Annex 3

Guidelines for the safe development and production of vaccines to human pandemic influenza viruses and influenza viruses with pandemic potential

Replacement of Annex 5 of WHO Technical Report Series, No. 941; and the WHO 2009 A(H1N1) update; and the WHO 2013 A(H7N9) update

Guidelines published by the World Health Organization (WHO) are intended to be scientific and advisory in nature. Each of the following sections constitutes guidance for laboratories that test candidate influenza vaccine viruses, vaccine manufacturers and national regulatory authorities (NRAs). If an NRA so desires, these WHO Guidelines may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these Guidelines are made only on condition that such modifications ensure that the risks of introducing influenza viruses into the community are no greater than would be the case if these WHO Guidelines are followed.

Abbreviations

ABSL	biosafety level
CVV	candidate vaccine virus
EID_{50}	egg infectious dose 50%
FFP	filtering face-piece
GISRS	(WHO) Global Influenza Surveillance and Response System
GMP	good manufacturing practice(s)
HA	haemagglutinin
HEPA	high-efficiency particulate air
HPAI	highly pathogenic avian influenza
IVPI	intravenous pathogenicity index
IVPP	influenza virus(es) with pandemic potential
LAIV	live attenuated influenza vaccine
LPAI	low pathogenic avian influenza
MDCK	Madin-Darby Canine Kidney (cells)
NA	neuraminidase
NRA	national regulatory authority
OIE	World Organisation for Animal Health
PFU	plaque forming unit(s)
PPE	personal protective equipment
PR8	influenza A/Puerto Rico/8/34 virus (A/PR/8/34)
RG	reverse genetics
$TCID_{50}$	tissue culture infectious dose 50%

1. Introduction

Careful risk assessment and strict biosafety and biosecurity precautions are needed in laboratory and manufacturing environments in order to ensure the safe handling of human pandemic influenza viruses, candidate vaccine viruses (CVVs) and influenza viruses with pandemic potential (IVPP) as the uncontrolled release of such viruses could have a significant impact on public health. In 2007, the WHO biosafety risk assessment and guidelines for the production and quality control of human influenza pandemic vaccines were published (*1*) in response to the pandemic threat posed by highly pathogenic avian influenza (HPAI) A(H5N1) viruses and the need to begin vaccine development. Since the publication of this WHO guidance, experience in the use of both IVPP and pandemic influenza viruses in the development and production of CVVs has increased globally. This experience includes the development and testing of CVVs derived by reverse genetics (RG) from HPAI viruses – a development reflected in these revised WHO Guidelines. Moreover, in response to the 2009 pandemic caused by the A(H1N1)pdm09 subtype virus and the emergence of low pathogenic avian influenza (LPAI) A(H7N9) viruses that are able to infect humans and cause severe disease with a high case fatality rate, the 2007 guidance was updated on two occasions by WHO (*2*, *3*). In addition, several WHO consultations – including the biannual WHO Vaccine Composition Meetings, the Global Action Plan for Influenza Vaccines (GAP) meetings and "switch" meetings[13] on influenza vaccine response at the start of a pandemic (*4*–*7*) – identified the testing timelines for CVVs as one of the bottlenecks to rapid vaccine responses. In light of these and other developments, requests were made to WHO by industry, regulators and laboratories of the WHO Global Influenza Surveillance and Response System (GISRS) to undertake a revision of the 2007 guidance.

In response, WHO convened a working group meeting on 9–10 May 2017 that was attended by experts, including representatives of WHO collaborating centres, WHO essential regulatory laboratories, national regulatory authorities (NRAs) for vaccine and biosafety regulation, manufacturers and the World Organisation for Animal Health (OIE). The working group reviewed cumulative experience, discussed the revision of the 2007 WHO guidance and reached a consensus on the outline and key elements of the revision (*8*). Subsequently, a draft revision was prepared and posted on the WHO website for public consultation. A WHO informal consultation was then held on 23–24 April 2018 to finalize the revision process (*9*). This document follows the risk assessment scheme used in the WHO biosafety guidance for pilot-lot vaccine

[13] Meetings relating to the issues involved in switching from seasonal influenza vaccine production to pandemic vaccine production in an emergency.

production (*10*). It also includes considerations relating to the greater scale of production needed to rapidly supply large quantities of vaccines, for which the risks are likely to be different to those for pilot lots. The document also takes into account the considerable experience gained from handling HPAI viruses and those classified as low virulence for avian species but highly virulent for humans.

2. Purpose and scope

These WHO Guidelines provide guidance to CVV-testing laboratories, vaccine manufacturers and NRAs on the safe development and production of human influenza vaccines in response to the threat of a pandemic. The document describes international biosafety expectations for pilot-scale and large-scale vaccine production and laboratory research. It is thus relevant to both vaccine development and vaccine manufacturing activities. It also specifies the measures to be taken to prevent or minimize the risk to workers involved in the development and production processes, and to prevent or minimize the risk of release of virus into the environment, including the risk of transmission to animals. Tests required to evaluate the safety of CVVs are also described. The document should be read in conjunction with the WHO *Laboratory biosafety manual* (*11*).

The guidance provided reflects greater knowledge of A(H5N1) subtype viruses (and other subtypes in general) and experience gained in the development and manufacture of vaccines against A(H5N1) viruses. Moreover, much has been learnt from experience with the A(H1N1)pdm09 virus, and from the production of vaccines against it. The guidance is also intended to apply to threats from any IVPP (for example, H2, H7 and H9 viruses) which may be virulent in humans. Manufacturers and laboratories handling HPAI viruses should consult their NRA to determine whether additional biosafety and biosecurity measures are required.

There is significant diversity in the pathogenicity of viruses used to make CVVs for the production of human vaccines and vaccines for other mammals. The transmission and pathogenicity of influenza viruses are multifactorial traits that are not completely understood (*12*). The haemagglutinin (HA) protein is the major virulence determinant of avian influenza viruses (*13*). Consequently, A(H5N1) HPAI viruses that 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 poultry. For viruses that are inherently less pathogenic for humans, the wild-type virus might be used directly for inactivated vaccine production (*14*). Thus, both reassortants derived by conventional reassortment and by RG – including those using synthetic nucleic acid as starting material (which may or may not be genetically modified) – and wild-type viruses are included in the scope of these Guidelines.

Although embryonated hens' eggs have traditionally been used to produce most influenza vaccines, cell culture techniques have also been successfully used for seasonal and pandemic vaccine production (*15*, *16*). The guidance provided in this document applies to current vaccine production technologies using either eggs or cell culture.

These Guidelines also cover both inactivated vaccines and live attenuated influenza vaccines (LAIVs). To date, most efforts to develop CVVs for pandemic vaccines have been focused on the development of inactivated vaccine. However, seasonal LAIVs derived from the A/Ann Arbor/6/60 virus have been licensed in North America and Western Europe, while LAIVs derived from the A/Leningrad/134/17/57 virus have been licensed in China, India, the Russian Federation and Thailand (*17*).

Technologies not covered by these Guidelines include new generation technology platforms that do not use live influenza vaccine viruses for production (for example, expressed recombinant proteins, virus-like particles, DNA- and RNA-based vaccines and vectored vaccines) – though some general principles may be applicable.

The guidance provided on containment measures in this document applies to all facilities and laboratories that handle live influenza viruses, including not only the CVV and vaccine manufacturing facilities but also the quality control laboratories of vaccine manufacturers, national control laboratories and other specialist laboratories. The transport of live virus materials within and between these sites must comply with international and national specifications (*18*).

