 1). The parental viruses determine the appropriate biocontainment level for conducting tests. The tests are described in the following sections.
CVVs that are genetically similar (i.e. have HA and NA derived from the same or a genetically very closely related wt virus and on the nominally same backbone) to a CVV that has already undergone complete safety testing may not require full testing; it may be sufficient to confirm sequence and genetic stability in this case.

5.1.1 Attenuation in ferrets

Ferrets were chosen because they have been used extensively as a good indicator of influenza virus virulence for humans (52). Typically, seasonal influenza viruses cause mild to no clinical signs in ferrets and virus replication is usually limited to the respiratory tract. PR8 virus has been assessed in ferrets and found to cause few or no clinical signs, and virus replication is limited to the upper respiratory tract. However, some wt HPAI viruses can cause severe and sometimes lethal infections (53, 54). Thus, in the absence of human data, the ferret is the best model to predict whether a virus will be pathogenic or attenuated in humans.

CVVs should be shown to be attenuated in ferrets in accordance with Table 1, except when virus-specific risk assessments suggest a different approach (ie, performance of ferret test where Table 1 does not require it or no ferret test where Table 1 requires it). These tests should be conducted in well-characterized ferret models standardised by the use of common reference viruses available from WHO CCs/ERLs for influenza. Detailed test procedures are described in Appendix 1. Attenuated viruses would be those that meet the attenuation criteria as defined in Appendix 1, with reference to the reference/standard viruses1. One or more laboratories may have ferret pathogenicity data on parental wt viruses that could be used by all testing laboratories as a further benchmark for comparison. Assessing the transmissibility of CVVs between ferrets is not required.

5.1.2 Pathogenicity in chickens

For CVVs derived from HPAI H5 or H7 parental viruses determining the chicken intravenous pathogenicity index (IVPI) is recommended and may be required by national authorities. The procedure should follow that described in the OIE guidance. Any virus with an index greater than 1.2 is considered an HPAI (55).

5.1.3 The ability to plaque in the presence or absence of added trypsin

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1 Until the establishment of reference viruses for pathogenicity testing, CVVs are expected to be compared to their respective wt parent virus; attenuation is then defined as relative to the pathogenicity of the wt virus.
HPAI viruses can replicate in mammalian cell culture in the absence of added trypsin, whereas LPAI viruses generally do not. This test is recommended for all CVVs derived from HPAI H5 or H7 parental viruses. It is recommended that these tests be established and characterized by use of known positive and negative control viruses.

5.1.4 The ability to cause chicken embryo death

HPAI viruses cause rapid chicken embryo death upon inoculation into eggs and this test has been used to complement in vivo IVPI assays (55). This test is recommended for all CVVs derived from H5 or H7 parental viruses.

5.1.5 Genetic sequence analysis and stability

Genetic sequencing is important to confirm identity and/or to verify the presence of attenuating and other phenotypic markers, (e.g., cold adaptation and temperature sensitivity in the case of LAIV CVVs). Genetic sequencing is recommended to verify retention (stability) of the markers of relevant phenotypic traits, where such markers are known, after 10 passages beyond production level in relevant substrates i.e., embryonated chickens’ eggs or cultured cells. These tests should be conducted on all CVVs, including wt CVVs.


