

Annex 4

Recommendations to assure the quality, safety and efficacy of influenza vaccines (human, live attenuated) for intranasal administration

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Recommendations published by WHO are intended to be scientific and advisory in nature. The parts of each section printed in type of normal size have been written in such a form that, should a national regulatory authority so desire, they may be adopted as they stand as definitive national requirements or used as the basis for such requirements. The parts printed in smaller type are comments and recommendations intended as guidance for manufacturers and national regulatory authorities that may benefit from additional information. It is recommended that any modifications be made only on condition that they ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the recommendations set out below. In order to facilitate the international distribution of vaccine made in accordance with these recommendations, a summary protocol for the recording of results of tests is given in Appendix 1. The terms “national regulatory authority” and “national control laboratory” as used in these recommendations always refer to the country in which the vaccine is manufactured.

Introduction

The WHO requirements for influenza vaccine (live) date from 1979 (1). The purpose of these updated recommendations is to provide vaccine manufacturers and national regulatory authorities with applicable considerations and guidance in developing specific processes for assuring the quality, safety and efficacy of influenza vaccines (human, live attenuated) for intranasal administration, including their nonclinical and clinical evaluation.

These recommendations apply to influenza vaccines (human, live attenuated) for intranasal administration using embryonated hen's eggs as substrates. It is expected that these vaccines will be produced using cell cultures as substrates and guidance is also provided for this eventuality. These recommendations are not specific for a particular form of influenza vaccine virus attenuation used to prepare the final influenza virus vaccine product. They should apply to the production and quality control of viruses intended for use in the manufacture of influenza vaccine (human, live attenuated) for intranasal administration, including reassortant viruses prepared either by classical reassortment methods or by reverse genetics techniques.

The recommendations (with possible modifications) are meant to apply to influenza vaccines (human, live attenuated) for intranasal administration, produced with seasonal vaccine strains for use during the inter-pandemic period as well as vaccines produced with strains for use during pandemics. However, these recommendations cannot anticipate every situation that may arise, and alternative considerations may be needed for specific public health circumstances. The first draft of this document was based on the requirements for influenza vaccines (live) from 1979 (1) and on the recommendations for the production and control of influenza vaccines (inactivated) (2). Sections on the preclinical, nonclinical and clinical evaluation were added to the updated recommendations to assure the quality, safety and efficacy of influenza vaccines (human, live attenuated) for intranasal administration. The section on testing animals for adventitious agents, which was included in the 1979 requirements, was removed from the updated recommendations as animal testing for that purpose is no longer generally recommended.

The recommendations in this document do not apply to the potential vector systems (i.e. other viral or bacterial hosts) that could be used to deliver the antigenic components of influenza viruses. Further recommendations may be developed in the future as additional strategies emerge for immunological control of influenza virus infections.

General considerations

Influenza is a significant cause of morbidity and mortality and each year has major social and economic impact throughout the world. Influenza viruses

undergo continuous evolutionary change, which makes control of influenza challenging. In order to assist national regulatory authorities and manufacturers in control efforts, WHO provides annual reviews of epidemiological information and recommendations on the influenza viruses to use in vaccines in countries of the northern and southern hemispheres (3).

Many people require medical treatment and/or hospitalization, and excess mortality often accompanies viral influenza epidemics; the vast majority of those affected are elderly. Because the elderly constitute the most rapidly increasing sector of the population in many countries, the epidemiology of viral influenza can be expected to change accordingly, especially in high-income countries. Although mortality is typically highest among the elderly, influenza epidemics originate in settings that bring together immunologically susceptible individuals who are capable of spreading infection rapidly throughout a community, such as school-aged children. Infants are particularly susceptible to the severe consequences of viral influenza infections, but children of all ages may experience complications of influenza, including pneumonia and death.

At present, the most generally available means of influenza prophylaxis is vaccination. Potential means of prevention, other than personal and societal hygienic measures, include antiviral medications. However, the indefinite nature of exposure to influenza virus, which could necessitate protracted compliance with an antiviral medication regimen, as well as issues of potential or real emergence of drug-resistant virus strains, has prompted strategies to reserve chemoprophylaxis for specific circumstances (4, 5).