Finally, risk assessments for vaccine manufacture will vary according to whether production occurs during an interpandemic phase or during a pandemic alert phase or pandemic phase (*19*). These Guidelines emphasize the steps required to identify and minimize risks in vaccine manufacture during the interpandemic phase, while indicating modifications that may be appropriate in other phases. It should be noted that a pandemic preparedness approach covering both inactivated influenza vaccines and LAIVs (formerly called "mock-up pandemic vaccines") during the interpandemic phase has been accepted by the European Medicines Agency (EMA) (*20*).

3. Terminology

The definitions given below apply to the terms as used in these WHO Guidelines. These terms may have different meanings in other contexts.

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

Airlock: an area found at the entrances or exits of rooms that prevents air in one space from entering another space. Airlocks generally have two

interlocked 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.

Backbone donor virus: an influenza virus that provides all or some non-glycoprotein internal genes to a reassortant virus. These genes may contain determinants of attenuation and/or confer high-growth/high-yield properties on the resulting reassortant virus.

Biosafety: the combination of physical and operational requirements and practices that protect personnel, the environment and the wider community against exposure to infectious materials during vaccine manufacture and quality control testing. Designation of laboratory facilities ranging from biosafety level (BSL) 1 to BSL4 and detailed principles for the operation of facilities at each level are set out in the WHO *Laboratory biosafety manual* (*11*) and in national or regional regulatory guidelines.

Biosafety committee: an institutional or organizational committee comprising individuals versed in the containment and handling of infectious materials.

Biosafety manual: a comprehensive document describing the physical and operational biosafety 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 who has responsibility for ensuring that the physical and operational practices required for the various levels of biosafety are carried out in accordance with the standard procedures of the institution.

Biosecurity: the protection and control of biological materials within laboratories and production facilities in order to prevent their unauthorized accessing, loss, theft, misuse, diversion or intentional release.

BSL2 or BSL3 enhanced: the use of additional physical and/or operational precautions, above those described for BSL2 or BSL3, based on a local risk assessment in consultation with the competent national authority and/or regulatory authority.

Bunded (area): an area that has either a permanent or a temporary barrier that is able to contain liquid and prevent leaks and spills from spreading contamination or damaging the facility (bunding).

Decontamination: a process by which influenza viruses are inactivated to prevent adverse health and/or environmental effects.

Egg infectious dose 50% (EID_{50}): the unit of infectious activity of a biological product or agent that causes infection in 50% of inoculated chicken embryos.

FFP2: a filtering face-piece (FFP) mask that provides high-level filtering capability against airborne transmissible microorganisms and generates an effective barrier to both droplets and fine aerosols to 94% efficiency.

FFP3: an FFP device (face-fitted mask) that provides high-level filtering capability against airborne transmissible microorganisms and generates an effective barrier to both droplets and fine aerosols to 99% efficiency (at 95 litres/minute air flow).

Fumigation: the process whereby a gaseous chemical is applied to sterilize or disinfect surfaces in an enclosed space.

Good manufacturing practice(s) (GMP): that part of quality assurance which ensures that products are produced and controlled consistently to the quality standards appropriate to their intended use and as required by the marketing authorization.

High-efficiency particulate air (HEPA) filter: a filter capable of removing at least 99.97% of all airborne particles with a mean aerodynamic diameter of 0.3 μm (previously high-efficiency particulate absorber of various efficiencies).

Highly pathogenic avian influenza (HPAI) viruses: avian influenza viruses causing systemic infection and mortality in chickens which, to date, are limited to H5 and H7 subtypes containing a cleavage site in HA with multiple inserted amino acids (also referred to in the literature as a "multibasic" or "polybasic" cleavage site – though other insertions have also been identified). The designation HPAI does not refer to the virulence of these viruses in human or other mammalian hosts.

Inactivation: the process of rendering an influenza virus nonviable by application of heat, chemicals (for example, formalin or β-propiolactone), ultraviolet irradiation or other means.

Intravenous pathogenicity index (IVPI): an indicator used to classify an avian influenza virus as HPAI or LPAI on the basis of mortality and morbidity over a 10-day period following intravenous inoculation of chickens with the virus.

Low pathogenic avian influenza (LPAI) virus: avian influenza viruses causing infections in poultry leading to no disease, mild disease or moderate disease (see also **IVPI**). LPAI viruses typically contain an HA with a single basic amino acid preceding the site of proteolytic cleavage (also referred to as a "monobasic" cleavage site). The designation LPAI does not refer to the virulence of these viruses in human and other mammalian hosts.

N95: a respiratory protective device designed to achieve a very close facial fit and very efficient filtration of airborne particles. The designation N95 means that the respirator blocks at least 95% of very small (0.3 μm) test particles when fitted correctly.

Primary containment: a system of containment, usually a biological safety cabinet or closed container, which prevents the escape of a biological agent into the work 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 and documented process for evaluating the potential risks that may be involved in a projected activity or undertaking.

Tissue culture infectious dose 50% ($TCID_{50}$): the unit of infectious activity of a biological product or agent that causes infection in 50% of inoculated tissue cultures.

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

4. Hazard identification

The hazards associated with vaccine manufacturing and laboratory testing of CVVs prepared from pandemic viruses and IVPP depend on: (a) the type of vaccine virus (reassortant or wild-type); (b) the method of production (egg-based, cell-culture-based or other); (c) whether it is an inactivated virus, live (attenuated) virus or recombinant virus-vectored vaccine; and (d) whether or not there are any deliberate modifications of the virus for attenuation or for enhanced immunogenicity and/or increased yield. Recombinant virus-vectored vaccines use replicating recombinant constructs based on viruses other than influenza virus (for example, modified vaccinia Ankara virus, adenovirus and vesicular stomatitis virus). The nature of their transgene, extent of virus shedding and the potential for recombination are outside the scope of the current document but are important factors that might need to be considered (*21*, *22*).

4.1 Candidate vaccine viruses

CVVs for both LAIVs and inactivated influenza vaccines are generally produced through reassortment with well-defined backbone donor viruses – for example, human viruses A/Puerto Rico/8/34 (PR8), A/Ann Arbor/6/60 or A/Leningrad/134/17/57. Wild-type viruses may also be used for vaccine production if recommended by WHO and approved by the NRA. Furthermore, new backbone donor viruses for reassortment are being developed and evaluated to enhance vaccine yields and other desirable properties. Although it is likely that a high-growth reassortant will provide the basis for future pandemic vaccine development, it is conceivable that a wild-type virus could be used.

4.1.1 Reassortants

The genome of the influenza A virus 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. HA is the major surface antigen of the virus, and antibodies directed against HA can protect from infection by neutralizing the virus. Antibodies to NA can inhibit viral infectivity at different points in the replication cycle and also have a role in protection from

disease (*23*). The remaining six RNA segments ("internal protein genes") encode internal structural and nonstructural viral proteins. The segmented structure of the genome allows for the exchange (reassortment) of individual RNA segments between influenza viruses upon coinfection of a single cell with two or more influenza viruses.

The conventional method for reassortment involves preparing CVVs by the co-inoculation of embryonated hens' eggs or tissue culture with a WHO-recommended wild-type virus and a backbone donor virus with a high-growth (yield) or attenuated phenotype. Co-inoculation allows for the reassortment of genetic segments between the two viruses. Antiserum against the surface glycoproteins of the backbone donor virus is then used to select a reassortant CVV, which must contain the HA gene of the WHO-recommended vaccine virus (but which normally contains both the HA and NA genes of the WHO-recommended vaccine virus). Amplification in eggs (or cultured cells) results in positive selection for the optimal combination of internal genes providing a high-yield reassortant virus. Several weeks are usually required for the production, validation and antigenic analysis of the reassortant (*7*). The use of a CVV in vaccine production requires approval by the NRA.