<table>
<thead>
<tr>
<th>Candidate vaccine virus</th>
<th>Tests needed on CVVs&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Proposed containment for vaccine production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reassortants&lt;sup&gt;b&lt;/sup&gt; and modified viruses derived from H5 and H7 HPAI&lt;sup&gt;c&lt;/sup&gt; viruses&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Ferret, chicken&lt;sup&gt;e&lt;/sup&gt;, sequence, plaquing, egg embryo, genetic stability</td>
<td>BSL2 Enhanced (pandemic influenza vaccine)</td>
</tr>
<tr>
<td>Reassortants&lt;sup&gt;b&lt;/sup&gt; and modified viruses derived from synthetic DNA representing H5 and H7 HPAI&lt;sup&gt;f&lt;/sup&gt; viruses</td>
<td>Ferret, sequence, plaquing, egg embryo, genetic stability</td>
<td>BSL2 Enhanced (pandemic influenza vaccine)</td>
</tr>
<tr>
<td>Reassortants and modified viruses derived from H5 and H7 LPAI&lt;sup&gt;g&lt;/sup&gt; viruses</td>
<td>Ferret, sequence, egg embryo, genetic stability</td>
<td>BSL2 Enhanced (pandemic influenza vaccine)</td>
</tr>
<tr>
<td>Reassortants and modified viruses derived from non-H5, H7 viruses</td>
<td>Ferret, sequence, genetic stability</td>
<td>BSL2 Enhanced (pandemic influenza vaccine)</td>
</tr>
<tr>
<td>Wild type H5, H7 HPAI viruses</td>
<td>Sequence, genetic stability&lt;sup&gt;n&lt;/sup&gt;</td>
<td>BSL3 Enhanced (pandemic influenza vaccine)</td>
</tr>
<tr>
<td>Wild type H5, H7 LPAI viruses</td>
<td>Ferret, sequence, genetic stability&lt;sup&gt;c, i&lt;/sup&gt;</td>
<td>BSL2 Enhanced&lt;sup&gt;1&lt;/sup&gt; (pandemic influenza vaccine)</td>
</tr>
<tr>
<td>Wild type non-H5, H7 viruses</td>
<td>Ferret, sequence, genetic stability&lt;sup&gt;c&lt;/sup&gt;</td>
<td>BSL2 Enhanced (pandemic influenza vaccine)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Test performed by WHO reference laboratory.

<sup>b</sup> With deletion of additional residues at HA connecting peptide

<sup>c</sup> Highly pathogenic avian influenza virus
This category refers to viruses derived by reverse genetics technology using as starting material wt HPAI virus (or RNA extracted from wt HPAI virus)

\(^2\)the requirement for performance of the chicken pathogenicity test (IVPI) is dependent on national regulatory requirements which are currently under review in some countries and may change

\(^3\)this category refers to viruses derived by reverse genetics technology using as starting material synthetic DNA; the nucleotide sequence of the synthetic DNA is equivalent or similar to wt HA and NA genes from HPAI viruses, with the exception that additional residues at the at HA connecting peptide have been removed

\(^4\)Low pathogenic avian influenza viruses.

\(^5\)if changes are identified pathogenicity tests may be required

\(^6\)if a virus specific hazard assessment identifies that additional control measures are appropriate, containment level may be increased, for example Asian-lineage H7N9, and additional pathogenicity testing required

6. Risk assessment and management

6.1 Nature of the work

Production of influenza vaccines in embryonated chicken eggs or cell culture require propagation of live virus. Modifications of some CVVs will result in viruses that are expected to be attenuated in humans (12, 21). Several production steps have the potential to generate aerosols containing live virus. The virus concentration in aerosols will depend on the production step and is highest during the harvest of infected allantoic fluid. Small amounts of virus containing liquid or very dilute virus suspensions are used during seed virus preparation and egg inoculation. Appropriate biosecurity and biosafety measures, such as use of laminar air flows, positive pressure laminar flow hoods, cleaning and decontamination of equipment, waste management and spill kits, will be in place to prevent accidental exposure in the work environment and the release of virus into the environment.

6.2 Health protection

6.2.1 Likelihood of harm to human health

Wild-type influenza viruses are able to infect humans and cause serious illness. Certain phenotypic features that are associated with virulence can be modified and the resulting CVV will have lower probability of causing harm to human health.
6.2.1.1 Reassortant CVVs

Reassortant CVVs containing a backbone of genes except segments 4 and 6 (HA and NA) from PR8, A/Ann Arbor/6/60 or A/Len/17 have been widely used for production of seasonal influenza vaccines and vaccines against the pandemic H1N1pdm09 influenza viruses. A significant body of data is available, suggesting that a reassortant virus composed of RNA segments coding for HA and NA derived from a pandemic virus and the genes coding for the internal and non-structural proteins ("internal genes") from PR8, A/Ann Arbor/6/60 or A/Len/17 will have only a low probability of causing harm to human health (12, 21).