Inactivated influenza vaccines, which function mainly by inducing IgG antibodies specific for influenza virus haemagglutinins, have a long and solid record of use. The interest in live influenza virus vaccines stems from their potential to permit simplified administration by intranasal drops or spray (6). In addition, they stimulate not only systemic humoral immunity but also local and systemic immune protective mechanisms, including mucosal IgA antibodies and cellular immunity. The possibility of controlling influenza virus infection and illness by the use of live attenuated virus vaccines given by the intranasal route was thus extensively investigated during the latter half of the twentieth century. Live influenza virus vaccines arising from studies of “cold-adapted” donor strains have been used as an effective public health tool in industrialized countries including the Russian Federation (7) and the United States of America (8). Although current cold-adapted vaccines are manufactured in embryonated hen’s eggs, there is ongoing research to develop influenza vaccines (human, live attenuated) using other methods of virus attenuation and produced in cell culture.

The principle of a live vaccine for controlling a viral infection has a sound basis and has been used in preventing other viral infectious diseases, such as poliomyelitis, measles, mumps and rubella. Attenuated poliovirus vaccines are given orally to infect the cells of the intestinal tract; this stimulates protective

immune responses that mimic those occurring after natural poliovirus infection. By analogy, it may be possible to initiate a benign influenza virus infection in the nasopharynx with an attenuated influenza virus strain to give protection against the prevalent wild-type influenza strains.

The successful deployment of live attenuated virus donor strains depends on ensuring an appropriate balance between attenuation and immunogenicity. The aim is to produce an attenuated virus that incorporates the key immunizing antigens and antigenic determinants of circulating wild influenza viruses but retains the stable genetic and phenotypic characteristics of the attenuated donor strain when given to susceptible individuals on a wide scale. The ideal candidate would provide strong strain-specific protection and broad cross-reactivity, stimulate all categories of protective immunity, produce few or no symptoms in the most susceptible hosts, and be able to infect all hosts in whom specific protective immunity is lacking. Pragmatically, compromise on one or more of these features may be required.

The continuing commercial development and public health use of influenza vaccines made from live attenuated influenza virus strains make it appropriate to review and update the WHO recommendations for such vaccines. Since the requirements for influenza vaccine (live) were published in 1979, there have been significant advances in influenza virus vaccine production. For example, current reverse genetics techniques allow the selection of a homogeneous predefined viral composition by using a system that reconstitutes influenza viruses from genetic codes specific for each of the eight influenza viral gene segments (9–11). Knowledge of the genetic markers associated with virus attenuation has also increased, allowing more stringent control of the vaccine. Additionally, considerable efforts have been devoted to pandemic planning to ensure that safe and effective vaccines can be produced quickly in response to a pandemic emergency (12, 13). It is therefore necessary to revise the previous guidance on live attenuated influenza vaccines in order to reflect new developments and current practices in the field. In accordance with current WHO policy, the revised document is renamed as “Recommendations”.

Part A. Manufacturing recommendations

A.1 Definitions

A.1.1 International name and proper name

The proper name of the vaccine shall be “influenza vaccine (human, live attenuated)”, translated into the language of the country of origin.

Use of the proper name should be limited to vaccines that satisfy the recommendations formulated below.

A.1.2 Descriptive definition

Influenza vaccine (human, live attenuated) is a preparation of live attenuated influenza virus originating from human or other species. Influenza vaccine (human, live attenuated) is an aqueous suspension, which may be lyophilized, and is intended for intranasal administration. Influenza vaccines (human, live attenuated) contain a strain or strains of influenza virus types A or B or a mixture of these two types, which have been grown individually in embryonated hen's eggs or in cell cultures. The influenza vaccines (human, live attenuated) shall be named "human" as they are to be administered to human beings.

A.1.3 International standards

No international reference preparations are currently available for quality control or release testing of influenza vaccine (human, live attenuated) for intranasal administration. Moreover, these influenza vaccines may differ between manufacturers such that infectivity tests and/or potency assays may not be standardized for universal application. Therefore, no recommendations based on the use of international reference preparations can be formulated at present for influenza vaccine (human, live attenuated) for intranasal administration.

Each manufacturer of influenza vaccines (human, live attenuated) for intranasal administration should provide preparations of reference live attenuated influenza viruses and specific antisera for use in tests of virus infectivity and/or potency specific to the live attenuated vaccine (see sections A.5.3.1, A.5.4.1 and A.7.3). The manufacturer should cooperate with national regulatory authorities to determine the acceptability of the proposed reference reagents.