An alternative to the conventional approach to reassortant development is the use of an RG methodology to produce a reassortant vaccine virus (*24*). This process usually incorporates into plasmids the six RNA segments encoding the internal proteins of a backbone donor virus and the two segments encoding the HA and NA from the WHO-recommended vaccine virus. The plasmids are subsequently transfected into cells, with or without additional helper plasmids, in order to generate the CVVs to be used for vaccine manufacturing. RG technology allows for the direct manipulation of the influenza gene segments and can be faster than the use of conventional reassortment. Moreover, if an HPAI virus is used in the RG process, the HA gene can be modified to remove the specific amino acid motif at the HA cleavage site that is known to convey high pathogenicity in poultry (*25*). The reassortant can thus be specifically designed to serve as a CVV without the capacity to cause high pathogenicity in birds. The RG system has been reported to produce a CVV within 9–12 days (*26*) but further analysis of the product takes additional time. The distribution and receipt of the WHO-recommended vaccine virus as a source of RNA for constructing RG HA and NA plasmids adds extra time to the reassortment process. These delays can be minimized if the reassorting laboratories use site-directed mutagenesis of existing plasmids containing the HA and NA genes of a related virus, or by the use of synthetic DNA (*27*, *28*).

4.1.2 Backbone donor viruses

Reassortant CVVs containing donor genes – with the exception of segments 4 and 6 (HA and NA) – from backbone donor viruses (PR8, A/Ann Arbor/6/60 or

A/Leningrad/134/17/57) have been widely used to produce seasonal influenza vaccines and pandemic A(H1N1)pdm09 vaccines. A substantial body of data indicates that reassortant viruses composed of RNA segments coding for HA and NA derived from a pandemic virus or IVPP and the RNA segments coding for the internal proteins from PR8, A/Ann Arbor/6/60 or A/Leningrad/134/17/57 will have only a low probability of causing harm to human health (*10*, *24*).

PR8 is a common backbone donor virus for generating reassortant vaccine viruses as it replicates to high titre in embryonated hens' eggs. Originally used in the late 1960s to produce "high-growth reassortants", the use of such reassortants as vaccine viruses increases vaccine yield many fold (*29*–*31*). Moreover, PR8 has undergone extensive passaging in mice, ferrets and embryonated hens' eggs. This has resulted in the complete attenuation of the virus, rendering it incapable of replicating in humans (*32*, *33*). Improved backbone donor viruses are being developed in order to enhance yields for CVVs used to manufacture inactivated influenza virus vaccines. These new donor viruses may be derivations of PR8 viruses but they may also have genes from viruses other than PR8, be synthetically generated and/or be optimized for specific HA and NA subtypes (*26*). Demonstration of the adequate attenuation of CVVs using new/improved backbone donor viruses will be needed before approval for use (see section 5).

Some countries have licensed seasonal LAIVs that use reassortants with a 6:2 gene constellation based on donor viruses such as A/Ann Arbor/6/60 and A/Leningrad/134/17/57. Clinical studies of some 30 different vaccine viruses over a period of more than 40 years have demonstrated that both A/Ann Arbor/6/60-based and A/Leningrad/134/17/57-based reassortant vaccine viruses are attenuated for humans (*34*, *35*). These donor viruses might also be used for developing pandemic influenza vaccines and an adequate level of attenuation has been shown for modified reassortant viruses of various subtypes (*36*). For each CVV derived from a new pandemic virus or IVPP, the level of attenuation should be verified by testing, as described below in section 5.1. A(H5N1)-specific LAIVs made from A/Ann Arbor/6/60 reassortants have been licensed for pandemic preparedness purposes in several countries.

4.1.3 Gene segments from wild-type viruses (WHO-recommended vaccine viruses)

The gene constellation of reassortant CVVs derived by traditional co-cultivation methods must be determined. Reassortants with 6:2 or 5:3 gene constellations containing the HA and NA genes of the wild-type strain are the most common – however, reassortants containing other gene combinations may also be considered. NRAs will provide guidance and give approval to acceptable gene constellations for use in influenza vaccines. It is also possible that a mutant (non-reassortant) wild-type virus could be selected that has improved growth characteristics.

Because of their potential association with pathogenicity, genes from the wild-type virus (especially the HA and NA genes) require particular attention.

4.1.3.1 Haemagglutinin cleavage site

Most HPAI viruses of the H5 and H7 subtypes contain sequences of basic amino acids at the cleavage sites separating their HA1 and HA2 domains. Elimination of these HA polybasic cleavage sites is associated with reduced virulence in mammalian and avian models and with a low IVPI. However, some wild-type LPAI viruses (for example, A(H7N9) viruses) have caused serious human disease despite causing few signs of illness in poultry (*37*).

For reassortants derived from HPAI H5 and H7 viruses by RG, the HA should be modified so that the amino acids inserted at the HA cleavage site are reduced to a single basic amino acid; 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 large-scale vaccine manufacture. Cleavage site modifications have consistently reduced pathogenicity for avian embryos and poultry (*38*). However, modifying the cleavage site does not guarantee low pathogenicity in humans and other mammalian species because of the presence of other virulence factors (*39*, *40*).

4.1.3.2 Receptor specificity

Preferential binding of the HA to α2,6-linked terminal sialic acid residues is associated with transmissibility of influenza viruses in humans (*41*, *42*). However, viruses that preferentially bind to α2,3-linked terminal sialic acid residues (for example, A(H7N9) and A(H5N1) viruses) do not transmit well between humans but may on occasion infect humans and cause serious illness (*43*). While receptor specificity must be considered as a factor in reducing the risk of virus transmissibility and of causing harm to human health, modifying it is insufficient for virus attenuation.

The hazards associated with reassortants depend in part on HA receptor specificity. If a reassortant has a preference for avian cell receptors (that is, α2,3-linked terminal sialic acid), the hazard to humans is considered to be lower. However, if a reassortant has a preference for mammalian cell receptors (α2,6-linkages; for example, the 1957 A(H2N2) pandemic virus) or possesses both avian and mammalian receptor specificities, there is a greater risk of transmissibility and human infection. For A/goose/Guangdong/1/96-lineage H5 reassortants, it is anticipated that the HA will retain a preference for α2,3-linked terminal sialic acid residues, and so their transmissibility between humans should be reduced. However, some HPAI A(H5N1) viruses (for example, from Egypt) have been reported to exhibit increased binding to α2,6 linkages while maintaining a preference for α2,3-linked terminal sialic acid residues (*44*, *45*).

It is anticipated that A(H5N1) reassortant viruses derived by RG according to WHO guidance (*46*) would be attenuated for humans compared to wild-type H5 viruses. Nevertheless, the human lower respiratory tract contains α2,3-linked sialic acid receptors and thus exposure to high doses of A(H5N1) viruses represents a risk of infection. Moreover, humans are immunologically naive to H5 and many other avian subtypes, and this too is an important risk factor.

It should be noted that influenza virus pathogenicity does not depend solely on HA but is a polygenic trait. The 1997 A(H5N1) virus had unusual PB2 and NS1 genes that influenced pathogenicity, whereas 2004 A(H5N1) viruses possessed complex combinations of changes in different gene segments that affected their pathogenicity in ferrets (*47*, *48*). Compared to HA, the NA protein of influenza viruses has a less prominent role as a virulence factor. It is known that a balance of HA (receptor binding) and NA (receptor destruction and virus release) activities is required for efficient viral replication (*49*, *50*). Further, specific adaptations in NA have been identified that facilitate transmission from wild aquatic birds to poultry. However, specific NA determinants for adaptation to, and virulence in, humans have so far not been found – though there is some evidence that the NA can mediate HA cleavage in A(H1N1) viruses (*51*, *52*). It is of note that resistance to the viral inhibitors oseltamivir and zanamivir is caused by specific mutations in either NA or HA. While it is acknowledged that there is a long history of safety for reassortants using PR8 or LAIV backbones, safety testing should be conducted as new CVVs are being produced (see section 5, Table A3.1 below) unless virus-specific risk assessments suggest a different approach.