Viruses that were rescued from plasmids will have the desired 6 "internal" genes of the helper virus, however this is not always the case for reassortants prepared by mixed infection and antibody selection ("classical reassortment"). Risks associated with RNA segments, other than segments 4 and 6, that were derived from the pandemic virus must be carefully evaluated.

Efforts have been made to modify backbones that were derived from PR8 or other viruses to achieve attenuation or to increase the yield during vaccine manufacturing. Until more field experience has become available, the potential of vaccine viruses that contain modified backbones to cause harm to human health needs careful assessment.

**HA cleavage site:** Most HPAI viruses contain a sequence of basic amino acids at the cleavage site separating the HA1 and HA2 subunit. HPAI viruses in which the basic amino acids have been removed from the HA by genetic engineering are likely to be attenuated. Modification of the HA cleavage site is associated with reduced virulence in animal models and is expected to result in attenuation in humans. Modification of the HA cleavage site only applies to HPAI H7 and H5 viruses. It should be noted that LPAI, e.g. H7N9 wt viruses also have caused serious human illness (56).

**Receptor specificity:** Preferential binding of the HA to α2,6 receptors is associated with transmissibility of pandemic influenza and circulating human viruses in humans (57,58). However, it must be noted that viruses with a preference to bind to α2,3 receptors, such as H7N9 viruses, have also been causing serious human illness (59). While receptor specificity should be considered during risk assessment as a factor in reducing the risk for a virus to cause harm to human health, it is not in itself sufficient for virus attenuation.

**Secondary reassortment:** It is conceivable that reassortment between a CVV, containing HA and NA from a pre-/pandemic virus, and a seasonal human wt influenza virus could occur.
during simultaneous infection of humans with both viruses. Such a reassortant may be replication-competent in humans, while having pre-/pandemic surface proteins. The likelihood that these events occur and lead to secondary reassortment is considered to be low. Laboratory and production facilities have biosafety control measures in place to prevent exposure of staff to live virus. In case of accidental exposure, it is unlikely that a CVV would replicate efficiently or transmit to human contacts. Virus shedding would be expected to be well below the titres considered to be needed for human infection; however, although there is no known precedent of secondary reassortment of using PR8 reassortants for vaccine manufacturing in more than 40 years, the public health consequences of such an event could be serious.

6.2.1.2 Wild-type CVVs of low pathogenicity (LP)
Pathogenicity of wt viruses with low in vivo pathogenicity, without multiple basic amino acids at the HA cleavage site is likely to be low in humans (60). However, it should be noted that some H7N9 viruses that are LP in poultry have caused severe illness in humans.

Transmissibility of wt viruses with avian receptor specificity is likely to be low in humans, whereas transmissibility of wt viruses with mammalian receptor specificity (e.g. human H2N2 and H9N2) is unknown.

In case of accidental exposure there is a risk of secondary reassortment with human viruses and the resulting reassortant viruses may be able to replicate in humans. Appropriate biosafety control measures will be in place during manipulation of these viruses to prevent exposure of staff.

6.2.1.3 Wild-type CVVs of high pathogenicity (HP)
The use of highly pathogenic wt CVVs is restricted to cell-culture-based production, which allows the use of closed systems. For diagnostic tests and vaccines for terrestrial animals, HPAI viruses should not be used. Instead, CVVs produced by reverse genetics and containing the haemagglutinin gene of an HPAI virus that had the cleavage site sequence altered to that of a LPAI H5/H7 virus are preferred (47). Appropriate biosecurity and biosafety measures during production, analytical testing and waste disposal are required to protect staff and prevent release of infectious virus into the environment.
6.2.1.4 Susceptibility of CVVs to neuraminidase inhibitors

Influenza viruses/CVVts that are sensitive to NA inhibitors (or other licensed drugs, once available) should be used for production if at all possible. Sequence verification may be enough to confirm drug susceptibility.