The infectivity tests and/or potency assays should be established and validated during vaccine development and approved by the national regulatory authority. Strains used for quality control or release testing of influenza vaccines (human, live attenuated) for intranasal administration should be preparations antigenically representative of viruses with surface antigens (haemagglutinin and neuraminidase) identical or closely related to the WHO-recommended vaccine strains (3).

A.1.4 Terminology

The definitions given below apply to the terms used in these recommendations. They may have different meanings in other contexts.

Adventitious agents. Contaminating microorganisms of the cell culture or line including bacteria, fungi, mycoplasmas and viruses that have been unintentionally introduced (14). WHO is developing further guidance on adventitious agents.

Attenuated donor virus strain. An attenuated influenza virus that provides the genes of attenuation for vaccine strains that can be shown to be safe during clinical trials in human beings (15).

Candidate influenza vaccine viruses for seasonal or non-highly pathogenic influenza A subtype viruses with pandemic potential. These influenza viruses, approved by WHO as suitable for making influenza vaccine, are typically prepared in vaccine virus reassortment laboratories by “classical” reassortment; however, reverse genetics techniques may also be considered in their preparation (16).

Candidate influenza vaccine viruses for H5N1 and other highly pathogenic influenza A subtype viruses. These influenza viruses, approved by WHO as suitable for making influenza vaccine, are prepared in vaccine virus reassortment laboratories by reverse genetics (16).

Cell bank. A collection of ampoules containing material of uniform composition stored under defined conditions, each ampoule containing an aliquot of a single pool of cells (14).

Cell seed. A quantity of well-characterized cells of human, animal or other origin stored frozen at or below -100°C in aliquots of uniform composition derived from a single tissue or cell, one or more of which would be used for the production of a master cell bank (14).

Classical genetics (classical reassortment). The process of finding and assembling a set of genes that affect a biological property of interest. In this process, mutants (reassortants) are generated by employing mutagens that accelerate the normal mutation rate or by growing the organism and allowing spontaneous mutations to occur. Mutants are selected for a particular biological property (phenotype) that differentiates them from the wild type. The location of the mutations responsible for the mutant phenotype is identified and analysed to determine the role of the altered DNA on the studied biological property (17).

Clinical evaluation of vaccines. Includes all the clinical trials and other clinical studies conducted in human beings pre- and post-licensure to determine the safe and effective use of vaccines intended for the control of specific diseases. Clinical evaluation is done in phases so that information is gathered in a coherent manner that respects the rights and dignity of all study participants and reduces the risks to participants, and that provides an understanding of the potential benefit of the vaccine under study (18).

Egg infectivity dose 50% (EID₅₀). The quantity of a virus suspension that will infect 50% of embryonated hen's eggs inoculated with the suspension (9).

Final bulk. The finished vaccine prepared from one or more monovalent pools present in the container from which the final containers are filled. It may contain one or more virus strains (2).

Final lot. A collection of sealed final containers that are homogeneous with respect to the risk of contamination during filling procedures (including lyophilization). A filling lot must therefore have undergone the filling procedures (including lyophilization) in one working session from a single final bulk (2).

Genetic reassortment. In genetic reassortment, genes from two or more influenza viruses are mixed in different combinations, resulting in hybrid viruses with genetic characteristics of each parent virus. This process occurs in nature but can also be achieved in a laboratory using “classical” reassortment or reverse genetics (16, 17).

High-growth reassortant viruses. Influenza viruses that have been genetically modified to grow better in embryonated hen’s eggs for optimal vaccine production (16).

Highly pathogenic influenza viruses. Influenza viruses (typically from an avian host) that cause at least 75% mortality when inoculated intravenously into 4–8-week-old chickens (19).

Influenza reference viruses. Wild-type influenza viruses selected by WHO as representative of important groups of influenza viruses on the basis of extensive antigenic and genetic studies and comparisons with viruses from many countries. As the influenza viruses evolve in nature, new reference viruses are selected (16).

Influenza virus subtype(s). Type A influenza viruses are further classified according to their combinations of haemagglutinin (H) and neuraminidase (N) antigens (i.e. specific proteins on the virus surface), e.g. H5N1. Sixteen H subtypes and nine N subtypes have been distinguished (16).