4.1.3.3 Secondary reassortment

It is conceivable that reassortment between a CVV containing HA and NA from an IVPP and a human wild-type seasonal influenza virus could occur during simultaneous infection of humans with both viruses. For secondary reassortants to be generated the following would need to happen:

- infection of a human (for example, vaccine production staff) with the CVV;
- simultaneous infection of the same human with a wild-type seasonal influenza virus; and
- reassortment between the CVV and the wild-type seasonal influenza virus.

Such a secondary reassortant may have properties distinct from the seasonal virus and might still be able to replicate in humans and spread from person to person. The likelihood of such secondary reassortment is considered to be low to negligible. However, laboratory and production facilities must have biosafety control measures in place to prevent the exposure of staff to live

reassortant viruses. In a case of accidental exposure, it is unlikely that a CVV would replicate efficiently or transmit to human contacts. In over 40 years of vaccine manufacturing, there have been no reported cases of influenza resulting from the secondary reassortment of CVVs.

4.1.4 Wild-type HPAI CVVs

The use of wild-type HPAI CVVs has been confined to cell-culture-based production, which for inactivated vaccines uses closed systems under high containment. Stringent biosafety and biosecurity measures are required during production, analytical testing and waste disposal in order to protect staff and prevent the release of infectious virus into the environment. CVVs produced by RG and demonstrated to be attenuated, as described in section 5 below, are preferred.

4.1.5 Other wild-type CVVs

Vaccines may also be produced from other wild-type CVVs (for example, swine and LPAI viruses).

The pathogenicity of these wild-type viruses for humans cannot be predicted; some A(H7N9) viruses that are of low pathogenicity in poultry have caused severe illness in humans (*53*). Although the transmissibility of wild-type viruses with avian receptor specificity is likely to be low in humans, the transmissibility of wild-type viruses with mammalian receptor specificity (for example, swine viruses) is largely unknown and is likely to depend on a number of factors, including population immunity.

Appropriate measures should be in place to prevent exposure of staff to CVVs derived from wild-type viruses because of the risk of secondary reassortment with circulating influenza viruses, as described in section 4.1.3.3.

4.1.6 Susceptibility of CVVs to NA inhibitors

CVVs that are sensitive to NA inhibitors or other licensed drugs should be used for vaccine production whenever possible. If the relationship between genotype and phenotype is well known, sequence verification may be sufficient to confirm the presence of genetic motifs known to be associated with drug susceptibility. Otherwise, susceptibility should be confirmed by phenotypic testing.

5. Safety testing of candidate vaccine viruses

CVVs can be developed by WHO GISRS laboratories, laboratories associated with WHO GISRS that have been approved by an NRA, and the laboratories of vaccine manufacturers. The following tests and specifications have been

developed on the basis of experience gained in evaluating CVVs derived from viruses of various subtypes. The safety testing required for different CVVs and their proposed containment levels are summarized in Table A3.1. The information summarized in this table should be considered as guidance – changes in the requirements may be determined on a case-by-case basis by WHO and/or national authorities. For CVVs developed from newly emerging IVPP, a WHO expert group will review the data obtained from safety testing and advise WHO. WHO will then provide further guidance on appropriate containment requirements through its expert networks such as WHO GISRS. Moreover, laboratories that generate CVVs will produce a summary report of all safety testing conducted on a given CVV. This documentation may be of assistance for importation/transportation purposes and is available on request from those laboratories.

The requirement to conduct or complete some or all of these tests prior to the distribution of a CVV may be relaxed on the basis of additional risk assessments. Such assessments should take into account evolving virological, epidemiological and clinical data, and national and international regulatory requirements for the shipment and receipt of infectious substances.

Animal tests with CVVs and IVPP should be conducted in animal containment facilities in accordance with the proposed containment levels shown in Table A3.1. For untested CVVs, the containment level to be used is the one shown for the respective wild-type virus. In specific cases – such as CVVs derived from synthetic DNA encoding modified HA genes (see footnote "c" in Table A3.1) from H5 and H7 HPAI viruses – the containment level may be lowered based on a virus-specific risk assessment. An appropriate occupational health policy should be in place.

5.1 Tests to evaluate pathogenicity of candidate vaccine viruses

The recommended tests for evaluating the pathogenicity of CVVs depend on the parental wild-type viruses (that is, WHO-recommended vaccine viruses) from which they are derived (Table A3.1). The nature of the parental viruses and the risks of the procedures involved will also determine the required containment level. The tests required to evaluate CVVs are described in the following sections. Some CVVs may not require complete safety testing if they are genetically similar to a CVV that has already been tested – that is, they have HAs and NAs derived from the same (or a genetically closely related) wild-type virus and are on the same (or a genetically closely related) backbone and have the same internal gene constellation. For these CVVs, it may be sufficient to confirm sequence and genetic stability as determined by a WHO expert group and/or by competent national authorities.

5.1.1 Attenuation in ferrets

Ferrets have been used extensively as a good indicator of influenza virus virulence in humans (*54*). Typically, seasonal influenza viruses cause mild-to-no clinical signs in ferrets, and virus replication is usually limited to the respiratory tract. PR8 viruses have also been assessed in ferrets and have been found to cause few or no clinical signs, with virus replication limited to the upper respiratory tract (*39*). Conversely, some wild-type HPAI viruses can cause severe and sometimes fatal infections in ferrets (*48*, *55*). Thus, in the absence of human data, the ferret is generally considered to be the best model for predicting pathogenicity and/or attenuation in humans. The mouse is not considered an appropriate model for the safety testing of influenza CVVs.

CVVs should be shown to be attenuated in ferrets in accordance with Table A3.1, except when virus-specific risk assessments suggest a different approach (for example, waiving the ferret test where Table A3.1 requires it). These tests should be conducted in well-characterized and standardized ferret models (for example, by using common reference viruses, when available, from WHO collaborating centres and/or essential regulatory laboratories for influenza). Detailed test procedures are described in Appendix 1 of these WHO Guidelines. One or more laboratories may have ferret pathogenicity data on parental wild-type viruses (that is, WHO-recommended vaccine viruses) that could be used by all testing laboratories as a further benchmark for comparison. Limiting the testing requirements for the wild-type viruses will help counteract the time delays associated with the export and import of IVPP and/or pandemic viruses. Assessing the transmissibility of CVVs between ferrets is not required because of the difficulties of standardizing this assay across laboratories (*56*, *57*).

5.1.2 Pathogenicity in chickens

For CVVs derived from HPAI H5 or H7 parental viruses, determination of the chicken IVPI is recommended and may also be required by national authorities. The procedure should follow that described in OIE guidance (*58*). Any virus with an index greater than 1.2, or that causes at least 75% mortality in inoculated chickens, is considered to be an HPAI virus (*58*).

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

HPAI viruses 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 the test be established and characterized using known positive and negative control viruses (*59*).

5.1.4 Gene sequencing

Gene sequencing is required for confirming virus identity and/or verifying the presence of attenuating and other phenotypic markers (for example, markers of cold adaptation and temperature sensitivity in the case of LAIV CVVs). HA and NA genes should be fully sequenced, with the extent of backbone gene segment sequencing dependent on the nature of the backbone donor viruses (for example, LAIV CVVs may require full sequencing to confirm the presence of attenuating mutations).