6.3 Environmental protection

6.3.1 Environmental considerations

Influenza A viruses are endemic throughout the world in some farm animals (pigs and horses) and some populations of wild birds, specifically birds of the families Anseriformes (ducks, geese and swans) and Charadriiformes (shorebirds) (61).

A number of influenza A viruses, such as H5, H7 and H9 can cause disease in domestic poultry. H5 and H7 can be highly pathogenic in poultry, whereas H9 is 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) (62). In big cats, the infections followed consumption of dead chickens infected with H5N1 viruses. Influenza A virus infection in dogs were caused by H3N8 or H3N2 viruses closely related to endemic equine and avian viruses, respectively. The H3N8 and H3N2 viruses continue to circulate in dogs in North America (63). Recently, LPAI avian A(H7N2) virus was identified as the cause of an outbreak of respiratory disease in domestic shelter cats in northeastern United States, indicating yet another mammalian host for avian influenza viruses (64).

It is expected that many prepandemic viruses will have avian receptor specificity and thus birds would theoretically be the species most susceptible. Several studies indicate that viruses with PR8 backbones are attenuated in chickens (65). A reassortant containing HA (with a single basic amino acid at the cleavage site) and NA from the 1997 Hong Kong H5N1 virus and the genes coding for the internal and non-structural proteins of PR8 was barely able to replicate in chickens and was not lethal (65). Similar studies have been performed with the 2003 Hong Kong H5N1 virus at the WHO Collaborating Centre, Memphis, USA (66), where the 6:2 PR8 reassortant did not replicate or cause signs of disease 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.

Hatta et al. (34) have demonstrated that acquisition of only one PR8 gene by an avian influenza virus can abolish virus replication in ducks.
It is likely that the temperature sensitive phenotype of the cold-adapted vaccine virus would also be attenuated in avian species due to the elevated body temperature of birds.

Pigs have both $\alpha_{2,3}$ and $\alpha_{-2,6}$-linked sialic acid receptors in abundance (67) and must be considered susceptible to most influenza viruses, including LAIV and CVVs with a backbone of PR8 genes.

### 6.4 Assignment of containment level

The production of influenza vaccine reassortant CVVs from highly pathogenic wt viruses should take place in BSL3 enhanced or BSL4 facilities, as advised by WHO (68) or national authorities. WHO Collaborating Centres, other specialized laboratories and some vaccine manufacturers provide characterized reassortant CVVs to all interested vaccine manufacturers who may develop vaccine seeds and vaccines from these materials.

In consideration of the hazards associated with egg and cell culture vaccine production and quality control with "classical" or "RG" derived reassortant or wt viruses of pandemic potential which have been demonstrated to be attenuated in ferrets and/or have demonstrated low pathogenicity in chickens where applicable, as specified in section 5.1, 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 (68). Any subsequent relaxation of the levels of containment to the standard used for seasonal production during the developing pandemic, should be authorized on a case-by-case basis by the national competent authorities after careful evaluation of the risks.

Special consideration should be given to hazards associated with cell culture vaccine production and quality control of HPAI or "classical" or "RG" reassortant pandemic viruses of unknown pathogenicity. The assigned containment level is BSL3 Large Scale manufacturing as defined below. This applies to both pilot-scale and large-scale production during the interpandemic phase and pandemic alert period (68). The parts of the facility where such work is done (both production and quality control) should additionally meet the OIE requirements for containment, which include not only biosafety and biosecurity but also requirements to limit the introduction and spread within animal populations (55). Any subsequent relaxation of the levels of containment during the developing pandemic, should be authorized on a case-by-case basis by the national competent authorities after careful evaluation of the risks.
In view of the open nature of large-scale egg-based vaccine production, it is not feasible to operate at BSL3 enhanced (pandemic influenza vaccine). Therefore egg-based vaccine production from HPAI H5 or H7 wt viruses is not recommended.

Containment conditions must be defined, based on an activity based risk assessment, taking into account the scale of manipulations, the titres of live virus, and whether an activity involves virus amplification. Biosafety control measures must be reconciled with rules and regulations governing the manufacture and testing of medicinal products known as good manufacturing practices (GMP) (44). It should be noted that biosafety control measures apply to manipulations involving live virus; they no longer apply once virus has been inactivated by a validated process.