Master cell bank. A quantity of fully characterized cells of human or other animal origin derived from the cell seed stored frozen at or below $-100\text{ }^{\circ}\text{C}$ in aliquots of uniform composition derived from the cell seed. The master cell bank is itself an aliquot of a single pool of cells generally prepared from a selected cell clone under defined conditions, dispensed into multiple containers and stored under defined conditions. The master cell bank is used to derive all working cell banks. The testing performed on a replacement master cell bank (derived from the same cell clone or from an existing master or working cell bank) is the same as for the initial master cell bank, unless a justified exception is made (2).

Master seed lot. The virus used to prepare the master seed lot is an attenuated influenza virus that combines the attenuating features of attenuated virus donor strain and the immunizing features of the wild-type virus reference strain. The master seed lot is a virus preparation that is antigenically representative of a WHO-recommended strain that has been processed at one time to ensure a uniform composition, is fully characterized, and may be used for the preparation of working seed lots or for production of vaccine. The national regulatory authority approves the master seed lot and its passage level.

Median tissue culture infective dose 50% (TCID₅₀). The quantity of a virus suspension that will infect 50% of tissue culture inoculated with the suspension.

Monovalent virus pool. A pool of a number of single harvests of a single virus strain processed at the same time (2).

Nonclinical evaluation of vaccines. All in vivo and in vitro testing performed before and during the clinical development of vaccines. The potential

toxicity of a vaccine should be assessed not only before the start of human trials but throughout clinical development (20).

Non-highly pathogenic influenza viruses. Influenza viruses (sometimes also termed low-pathogenic) that cause less than 75% mortality when inoculated intravenously into 4–8-week-old chickens (19).

Novel (new) subtype of human influenza A virus. Refers to human influenza viruses with haemagglutinin and/or neuraminidase antigens that are distinct from seasonal influenza viruses and with the potential to cause a pandemic (16).

Plaque-forming unit (pfu). The smallest quantity of a virus suspension that will produce a plaque in monolayer cell cultures (21).

Preclinical evaluation of vaccines. All in vivo and in vitro testing carried out before the first testing of vaccines in humans. This is a prerequisite for the initiation of clinical trials and includes product characterization, proof of concept/immunogenicity studies and animal safety testing (20).

Production cell cultures. A collection of cell cultures, used for biological production, that have been prepared together from one or more containers from the working cell bank or, in the case of primary cell cultures, from the tissues of one or more animals (14).

Reverse genetics. The technique of determining a gene's function by first sequencing and then mutating it, and identifying the nature of the change in the phenotype (17).

Single harvest. A quantity of virus suspension harvested from the growth substrate inoculated with the same virus strain and incubated and harvested together in one session (14).

Specific pathogen-free (SPF). Used in relation to animals that have been shown by the use of appropriate tests to be free from specified pathogenic microorganisms, and also refers to eggs derived from SPF birds (22).

Specific antibody-negative (SAN). Used in relation to animals that have been shown by the use of appropriate tests to be free from antibodies to specified avian pathogenic microorganisms, and also to eggs derived from SAN birds (22).

WHO-recommended viruses for vaccine use. Wild-type influenza viruses recommended by WHO as the basis for an influenza vaccine (16).

Wild-type influenza viruses. Influenza viruses that have been cultured either in eggs or in cells (i.e. isolated) directly from clinical specimens and that have not been modified (16).

Wild-type reference virus strain. An influenza virus that has been selected to antigenically represent the circulating viruses against which the vaccine should protect recipients (15).

Working seed lot. A quantity of fully characterized virus of uniform composition derived from a master seed lot by a number of passages not exceeding the maximum approved by the national regulatory authority. The working seed lot can be used for production of vaccines (2).

Working cell bank. A quantity of cells of uniform composition derived from the master cell bank at a finite passage level, dispensed in aliquots into individual containers appropriately stored, usually frozen at or below -100°C , one or more of which would be used for production purposes. All containers are treated identically and, once removed from storage, are not returned to the stock (14).

A.2 **Background on influenza vaccine (human, live attenuated) production**

Even before inactivated influenza vaccines entered commercial use, there was interest in live attenuated vaccines. Motivation for their development included mimicking the immune responses to natural influenza virus infection.