5.1.5 Genetic stability

The genetic stability of CVVs is generally assessed after 6–10 passages in relevant substrates (that is, embryonated hens' eggs or cultured cells). Subsequent sequence analysis can verify the retention (stability) of the markers of relevant phenotypic traits related to pathogenicity, where such markers are known. These tests should be conducted on all CVVs (including wild-type CVVs) prepared from pandemic viruses and IVPP. It may be possible to ship viruses to manufacturers before genetic stability has been fully established by the reassorting laboratories.

Table A3.1
Required safety testing of CVVs and proposed containment levels for vaccine production

Category of CVV	Tests needed on CVVs[a]	Proposed containment level for vaccine production[b]
Modified reassortant viruses based on H5 and H7 HPAI viruses[c]	Ferret (5.1.1); chicken (5.1.2);[d] plaquing (5.1.3); sequence (5.1.4); genetic stability (5.1.5)[e]	BSL2 enhanced (6.5.1)
Reassortant viruses derived from H5 and H7 LPAI viruses	Ferret (5.1.1); sequence (5.1.4); genetic stability (5.1.5)[e]	BSL2 enhanced (6.5.1)
Reassortant viruses derived from non-H5 or non-H7 viruses	Ferret (5.1.1); sequence (5.1.4); genetic stability (5.1.5)[e]	BSL2 enhanced (6.5.1)
Wild-type H5, H7 HPAI viruses	Sequence (5.1.4); genetic stability (5.1.5);[e] also see footnote f	BSL3 enhanced (6.4)
Wild-type H5, H7 LPAI viruses	Ferret (5.1.1); sequence (5.1.4); genetic stability (5.1.5);[e] also see footnote g	BSL2 enhanced (6.5.1)[g]

Table A3.1 *continued*

Category of CVV	Tests needed on CVVs[a]	Proposed containment level for vaccine production[b]
Wild-type non-H5 or non-H7 viruses	Ferret (5.1.1); sequence (5.1.4); genetic stability (5.1.5);[e] also see footnote g	BSL2 enhanced (6.5.1)[g]

[a] Tests to be performed by a WHO GISRS or other approved laboratory.

[b] The proposed containment levels may be changed (to higher or lower containment) based on a specific risk assessment.

[c] This category refers to viruses derived by RG technology such that the additional amino acids at the HA cleavage site are removed.

[d] 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.

[e] Genetic stability testing should be performed. However, it should not delay the distribution and use of the CVV by manufacturers and can be performed subsequently.

[f] Testing in ferrets and chicken (IVPI) is not required because the highly pathogenic phenotype is already known and the highest containment level is required to work with these viruses.

[g] If a virus-specific hazard assessment identifies that additional control measures are appropriate, the containment level may be increased. The requirements for this level may be specific to a particular facility and should be assessed on a case-by-case basis by the relevant competent national authority.

6. Risk assessment and management

6.1 Nature of the work

Influenza vaccine production in embryonated hens' eggs or cell culture requires the propagation of live virus. In most cases, the generation of CVVs will result in viruses that are expected to be attenuated in humans (*10*, *24*). Several steps in the manufacturing process have the potential to generate aerosols containing live virus. The virus concentration in aerosols will depend on the specific production step and will be highest during the harvesting of infectious allantoic fluid and much lower during seed virus preparation and egg inoculation (which involve either small amounts of liquid containing virus or very dilute virus suspensions). Appropriate biosafety measures (for example, the use of laminar air flows, biological safety cabinets with HEPA filtration, cleaning and decontamination of equipment, waste management and spill kits) must be in place to prevent accidental exposure in the work environment and the release of virus into the general 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 – however, many of the viruses used for producing vaccines are CVVs in an

attenuating donor backbone (for example, A/PR/8/34, A/Ann Arbor/6/60 or A/Leningrad/134/17/57) and so the resulting CVV will have a low probability of causing harm to human health.

Vaccine manufacture requires adherence to both GMP and appropriate biosafety requirements for biological products, as well as to related national regulations, technical standards and guidelines. GMP protects the product from the operator, and protects the operator and the environment from the infectious agent, thus reducing the risk of any hazard associated with production. Reassortants derived from PR8 backbone donor viruses have been used routinely for producing inactivated influenza vaccines for over 40 years. This work usually requires thousands of litres of infectious egg allantoic fluid, which can create substantial aerosols of reassortant virus within manufacturing plants. Although manufacturing staff may be susceptible to infection with these virus aerosols, there have been no documented or anecdotal cases of work-related human illness resulting from occupational exposure to the reassortant viruses described above. Similarly, reassortants derived from the A/Ann Arbor/6/60 and A/Leningrad/134/17/57 viruses have been used for the production of LAIV for many years with no reported cases of work-related human illness related to these viruses. While no study has yet been undertaken to detect asymptomatic infections caused by either PR8-derived or live attenuated viruses, the attenuation status of these CVVs continues to be supported by their excellent safety record.

The use of pandemic CVVs that express avian influenza genes may lead to potential consequences for agricultural systems. For example, if influenza A H5 or H7 viruses or any influenza A virus with an IVPI greater than 1.2 are introduced into poultry (*60*) then OIE must be notified of the presence of infection, and this could lead to the implementation of biosecurity measures aimed at preventing the spread of disease (*58*, *60*). Moreover, infection of birds other than poultry (including wild birds) with influenza A viruses of high pathogenicity must also be reported to OIE.

6.2.2 Vaccine production in eggs

Influenza vaccine has been produced in embryonated hens' eggs since the early 1940s. Much experience has been gained since then, with some facilities 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 production stages and/or laboratory quality control activities prior to virus inactivation. During egg inoculation only small amounts of liquid containing virus or very dilute virus suspensions are used. When the eggs are opened to harvest the allantoic fluid, the open nature of this operation may result in hazardous exposure to aerosols and spills. Following this step, the allantoic fluid is handled in closed vessels and so the hazards arising

from live virus during subsequent processing and virus inactivation (if used) are less than during virus harvest. The collection and disposal of egg waste is potentially a significant environmental hazard. Ensuring the safe disposal of the waste from egg-grown vaccines, both within the plant and outside, is therefore critical and procedures should comply with the guidance and/or requirements of competent national authorities.

6.2.3 Vaccine production in cell culture

For pandemic influenza vaccines produced in cell cultures, the biosafety risks associated with manufacturing will depend primarily on the nature of the cell culture system. Closed systems such as bioreactors usually present little or no opportunity for exposure to live virus during normal operation – however, additional safety measures must be taken during procedures for adding samples to the bioreactor or removing them from it. Virus production in roller bottles and cell culture flasks may allow for exposure to live virus through aerosols, spills and other means. Additional risks can be associated with the inactivation and disposal of the large quantities of contaminated liquid and solid waste (including cellular debris) generated by this method.

During passage in mammalian cells, it is possible that genetic mutations may be selected for in pandemic and IVPP CVVs that render them more adapted to humans. These changes are most likely to occur within or close to the receptor-binding domain of the HA glycoprotein. Sequence analysis may detect such changes, but whether these changes affect the ability of a mutant virus to cause infection in humans is not well established. In one study, an attempt to de-attenuate a PR8 virus by multiple passage in organ cultures of human tissue failed (*32*). Another study showed that human viruses with α2,6 receptor specificity were likely to mutate to α2,3 receptor specificity upon passage in Madin-Darby Canine Kidney (MDCK) cells thus making them less likely to be infectious for humans (*61*). Overall, the hazards arising from the inherent properties of a reassortant or wild-type virus are likely to be greater than the probability of the virus adapting to a more human-like phenotype in cell culture.

6.2.4 Hazards from the vaccine

Inactivated pandemic influenza vaccines present no biosafety risks beyond the manufacturing process, provided that the results of the inactivation steps show complete virus inactivation, rendering the virus incapable of replication.