6.5 Environmental control measures

Appropriate containment measures to prevent release of live virus into the environment must be in place.

Local biosafety/biosecurity regulations provide guidance on the disposal of potentially infectious waste. Notably, contaminated waste from current production facilities may reach high virus titres. All decontamination methods should be validated. If possible, decontamination of waste should take place on site. Where this is not possible, it is the responsibility of each manufacturer to ensure that procedures are in place to safely contain material during transport prior to decontamination off site. Guidance on regulations for the transport of infectious substances is available from WHO (18) and national competent authorities. In all cases the procedures must be validated to ensure that they function at the scale of manufacturing.

Medical surveillance program of staff should be established prior to vaccination.

Procedures should be in place to provide antiviral medicines, in case the situation warrants it, e.g. in case of accidental exposure. Where antiviral medicines are only available as prescription medicines, care should be taken to have stocks available in pharmacies.

Suitable PPE must be available to prevent exposure of staff to live virus. In order to further reduce the risk of secondary reassortment in case of accidental exposure, the use of seasonal influenza vaccines should be recommended to staff. For the same reason, the use of a pre-/pandemic vaccine can be considered if available and if marketing authorization has been received.
Each manufacturer should also assess the risk of contamination of birds, horses, pigs or other susceptible animals based on the likelihood of their being in the vicinity of the manufacturing plant. Following occupational exposure, staff or other personnel entering the 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. If conjunctivitis or respiratory signs indicating the potential development of influenza infection or disease develop during this 14 day period this period, quarantine time should be extended to 14 days after the symptoms have resolved.

Stringent measures to control rodents, other mammals and birds should be in place.

6.5.1 Specifications for "BSL2 enhanced (pandemic influenza vaccine) facilities"

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 (1).

6.5.1.1 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, the environment and the population at large. 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
- use of high-efficiency particulate air (HEPA) filtration of air prior to exhaust into public areas or the environment
- use of positive pressure barrier and/or negative pressure in-line sinks prior to exhausting to areas where no live virus is handled

In addition, the following decontamination procedures should take place:

- decontamination of all waste from BSL2 enhanced (pandemic influenza vaccine) areas
- decontamination of all potentially contaminated areas at the end of a production campaign through cleaning and validated decontamination, for example gaseous fumigation.
6.5.1.2 Personal protection

- Full-body protective laboratory clothing (for example, Tyvek® disposable overalls)
- If activities cannot be contained by primary containment and open activities are being conducted, the use of respiratory protective equipment, such as N95, FFP3 (69) 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.
- All personnel, including support staff and others who may enter the production or QC areas where pandemic viruses may be handled, should be instructed, in a written document to which they sign their agreement, not to have any contact with susceptible animals such as ferrets or farm animals, in particular birds, horses or pigs, for 14 days after departure from the facility where vaccine has been produced. 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. Currently the risks involved in contact with household dogs and cats are not considered to be significant, but the available scientific evidence is sparse.
- Wherever possible, it is strongly recommended that staff should be prophylactically vaccinated with seasonal inactivated influenza vaccines.
- It is anticipated that pilot lots of pandemic virus vaccine will have already been produced before large scale vaccine production is attempted. Experimental vaccines inducing protective antibody levels are recommended for use by staff before large scale vaccine production commences, provided they are available and marketing authorization has been received.
- Procedures should be in place to provide antiviral treatment in case the situation warrants it (e.g. accidental exposure).

6.5.1.3 Monitoring of decontamination

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

6.5.2 Specifications for "BSL3 Large Scale Manufacturing"

It is assumed that ferret pathogenicity testing will be conducted on all strains of unknown pathogenicity even given the assumptions above (Section 5.1) related to the low probability of a PR8 reassorted virus being pathogenic in humans. This assumption is based on the experience currently held relating to reassortants of HA subtypes other than H1 & H3; i.e., H5, H7, and H9.