A number of candidate attenuated virus donor strains have been examined over the years, including A/Puerto Rico/8/34 (H1N1), A/Okuda/57 (H2N2) (attenuated by simple serial passage), avian–human reassortant viruses (attenuated by host range characteristics of the avian donor strains), and temperature-sensitive mutants (attenuated by chance recovery of viruses, serial passage, or introduction of mutations by directed mutagenesis) (23, 24). Many candidates, however, have been withdrawn from consideration during clinical development. For example, reassortants prepared with A/Puerto Rico/8/34 are not always well attenuated and introduction of a temperature-sensitive mutation without other stabilizing mutations resulted in a virus strain prone to reversion to a non-attenuated form, which may occur after replication in a vaccine recipient.

Thus far, the most successful strategy for preparing attenuated virus donor strains has been the development of cold-adapted attenuated influenza viruses by serial passage at sequentially lower temperature, which produced mutations in multiple gene segments (25–29). The presence of multiple mutations involving several influenza virus gene segments appears to contribute to the stability of the live attenuated virus genomes and to lower the probability of a reversion to virulence. Methods other than cold adaptation are also being explored and are in the early stages of evaluation (30–32). If new live influenza vaccines are approved for human use, these present recommendations may be revised.

Influenza vaccines contain the antigens of one or more influenza A and B viruses that represent the wild-type influenza viruses prevalent in human populations. Influenza A viruses are separated into subtypes based on structurally and antigenically distinct haemagglutinins and neuraminidases. Influenza B viruses are not separated into subtypes, but they do have genetic lineages of haemagglutinins and neuraminidases, which may be antigenically distinguishable. Influenza A subtypes and influenza B lineages undergo progressive evolutionary changes of haemagglutinin and neuraminidase antigens (antigenic drift), which may reduce the efficacy of vaccines when the

vaccines are inadequately matched to the prevalent viruses. For influenza A virus subtypes not previously circulating in human populations (antigenic shift), it is expected that vaccines will be effective only if they incorporate the antigens of the novel influenza A subtype.

The composition of influenza vaccines (human, live attenuated), like that of other influenza vaccines, is constantly under review to optimize the protective efficacy against prevalent epidemic strains. Accordingly, WHO publishes recommendations twice a year concerning the strains to be included, so that virus strains antigenically matched to circulating strains are included in vaccines manufactured for distribution in the northern and southern hemispheres (3).

Antigenic modifications in the haemagglutinin and neuraminidase molecules typically involve variation in surface amino acid residues in the region of the molecule furthest from the viral envelope. Future antigenic variations cannot be predicted because the mechanism of selection of antigenic variants (antigenic drift) is not known and several evolutionary pathways appear possible. Antigenic shifts (the appearance of new influenza A haemagglutinin subtypes) are also unpredictable.

In addition to antigenic drift and shift, there is another type of variation among influenza viruses caused by the preferential growth of virus subpopulations in different host cells in which the virus is cultivated. Influenza viruses grown in embryonated hen's eggs often exhibit genetic, antigenic and biological differences from those isolated and maintained in mammalian cells. Sequence analyses of the haemagglutinin genes of egg-adapted variants show that human influenza viruses grown in eggs are less likely to maintain fidelity to the original sequence than the same viruses grown in mammalian cells. It is therefore important in vaccine preparation to ensure that antigenic changes in the haemagglutinin molecule do not impair the protective effects of the vaccine.

There is a long history of safety for egg-grown influenza virus vaccines (33, 34). However, it is known that influenza viruses grown in embryonated hen's eggs can be contaminated with other viral agents. Although the use of eggs from flocks husbanded to meet agricultural health criteria (35) for freedom from specific pathogens may reduce the chances of introduction of a microbial agent, adventitious agents can be introduced from any egg source. The recommendations in this document have been revised in view of the findings with egg-grown viruses, the increasing use of mammalian cells for virus isolation and vaccine production, and the improved methods of detecting adventitious agents (14, 36). WHO is developing further guidance on testing adventitious agents.