In an interpandemic or pandemic alert phase, pilot-scale live attenuated pandemic influenza vaccines may be developed for clinical evaluation. The biosafety risks associated with virus shedding or other unintentional release of virus into the environment following vaccination should be carefully assessed. Based on this risk assessment, subjects participating in clinical trials in the

interpandemic or pandemic alert phases should be kept in clinical isolation. If this is not done then indirect hazards for humans could arise.

While it is very unlikely that an LAIV will be harmful to humans, an indirect potential hazard may exist through secondary reassortment with a human or animal influenza virus, as discussed in section 4.1.3.3.

Evidence to date indicates that the probability of generating secondary reassortants is very low (*62*). Moreover, containment procedures have significantly improved over the last 40 years and production staff can be vaccinated to reduce the chances of acquiring an infection with a circulating wild-type seasonal influenza virus, thus minimizing the risk of secondary reassortment. In addition, appropriate personal protective equipment (PPE) can also be provided.

6.3 Environmental protection

6.3.1 Environmental considerations

Influenza A viruses are enzootic or epizootic in some farm animals (poultry, pigs and horses) and in some populations of wild birds – particularly birds of the families Anseriformes (ducks, geese and swans) and Charadriiformes (shorebirds) (*63*).

Several avian influenza A viruses (especially H5 and H7 subtypes) can be highly pathogenic in poultry. In addition, sporadic infections by influenza A viruses have been reported in other species, including farmed mink, wild whales, seals, captive populations of wild cats (tigers and leopards) (*64*) and domestic cats (*65*) and dogs (*66*). In big cats, infection has been reported following the consumption of dead chickens infected with A(H5N1) viruses.

It is expected that many IVPP will have avian receptor specificity and thus birds would theoretically be most susceptible. Many studies indicate that viruses with PR8 backbones are attenuated in chickens. For example, a reassortant containing an HA with a single basic amino acid at the cleavage site, an NA from the 1997 Hong Kong A(H5N1) virus and the genes coding for the internal protein genes of a PR8 virus was barely able to replicate in chickens and was not lethal (*67*). Similarly, a 6:2 PR8 reassortant that contained a 2003 Hong Kong A(H5N1) HA did not replicate or cause signs of disease in chickens (*39*). The removal of the multiple basic amino acids at the HA cleavage site in these H5/PR8 reassortants probably played a major role in reducing the risk for chickens.

It is likely that the temperature-sensitive phenotype of cold-adapted vaccine viruses would limit replication of these viruses in avian species due to the higher body temperature of birds. Pigs, however, have both α2,3- and α2,6-linked sialic acid receptors in abundance (*68*) and, in the absence of direct evidence to the contrary, must be considered susceptible to most influenza A viruses, including LAIV and PR8 reassortants.

6.4 Assignment of containment level

Definition of the required containment conditions must be 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 under GMP (*69*). It should be noted that biosafety control measures apply to manipulations involving live virus – such measures no longer apply once a virus has been inactivated by a validated process.

The generation of reassortant CVVs from HPAI viruses typically takes place in BSL3 facilities, as advised by WHO (*46*), with additional enhancements (BSL3 enhanced). The use of additional physical and/or operational precautions, above those described for BSL3 (*11*), should be based on a local risk assessment in consultation with the competent national authority and/or regulatory authority. The specific enhancements will vary from facility to facility and will depend on the design of the facility and operational procedures employed. Such enhancements may include the use of dedicated laboratory clothing, availability of shower facilities, final HEPA filtration of laboratory exhaust air, laboratory effluent decontamination and a quarantine plan.

Special consideration should be given to the hazards associated with the cell-culture production and quality control of vaccines made from HPAI wild-type CVVs. In view of the open nature of large-scale egg-based vaccine production, it is not feasible to operate in BSL3 enhanced conditions. Therefore, egg-based vaccine production from HPAI wild-type viruses is not recommended.

Because of the hazards associated with egg- and cell-culture vaccine production and quality control involving the use of conventional or RG-derived reassortant CVVs that are known to be attenuated (see section 5.1), the production facility should have a BSL2 enhanced containment level, as specified in section 6.5.1. Under defined circumstances, CVVs for which safety testing has not yet been completed may be used in production facilities that comply with containment level BSL2 enhanced with additional controls, as specified in section 6.5.2, with the approval of the NRA. The parts of the facility where such work (both production and quality control) is carried out should meet national and OIE requirements for containment. These requirements include biosafety and biosecurity requirements and environmental controls that limit the introduction into, and spread within, animal populations (*58*). The requirements to be met should be agreed upon with WHO and competent national and regional authorities (*19*, *70*). This guidance applies to both pilot-scale and large-scale production during the interpandemic and pandemic alert phases (*19*). Any subsequent relaxation of the containment level to the standard used for seasonal vaccine production must be authorized by the competent national authorities on a case-by-case basis after evaluating the risks.

6.5 Environmental control measures

Containment measures to prevent the release of live virus into the environment should be established on the basis of a risk assessment specific to the virus, the production system and relevant biosafety guidelines – either those of NRAs or of WHO.

Local biosafety and biosecurity regulations provide guidance on the disposal of potentially infectious waste. In particular, contaminated waste from production facilities may reach very high virus titres. All decontamination methods should be validated at regular intervals as required by the competent national authority. If possible, decontamination of waste should take place on site. Where this is not possible, it is the responsibility of the manufacturer to contain material safely during transport prior to off-site decontamination. Guidance on regulations for the transport of infectious substances is available from WHO (*18*) and from competent national authorities. In all cases, procedures must be validated to ensure that they function at the scale of manufacturing. Stringent measures to control rodents, other mammals and birds must also be in place.

Each manufacturer should also assess the risk of exposing birds, horses, pigs or other susceptible animals if they are likely to be in the vicinity of the manufacturing plant. Following potential occupational exposure to live virus, staff or other personnel should avoid visiting pig, horse or bird facilities (for example, farms, equestrian events and bird sanctuaries) for at least 14 days following exposure. If conjunctivitis or respiratory signs and symptoms suggest that influenza might develop during this 14-day period, the quarantine period should be extended to 14 days (twice the expected time for virus shedding) after the signs and symptoms have resolved (*71*).

6.5.1 Specifications for BSL2 enhanced production facilities

In addition to the principles for BSL2 facilities specified in the WHO *Laboratory biosafety manual* (*11*), the specifications for BSL2 enhanced facilities include those described below.

6.5.1.1 Facility

The facility should be designed and operated in such a way as to protect the vaccine, the staff producing and testing the vaccine, the environment and the population at large. Different solutions may be needed according to the risks inherent in the operation(s) conducted in the area. Specialized engineering solutions will be required that may include the following:

- Use of relative negative pressure biosafety cabinets;
- HEPA filtration of air prior to its exhaust into public areas or the environment;

- Room pressure cascades designed to contain live virus safely while also protecting the product. A net negative pressure between the atmosphere and areas where live virus is handled can be separated by an area (barrier) of positive pressure higher than the pressure both in the atmosphere and in areas where live virus is handled. Alternatively, a negative-pressure barrier can be built where live virus is trapped and then removed by HEPA filtration before it can escape into the atmosphere.

In addition, the following decontamination procedures should take place:

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

6.5.1.2 Personal protection

A range of personal protection measures should be in place, including the following:

- Full-body protective laboratory clothing (for example, Tyvek® disposable overalls) should be available.
- If activities cannot be contained by primary containment and open activities are being conducted, the use of respiratory protective equipment which can be checked for close facial fit – such as FFP2 (for example, N95) or FFP3 (*72*) respirators – is required. Appropriate minimum 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 quality control areas where CVVs, pandemic viruses and IVPP are handled, should sign a written document in which they agree not to have any contact with susceptible animals (for example, ferrets or farm animals, especially birds, horses and pigs) for 14 days after leaving the facility where vaccine has been produced. If conjunctivitis or respiratory signs and symptoms suggest that influenza might develop during this 14-day period, the quarantine period should be extended to 14 days (twice the expected time for virus shedding) after the signs and symptoms have resolved (*71*). Currently the risks involved in contact with household dogs and cats

are not considered to be significant, but the scientific evidence on this risk is sparse.