Under these circumstances, a facility will need to meet the requirements for protection of personnel handling potentially dangerous micro-organisms found in the WHO Laboratory Biosafety Manual, Third edition (1), which lays out a Risk Survey to be undertaken prior to rating a laboratory space as either BSL-1, 2 or 3. Similar requirements are found in the European Directive 2000/54/EC (2007) (70) on the protection of workers from risks related to exposure to biological agents at work and the US CDC’s Biosafety in Microbiological and Biomedical Laboratories Guide (Edition 5) (71).

Following review of the requirements for Biosafety at Level BSL3 from the sources noted above, it is proposed that a facility meeting the criteria detailed below, and with the noted operator protection in place, would be suitable to manufacture vaccine, at large scale using a virus seed prepared by RG or classical reassortant methodology, whilst pathogenicity remains unknown.

6.5.2.1 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:

- Appropriate signage and labeling related to the activities being undertaken when a virus of unknown pathogenicity is in use.
- The facility must be designed and constructed as a contained GMP space. The surfaces and finishes must comply with GMP requirements which will ensure they are appropriately sealed and easily cleaned and decontaminated.
• The air cascades within the facility should be such that any live virus is contained within the work zones in which it is used. All work with infectious virus must be conducted within these contained zones.

• Access to the contained areas must be gained via double door entry airlocks. The airlocks should operate at a pressure either lower or higher than that on either side. In this way the airlocks become either a sink or a pressure barrier, maintaining an airflow containment of the facility. Note that in the case where the airlocks provide a low pressure ‘sink’ it will be required that the entry and exit doors be interlocked or alarmed with a suitable delay or alarm system to prevent both being opened at the same time. The air pressure cascade within the negatively pressure ‘contained’ zone should allow for compliance with GMP (i.e. higher pressures in cleanest zones) requirements for clean rooms.

• All supply and exhaust air must be passed through HEPA filtration, again maintaining the required containment and GMP conditions. The air conditioning systems within the facility must be the subject of rigorous risk assessment related to potential failure modes and ‘fail safe’ systems implemented where deemed necessary. The facility should be under constant monitoring related to environmental factors including the maintenance of appropriate room pressure differentials. Should there be any undesirable fluctuation the situation should be alarmed and appropriate action taken.

• All equipment to be reused is either cleaned in place, decontaminated by means of autoclaving or otherwise cleaned and decontaminated by validated, dedicated systems prior to washing for reuse.

• Areas of potential liquid spill should be assessed and bunded (dammed) to ensure containment of any spill. This should include waste treatment plants and processes. Procedures must be in place such that spills are contained, cleaned and the contaminated materials properly disposed so as not to compromise the integrity of the facility.

• Materials entry to the ‘contained’ zone should be via separately HEPA filtered, interlocked, double ended Pass Through Cabinets (PTC) or double-ended autoclaves.

• All facility waste, including egg waste, should be discarded via validated on-site waste effluent systems or by autoclaving. The validation of waste systems should specifically include viral inactivation testing. Any items which follow the process from the external environment, through the manufacturing process and are returned to the external environment must be the subject of particular attention (e.g. the plastic egg maché).
Dedicated washing and decontamination of equipment and/or procedures must be provided which, again, must be fully validated for viral inactivation.

6.5.2.2 Personal protection

- All clothing worn outside the facility should be replaced by manufacturing facility garments upon entry into the facility.
- It should be noted that gowning requirements in viral zones should always include full suit, overshoes, eye protection and double gloves.
- The provision of full hood powered air-purifying respirators (PAPR) for all personnel working within the containment areas of the manufacturing facility. These powered hoods are to be worn at all times when the facility is in operation under these enhanced biosafety requirements. Figure 1 shows the type of operator protection to be provided.