There have been several influenza A virus pandemic alerts since 1997 (H5N1 subtype in 1997, 2003, 2005 and later years; H7N7 in 2003; and H9N2 in 1999) when avian influenza A viruses caused illness often serious enough to require hospitalization and to cause death in infected humans. In addition, the 2009 experience with pandemic influenza A (H1N1) demonstrates the

potential for non-avian influenza viruses to cause significant morbidity and mortality. These events illustrate the need for some flexibility and a variety of strategies for the production and clinical use of a vaccine in response to a pandemic. For example:

- it may be necessary to generate a vaccine virus from a highly pathogenic virus by reverse genetics;
- monovalent vaccines may be preferred; and
- two vaccine doses may be needed by all vaccine recipients.

Reflecting the special needs of an influenza pandemic, WHO has developed recommendations to assure the quality, safety and efficacy of the vaccine for pandemic situations (12).

The use of reverse genetics for vaccine virus development is relevant to both inter-pandemic and pandemic vaccines (15). Reverse genetics technology has already been introduced as a method for generating reassortants for manufacturing commercial influenza vaccines (human, live attenuated). It allows necessary genetic modifications such as removal of virulence motifs, and is therefore also used to produce reassortants for use in vaccines in the event of a pandemic. Reverse genetics technology involves transfecting mammalian cells with plasmids coding for influenza virus genes in order to produce a virus reassortant. Production of reassortants in this way is similar in concept to classical reassorting methods, but there are some important differences.

- The source of the influenza virus haemagglutinin and neuraminidase genes for reverse genetics is reduced in importance since the process of extraction of nucleic acid eliminates concerns about adventitious agents: an egg isolate, an isolate in cells not approved for human vaccine production, or a clinical specimen may all be adequate to provide the nucleic acid needed for the start of reverse genetics.
- The reverse genetic reassortant virus is generated in mammalian cells acceptable to national regulatory authorities.
- In some countries, a reassortant produced using reverse genetics is classified as a “genetically modified organism” and the vaccine should comply with national regulations or with WHO’s Biosafety risk assessment and guidelines for the production and quality control of human influenza pandemic vaccines (13).

Development of a new candidate attenuated donor virus should apply the knowledge gained in developing safe and immunogenic vaccines. Significant information has accumulated to indicate that not all attenuation methods result in donor vaccine virus strains of acceptable stability. For example, it is known that

an attenuated donor virus strain based on a mutation conferring temperature sensitivity alone can be expected to revert to a virulent form, even after a single passage in human recipients (26). Attenuation due to multiple mutations appears to be more stable and probably more useful for long-term implementation of influenza vaccines (human, live attenuated). Genetic stability and retention of key phenotypic features of the attenuated donor strain are extremely important quality characteristics. Assurance that reversion to virulence is unlikely to occur in humans should be established in preclinical and clinical studies and monitored carefully during the post-marketing period.

Multiple strategies for deriving candidate master seed and working seed viruses may be considered for seasonal vaccines and vaccines against influenza A subtype viruses not classified as highly pathogenic (e.g. pandemic influenza A (H1N1) 2009 virus). Only reverse genetics is appropriate for candidate vaccine viruses derived from highly pathogenic avian influenza viruses. However, both classical reassorting methods and reverse genetics techniques represent controlled methods to be considered for developing seed viruses once a satisfactory attenuated virus donor strain is obtained.

Classical reassortment between the attenuated virus donor strain and the new wild-type virus requires selection steps that may not always be readily successful in providing the construct of choice. However, classical reassortment can be an effective and relatively rapid method of producing attenuated seed viruses with wild parent surface antigens. General experience with live virus vaccines shows that candidate seed viruses derived by classical reassortment should be cloned at least three times by limiting dilution passage in SPF-SAN embryonated eggs or plaque purified in qualified cells to ensure purity of the desired attenuated seed virus. Reverse genetics methods permit a directed and more defined preparation of the desired reassortant virus. These methods also allow elimination of the highly pathogenic phenotype of avian influenza viruses, since the major molecular determinants of pathogenicity (multiple basic amino acids at the haemagglutinin cleavage site) can be removed during the preparation of the plasmids used to produce the reverse genetics reassortant. Neither reverse genetics nor classical reassortment eliminates the requirement for careful genetic and phenotypic assessment of the potential master seed and working seed viruses to ensure retention of attenuation.