- It is strongly recommended that staff should be vaccinated with seasonal influenza vaccines.
- If vaccines targeting the virus in production are available and marketing authorization has been received, vaccination with these vaccines is recommended for staff before large-scale vaccine production commences.
- A medical surveillance programme for staff should be established prior to manufacturing activities. Antiviral medicines should always be available in case of accidental exposure. Where these medicines are available only on prescription, access to prescribing doctors/hospitals and to stocks of medication should be ensured.

6.5.1.3 Monitoring of decontamination

Cleaning and decontamination methods must be validated and reviewed periodically as part of a master plan to demonstrate that the protocols, reagents and equipment used are effective in inactivating pandemic influenza virus on all surfaces, garments of personnel, waste materials and storage containers. Once decontamination protocols for influenza virus have been fully described and validated, there is no need to conduct a separate validation study for each new influenza 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 virus.

6.5.2 Specifications for BSL2 enhanced production facilities with additional controls

It is assumed that ferret pathogenicity testing will be conducted on all CVVs of unknown pathogenicity, even given the assumptions outlined above (see section 5.1) regarding the low probability that a PR8 reassortant virus or LAIV is pathogenic for humans. This assumption is based on current experience in relation to reassortants of HA subtypes other than H1 and H3 (that is, H5, H7 and H9). A facility must meet the requirements for protecting personnel who handle potentially dangerous microorganisms. The WHO *Laboratory biosafety manual* (*11*) includes a risk survey that can be undertaken prior to rating a laboratory space as BSL1, BSL2 or BSL3. Similar requirements can be found in Directive 2000/54/EC of the European Parliament and of the Council (70) on the protection of workers from risks related to exposure to biological agents at work, and the United States Department of Health and Human Services *Biosafety in microbiological and biomedical laboratories* (5th edition) (*72*).

Large-scale vaccine manufacturing using a CVV before its safety testing is complete can be considered if justified by evolving virological, epidemiological and clinical data, and if it meets national and international regulatory requirements regarding the shipment, receipt and handling of infectious substances.

A facility that meets the criteria detailed below in section 6.5.2.1 and that has the noted operator protections in place could be considered suitable for manufacturing vaccine at large scale using a CVV prepared by RG or a conventional reassortant methodology before safety testing is complete, with the approval of the NRA.

6.5.2.1 Facility

The facility should be designed and operated in such a way as to protect the vaccine, the staff producing and testing the vaccine, the environment and the population at large. This will require specialized engineering solutions that may include the following:

- Appropriate signage and labelling must be in place regarding the activities being carried out when a virus is in use while the safety testing is being completed.
- The facility must be designed and constructed as a contained GMP space. The surfaces and finishes must comply with GMP requirements (*69*) that ensure they can be sealed and easily cleaned and decontaminated.
- The air cascades within the facility should be such that any live virus can be contained within the work zones in which it is being used. All work with infectious virus must be conducted within these contained zones.
- Access to the contained areas must be via double-door entry airlocks. The airlocks should operate at a pressure that is either lower or higher than that on either side. In this way the airlocks become either a "sink" or a pressure barrier, containing the flow of air within the facility. In cases where the airlocks provide a low-pressure sink, the entry and exit doors should be interlocked or fitted with a suitable delay or alarm system to prevent both being opened at the same time. It is also acceptable if the airlocks are part of a series of increasing negative pressure. The air pressure cascade within the negative-pressure contained zone should comply with GMP requirements (that is, higher pressures in cleanest zones) for clean rooms.
- All supply and exhaust air must be passed through HEPA filtration while maintaining all required containment and GMP conditions.

Air-handling systems within the facility must be rigorously assessed to ensure that they protect against potential failure. Fail-safe systems must be installed wherever necessary. The facility should be constantly monitored to ensure that appropriate room pressure differentials are maintained.

- All reusable equipment should be cleaned in place, decontaminated by means of autoclaving, or otherwise cleaned and decontaminated by validated, dedicated systems prior to reuse.
- Areas of potential liquid spill, including waste-treatment plants and processes, should be assessed and bunded to ensure that any spill is contained. Procedures must be in place to ensure that spills are contained, areas are cleaned and contaminated materials are properly disposed of in order not to compromise the integrity of the facility.
- The entry of materials into contained zones should be via separately HEPA-filtered, interlocked, double-ended "pass-through cabinets" or double-ended autoclaves.
- All facility waste, including egg waste, should be discarded via validated on-site waste-effluent systems or following decontamination by autoclaving. Any items which pass from the external environment to the manufacturing process and are later returned to the external environment (for example, egg trays) must receive special attention. Dedicated procedures for the washing and decontamination of equipment must be in place and fully validated.

6.5.2.2 Personal protection

- All clothing worn outside the facility should be replaced by manufacturing-facility garments on entry into the facility.
- Gowning in areas in which live virus is handled should always include full suit, overshoes, eye protection and double gloves.
- Suitable PPE (full hood powered air-purifying respirators based on the risk assessment) should be provided for all personnel working in containment areas within the manufacturing facility. The hoods should be worn at all times when the facility is in operation under these enhanced biosafety requirements.
- All facility clothing is to be removed on exit, with soiled clothing removed from the facility via a decontamination autoclave or similar method. The surfaces of respirator hoods should be decontaminated.
- Specific procedures should be developed and implemented for the operation of the facility under enhanced biosecurity conditions.

- It is strongly recommended that staff should be vaccinated with a seasonal influenza vaccine. In the case of pandemic viruses and IVPP, and before large-scale vaccine production is attempted, pilot lots of vaccine may already have been produced. If they are available and if marketing authorization has been received, vaccination of staff against the virus being produced is recommended before large-scale production begins.
- Procedures should be in place to provide antiviral treatment whenever warranted (for example, following accidental exposure).
- On-site occupational health and safety and medical support should be maximized by providing medical consultation and training in recognizing influenza-like symptoms, along with out-of-hours referral to medical facilities with quarantine capabilities.
- It is recommended that staff should take showers on exiting the facility. Showers are mandatory for staff who may have been accidently exposed to vaccine virus.
- All personnel, including support staff and others who may enter the production or quality control areas where CVVs, pandemic viruses and IVPP are handled, should sign a written document in which they agree not to have any contact with susceptible animals (for example, ferrets or farm animals, especially birds, horses and pigs) for 14 days after leaving the facility where vaccine has been produced. If conjunctivitis or respiratory signs and symptoms suggest that influenza might develop during this 14-day period, the quarantine period should be extended to 14 days (twice the expected time for virus shedding) after the signs and symptoms have resolved (*71*). Currently the risks involved in contact with household dogs and cats are not considered to be significant, but the scientific evidence on this risk is sparse.

6.6 Biosafety management and implementation within a vaccine production facility

6.6.1 Management structure

The implementation of these WHO Guidelines requires that the institution employs a biosafety officer who is knowledgeable about large-scale virus production and containment but whose reporting responsibilities are independent of the production unit. The biosafety officer is responsible for overseeing the implementation of biosafety practices, policies and emergency procedures within the company or organization and should report directly to the highest

management level. A biosafety officer is needed in addition to a qualified person who, in some countries, has overall responsibility for a medicinal product.