![Figure 1: Operator Protection](image)

- All facility clothing is to be removed on exit, with soiled clothing transferred out of the facility via a decontamination autoclave or similar method. The respirator hoods may be decontaminated with 70% alcohol or similar and stored in the ‘grey’ zone of the entry/exit airlock.
- Specific operational procedures should be developed and implemented for operation under enhanced biosecurity conditions.
- Wherever possible it is strongly recommended that staff should be prophylactically vaccinated with a seasonal influenza vaccine. In the case of pandemic strains, it is
anticipated that before large scale vaccine production is attempted, pilot lots of vaccine will
have already been produced. If available and if marketing authorization has been received,
experimental vaccines inducing protective antibody levels are recommended for use by staff
before large scale vaccine production commences

- Procedures should be in place to provide antiviral treatment in case the situation warrants it.
  (e.g. accidental exposure).
- Daily temperature monitoring before commencing work (with an exclusion policy for ill
  workers) where this is possible
- On-site Occupational Health and Safety and medical support should be maximized with
  such additional measures as:
  - Medical consultation / training in recognition of ‘flu-like’ symptoms
  - Out of hours referral plan to medical facilities with quarantine facilities
- Taking a full body shower upon exit from the BSL3 Large Scale Manufacturing
  containment facility is recommended. It is mandatory following situations when staff may
  have been exposed to vaccine virus.
- All personnel, including support staff and others who may enter the production or QC areas
  where pandemic viruses may be handled, should be instructed, in a written document to
  which they sign their agreement, not to have contact with animals, in particular farm
  animals 14 days following departure from the facility where vaccine has been produced.
  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. Currently the risks involved in contact with household
dogs and cats are not considered to be significant, but the available scientific evidence is
sparse.

6.6 Biosafety management and implementation within a vaccine production facility

6.6.1 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 virus 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 virus production and quality control that is responsible for reviewing the biosafety status within the company and for coordinating preventive and corrective measures. The institutional biosafety officer must be a member of the Committee. The chairperson should be independent of both the production and quality control functions. The Committee should report to the executive management of the manufacturing company to ensure that adequate priority and resources are made available to the Committee to implement the required measures.

6.6.2 Medical surveillance

Occupational health departments at vaccine manufacturers of pandemic virus influenza vaccines should provide training in recognizing the clinical signs of influenza infection to company physicians, nurses and vaccine manufacturing staff including supervisors, who must make decisions on the health of personnel associated with the manufacture and testing of pandemic virus influenza vaccine. Local medical practitioners caring for personnel from 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. Manufacturers should ensure staff understand that they have an obligation to seek medical attention and to report any influenza-like illness to the occupational health department or equivalent. Manufacturers should ensure that procedures are in place to provide antiviral treatment if the situation warrants it (e.g. accidental exposure) and have defined means or arrangements of advising staff with any influenza–like illness as necessary.

6.6.3 Implementation

A detailed and comprehensive risk analysis should be conducted to define possible sources of contamination of 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, volume 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, or equivalent must be created and implemented to fully describe the biosafety aspects of the production process and of the quality control activities. It should define 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 biannually.

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.
Authors and acknowledgements

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3. WHO H1N1 updates (2009):


   http://www.who.int/influenza/resources/publications/influenzavaccineresponse_meeting02/en/.
10. WHO Global Action Plan for Influenza Vaccines (GAP)
http://www.who.int/influenza_vaccines_plan/en/


Appendix 1

Testing for attenuation of influenza vaccine viruses in ferrets

Laboratories conducting testing for attenuation of influenza viruses in ferrets should make use of a panel of standard/or reference viruses (‘pathogenicity standards’ in the following section) and defined experimental outcomes for pathogenicity testing. The pathogenicity standards (to be established by WHO laboratories) serve as benchmarks for the pathogenicity test in ferrets and delineate the expected outcomes. The use of these standards will ensure that attenuation of CVVs is being measured against common parameters independently of subtype. The CVV to be tested must show parameters of pathogenicity below the predefined values of a high pathogenicity standard and in line with those of an attenuation standard in order to be designated as attenuated. Comparative attenuation in comparison with the parental wild-type virus is not necessary in this case. However, laboratories that have the capacity to evaluate attenuation of a CVV compared with the parental wild-type virus can continue to do so. To account for the expected experimental variability of results across different laboratories, the pathogenicity standards should be tested in ferrets at each testing laboratory according to the experimental protocol shown below when establishing the ferret model for pathogenicity testing and at regular intervals thereafter. The outcomes of these tests should fall within the limits described for the pathogenicity standards. In cases of discrepancy, a review of the ferret model should be conducted and advice should be sought from experienced WHO laboratories.