Influenza viruses present a challenge for vaccine preparation as they exhibit continuous antigenic change in surface antigens of circulating influenza virus strains. Both inactivated and live influenza virus vaccines can confer a degree of cross-protection against related virus strains within a common haemagglutinin or neuraminidase subtype. For either type of vaccine, the use of a vaccine strain with haemagglutinin and neuraminidase antigenically identical to the naturally prevalent virus strain is expected to provide optimum protective

efficacy. In practice, however, it may not always be possible for the haemagglutinin and neuraminidase to be identical with influenza viruses, since further evolution may occur during the several months needed for vaccine preparation.

The interval between the appearance of an influenza virus variant and its spread throughout the world may be only a matter of months, and the development of an appropriate vaccine virus strain must therefore be rapid. The time available for the yearly preparation and testing of new influenza vaccine (human, live attenuated) lots must be carefully estimated and planned; some flexibility may be possible in completing control tests for routine production lots. Clinical studies, if undertaken, must be focused but the information they can provide about potential vaccine performance is likely to be severely limited.

Where appropriate, the technologies and experience acquired over several decades in the production and control of all live virus vaccines should be applied to influenza vaccines (human, live attenuated) for intranasal administration. As for all live vaccines, the substrate on which the virus is propagated is critically important to maintaining the consistency and safety of the vaccine product. Influenza vaccines (human, live attenuated) for intranasal administration are usually produced in 9–11-day-old vaccine-quality embryonated eggs; a large measure of safety can be assured by sourcing the eggs from closed-layer flocks that are continuously monitored for known specific pathogenic agents and antibodies against them. These SPF-SAN layer flocks are now available in several countries, and the eggs – or cell cultures derived from them – have been widely used in the production of a number of vaccines, including measles and mumps vaccines (37).

The production of influenza vaccines (human, live attenuated) using cell culture is currently under active research. When considering the use of animal cells as *in vitro* substrates for the production of biologicals, specific WHO guidelines should be taken into account (14, 36). Since live influenza vaccines depend on the viability of the virus in filled containers, the storage conditions as well as the short- and long-term stability of liquid and lyophilized products should be established by rigorous studies similar to those undertaken in the preparation of other live virus vaccines (38).

Apart from addressing the technical challenges associated with the rapid development of suitable attenuated vaccine seed viruses, manufacturers and national regulatory authorities should cooperate to define the need for special administrative arrangements for registration and licensing and to establish the nature of data needed for review of the vaccine product preparation and use. Close collaboration between manufacturers and the national regulatory authorities is required, particularly during development, production and testing of the initial batches of live vaccines. The time for considering the risks and benefits of the vaccines and for completing licensing procedures should be as short as practically possible if appropriate influenza vaccines (human, live attenuated) for intranasal administration are to be available soon after the emergence of a variant.

In a public health emergency, i.e. influenza pandemic, the abnormal vaccine demand may create difficulties if all tests recommended in the WHO guidelines on regulatory preparedness for human pandemic influenza vaccines (12) are to be carried out. Decisions to modify these requirements in the interests of public health during such an emergency are the responsibility of national regulatory authorities. Since progress in the development and implementation of live attenuated influenza vaccines may result in improvements or, conversely, in additional concerns to be addressed, the WHO Recommendations to assure the quality, safety, and efficacy of influenza vaccines (human, live attenuated) for intranasal administration are expected to need updating from time to time.

A.3 General manufacturing recommendations

The general requirements for manufacturing establishments contained in WHO good manufacturing practices for biological products (39) should apply to establishments manufacturing influenza vaccine (human, live attenuated) for intranasal administration, with the addition of the following.

A.3.1 Procedures and facilities

Details of standard operating procedures for the production and testing of influenza vaccines adopted by a manufacturer, together with evidence of appropriate validation of the production process, should be submitted to the national regulatory authority for approval. Proposals for modification of the manufacturing/control methods should also be submitted to the national regulatory authority for approval.

Production areas should be cleaned, disinfected and/or decontaminated by validated procedures before being used for the manufacture of influenza vaccines (human, live attenuated) for intranasal administration. The areas where processing of live attenuated influenza vaccines takes place, and the procedures used for manufacturing, should be designed to ensure that it is impossible to contaminate the influenza vaccine (human, live attenuated) for intranasal administration with another product. It is considered that filling of influenza vaccine (human, live attenuated) for intranasal administration could occur on a campaign basis in the same facility used for filling other vaccines provided that the manufacturer develops and performs a risk analysis and evaluation and puts in place validated procedures for risk control (13).