A biosafety committee that includes representatives of the vaccine production and quality control units should be responsible for reviewing the biosafety status of the company and for coordinating preventive and corrective measures. The institutional biosafety officer must be a member of the committee. The committee chairperson should be independent of both the production and quality control units. The biosafety committee should report to the executive management of the manufacturing company to ensure that adequate priority is given to the implementation of the required biosafety measures, and that the necessary resources are made available.

6.6.2 Medical surveillance

Manufacturers of vaccines to protect against human pandemic influenza viruses and IVPP should provide training to their occupational health professionals in recognizing the clinical signs and symptoms of influenza. Company physicians, nurses and vaccine manufacturing supervisors and staff must make decisions on the health of personnel who are associated with the manufacturing and testing of these vaccines. Local medical practitioners caring for personnel from the manufacturing site should receive special training in the diagnosis and management of pandemic influenza infection and should have access to rapid influenza diagnostic kits and to a laboratory that performs molecular diagnosis of influenza (for example, using real-time polymerase chain reaction). Any manufacturer starting large-scale production should have documented procedures – including diagnostic procedures and prescribed treatment protocols – for dealing with influenza-like illness affecting the staff and their family members. Manufacturers should ensure that staff understand their obligation to seek medical attention for any influenza-like illness and to report it to the occupational health department or equivalent. Manufacturers should ensure that antiviral treatment is available if warranted (for example, in the case of accidental exposure) and should have defined arrangements for advising staff with any influenza-like illness.

6.6.3 Implementation

A 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. The 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 necessary to reduce the risk to workers and to the environment should be considered as part of this analysis, and the results should be documented.

A comprehensive biosafety manual or equivalent document must be published and implemented. The manual should fully describe the biosafety aspects of the production process and quality control activities. It should define such items as emergency procedures, waste disposal, and the safety practices and procedures that were identified in the risk analysis. The manual must be made available to all staff working in the production and quality control units, and at least one copy must be present in the containment area(s). The manual should be reviewed at least every 2 years.

Comprehensive guidelines outlining the response to biosafety emergencies, spills and accidents should be prepared and should be made available to key personnel both for information and for coordination with emergency response units. Rehearsals of emergency response procedures are helpful. The guidelines should be reviewed and updated at a defined frequency (for example, annually).

Implementation of the appropriate biosafety level in the production and testing facilities should be verified through an independent assessment. National requirements concerning verification mechanisms should be in place and must be followed.

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Appendix 1

Testing for attenuation of influenza CVVs in ferrets

Laboratories testing for the attenuation of influenza CVVs in ferrets should make use of a panel of standard/reference viruses (referred to as "pathogenicity standards" in the following sections) with 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 the attenuation of CVVs is being measured against common parameters independently of subtype. In order to be designated as attenuated, the CVV to be tested must show parameters of pathogenicity that are below the predefined values of a high pathogenicity standard and are in line with the values of an attenuation standard. Comparative attenuation with the parental wild-type virus is not necessary in this case. However, laboratories that have the capacity to evaluate the attenuation of a CVV compared with the parental wild-type virus can continue to do so. To minimize the expected experimental variability of results across different laboratories, the pathogenicity standards can 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 results 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 carried out and advice should be sought from experienced WHO laboratories.

Test virus

The 50% egg or tissue culture infectious dose (EID_{50} or $TCID_{50}$) 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 10^6 to 10^7 EID_{50}, $TCID_{50}$ or PFU of virus and diluted not less than 1:10. Where possible, the pathogenic properties of the donor PR8 virus 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 A3.1 (see section 5 above). For untested CVVs, the containment level to be used for the ferret safety test is the one shown

for the respective wild-type virus. In specific cases, such as for CVVs derived from synthetic DNA representing H5 and H7 HPAI viruses, the containment level may be lowered based on a virus-specific risk assessment. An appropriate occupational health policy should be in place.

Experimental procedure

Outbred ferrets 4–12 months of age that are serologically negative for currently circulating influenza A and B viruses and for the test virus strain are anaesthetized by either intramuscular administration of a mixture of sedatives – for example, ketamine (25 mg/kg) and xylazine (2 mg/kg) and atropine (0.05 mg/kg) – or by suitable inhalant anaesthetics. A standard virus dose of 10^7 to 10^6 EID_{50} (or $TCID_{50}$ or PFU) in 0.5–1 ml of phosphate-buffered saline is used to inoculate animals. The dose should be the same as that used for pathogenicity studies with the wild-type parental virus, if used, or the pathogenicity standards previously characterized 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 4–6 ferrets should be inoculated. One group of 2–3 animals should be euthanized on day 3 or day 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 or established standards, it is recommended that additional lung samples be collected and processed with haematoxylin and eosin staining for histopathological evaluation. The remaining brain tissue should be collected for histopathological evaluation in the event that infectious virus is detected in this tissue. The remaining animals are observed for clinical signs, which may include weight loss, lethargy (based on a previously published index) (*1*), respiratory and neurological signs, and increased body temperature. Collection of nasal washes from animals anaesthetized 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 7 days. At the termination of the experiment on day 14 after inoculation, a necropsy should be performed on at least two animals and organs should be collected. If signs of substantial gross pathology are observed (for example, lung lesions), the organ samples should be processed for histopathological evaluation as described above.

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, and histopathology of the lungs should demonstrate attenuation

when compared to wild-type viruses or established 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 some seasonal A(H3N2) viruses (*2*) where virus was detected in the olfactory bulb. Consequently, if virus is detected in the anterior or posterior regions of the brain (excluding the olfactory bulb) the significance of such a finding may be confirmed by performing immunohistochemistry to detect viral antigen and/or histopathological analysis of brain tissue collected on day 3 or day 4 and on day 14 after inoculation. The detection of viral antigen and/or neurological lesions in brain tissue would confirm virus replication in the brain. The presence of neurological signs and confirmatory viral antigen and/or histopathology in brain tissue would indicate a lack of suitable attenuation of the CVV.

A model summary table for reporting test results is provided in Table A3.A1.1 with the intention of harmonizing data reporting between laboratories testing for the attenuation of influenza CVVs in ferrets.

Table A3.A1.1
Summary of results in ferrets infected intranasally with CVV

Virus	Dose (EID_{50})[a]	Number of animals	Number of animals with clinical signs to day 14 post-inoculation				Mean maximum % weight loss	Respiratory tract viral titres (log_{10} EID_{50}/ml or $TCID_{50}$)[b]		Lung lesions (day 3/4)[c, d]	Lung lesions (day 14)[c, d]	Detection of virus in other organ[e]
			Lethargy	Respiratory	Weight loss	Other (e.g. fever)		Mean peak nasal wash or nasal turbinate	Lung			
CVV												
Reference virus(es)												

[a] Indicate whether dose is expressed as EID_{50}, $TCID_{50}$ or PFU.

[b] Indicate whether respiratory viral titres are expressed as EID_{50}, $TCID_{50}$ or PFU per ml or g. Give lower limit of detection.

[c] Score gross pathological lung lesions as: – (absent); + (≤ 20%); ++ (> 20% but < 70%); or +++ (> 70%).

[d] Indicate outcome of any histopathological evaluation.

[e] Indicate organ or not detected.

References

1. Reuman PD, Keely S, Schiff GM. Assessment of signs of influenza illness in the ferret model. J Virol Methods. 1989;24(1–2)27–34 (abstract: https://www.sciencedirect.com/science/article/pii/0166093489900049, accessed 6 February 2019).
2. Zitzow LA, Rowe T, Morken T, Shieh W-J, Zaki S, Katz JM. Pathogenesis of avian influenza A (H5N1) viruses in ferrets. J Virol. 2002;76(9):4420–9 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC155091/, accessed 6 February 2019).