Test virus

The 50% egg or tissue culture infectious dose (e.g. EID$_{50}$, TCID$_{50}$) or plaque-forming units (PFU) of the reassortant CVV or pathogenicity standard will be determined. The infectivity titres of viruses should be sufficiently high to allow infection with $10^7$ to $10^6$ EID$_{50}$, TCID$_{50}$ or PFU of virus and diluted not less than 1:10. Where possible, the pathogenic properties of the donor PR8 should be characterized thoroughly in each laboratory.

Laboratory facility

Animal studies with the CVV and the pathogenicity standards should be conducted in animal containment facilities in accordance with the proposed containment levels shown in Table 1. For untested CVVs, the biocontainment level to be used for the ferret safety test is the one shown for the respective wild-type virus, except for ‘Reassortants and modified viruses derived...
from synthetic DNA representing H5 and H7 HPAI viruses’, for which the containment level proposed in Table 1 (BSL2 enhanced) can be used (1). An appropriate occupational health policy should be in place (2).

**Experimental procedure**

Outbred ferrets 4-12 months of age that are serologically negative for currently circulating influenza A and B viruses and the test virus strain are anesthetized by either intramuscular administration of a mixture of sedatives (e.g. ketamine (25 mg/kg) and xylazine (2 mg/kg) and atropine (0.05 mg/kg)) or by suitable inhalant anesthetics. A standard virus dose of $10^7$ to $10^6$ EID50 (or TCID50 or PFU) in 0.5 to 1 ml of phosphate-buffered saline is used to inoculate animals. The dose should be the same as that used for pathogenesis studies with the wild-type parental virus, if used, or the pathogenicity standards previously characterised and regularly assessed in the laboratory. The virus is slowly administered into the nares of the sedated animals, reducing the risk of virus being swallowed or expelled. A group of four to six ferrets should be inoculated. One group of two to three animals should be euthanized on day 3 or 4 after inoculation and samples should be collected for estimation of virus replication from the following organs: spleen, intestine, lungs (samples from each lobe and pooled), brain (anterior and posterior sections sampled and pooled), olfactory bulb of the brain, and nasal turbinates. If gross pathology demonstrates lung lesions similar to those observed in wild-type viruses, additional lung samples may be collected and processed with haematoxylin and eosin (H&E) staining for histopathological evaluation. The remaining animals are observed for clinical signs, which may include weight loss, lethargy [based on a previously published index (3)], respiratory and neurologic signs, and increased body temperature. Collection of nasal washes from animals anesthetized as indicated above should be performed to determine the level of virus replication in the upper airways on alternate days after inoculation for up to seven days. At the termination of the experiment on day 14 after inoculation, a necropsy should be performed on at least two animals and organs collected. If signs of substantial gross pathology are observed (e.g. lung lesions), the organ samples should be processed as described above for histopathology.

**Expected outcome**

Clinical signs of disease such as lethargy and/or weight loss should be within the predefined ranges of acceptable pathogenicity defined by the pathogenicity standards. Viral titres of the vaccine strain in respiratory samples should be within the ranges of acceptable virus replication.
defined by the pathogenicity standards. Replication of the CVV should be restricted to the respiratory tract. Virus isolation from the brain is not expected. However, detection of virus in the brain has been reported for seasonal H3N2 viruses (4); this is due to detection of virus in the olfactory bulb. Therefore, should virus be detected in the anterior or posterior regions of the brain (excluding the olfactory bulb) the significance of such finding may be confirmed by performing immunohistochemistry to detect viral antigen and/or histopathological analysis of brain tissue collected on day 14 after inoculation. Detection of viral antigen and/or neurological lesions in brain tissue would confirm virus replication in the brain. Presence of neurological signs and confirmatory viral antigen and/or histopathology in brain tissue would indicate a lack of suitable attenuation of the CVV.

References for Appendix 1