Facilities for vaccine production should be constructed with adequate containment features to accommodate the candidate influenza vaccines derived from the wild-type influenza viruses; WHO's Biosafety risk assessment and guidelines for the production and quality control of human influenza pandemic vaccines (13) should be followed. Standard operating procedures must be developed for dealing with emergencies involving accidental spillage, leakage

or other dissemination of virus. High levels of biocontainment are required for work with the highly pathogenic wild-type influenza viruses that may be used in generating master and working seed viruses.

The production of influenza vaccines (human, live attenuated) for intranasal administration should be conducted by staff who have not handled other infectious microorganisms or animals on the same working day. Staff must practise good sanitation and health habits and personnel with respiratory or other apparent infectious illness should be excluded. Particular attention should be paid to the recommendations given in WHO good manufacturing practices for biological products (39) regarding the training and experience of personnel in charge of production and testing and of those assigned various responsibilities in the manufacturing establishment. Personnel employed in the production and control facilities should be adequately trained and protected against accidental infection with influenza virus according to guidance in the *WHO biosafety manual* (40) and in *Biosafety guidelines for personnel engaged in the production of vaccines and biological products* (41). Protection of personnel and containment measures should also follow Biosafety risk assessment and guidelines for the production and quality control of human influenza pandemic vaccines (13).

Manufacturers and national regulatory authorities should consider whether the influenza vaccine (human, live attenuated) for intranasal administration presents any significant environmental, agricultural, or human risks. WHO's Biosafety risk assessment and guidelines for the production and quality control of human influenza pandemic vaccines (13) provides a detailed strategy to minimize the risks of introducing influenza virus strains into the community.

A.3.2 Eggs and cell cultures

Fertile eggs are currently the preferred substrate for vaccine production, with an estimated 600 million eggs being used annually for this purpose worldwide. In general, two kinds of vaccine-quality embryonated eggs are available for the production of influenza vaccines (human, live attenuated) for intranasal administration – SPF-SAN and non-SPF eggs (42).

Only vaccine-quality embryonated hen's eggs obtained from layer flocks meeting the health surveillance requirements of the relevant national animal health authority and the national regulatory authority for the production of influenza vaccine (human, live attenuated) for intranasal administration should be introduced into or handled in the production area.

General requirements for animal health surveillance have been established by the World Organisation for Animal Health (35). Internationally accepted requirements on hygiene and disease security procedures in poultry breeding flocks and hatcheries have also been established (43). The relevant national animal health authority and the national regulatory

authority in Member Countries and Territories of the World Organisation for Animal Health (OIE) are bound to follow the OIE *Terrestrial animal health code* (35, 43). The relevant national animal health authority and the national regulatory authority should work together to establish national animal health requirements for layer flocks from which vaccine-quality embryonated eggs are obtained for production of influenza vaccines (human, live attenuated) for intranasal administration.

Use of vaccine-quality SPF-SAN embryonated eggs

The use of vaccine-quality SPF-SAN embryonated eggs is encouraged for the manufacture of influenza vaccines (human, live attenuated) for intranasal administration but this does not eliminate the need for adventitious agent (as defined in section A.1.4.) testing.

The animal health requirements for SPF-SAN layer flocks are similar across regions and countries (35, 43–45). Hens and roosters in SPF-SAN layer flocks are kept under strictly isolated conditions to guarantee freedom from the avian pathogens (SPF layer flocks) and antibodies (SAN layer flocks) against the avian pathogens that are laid down in the national animal health and regulatory requirements. These flocks are not vaccinated against avian pathogens and must be kept in filtered-air positive-pressure poultry housing in isolation from commercial poultry (37, 46).

A health surveillance programme in SPF-SAN layer flocks is strictly followed and tests are performed regularly to ensure the SPF and SAN status. In some countries, SPF-SAN layer flocks are monitored weekly for quality control: all birds are bled when an SPF-SAN layer flock is established, and thereafter a percentage of the birds are bled at specified intervals. The sera are screened for antibodies to the relevant avian pathogens. These pathogens may also be detected in the flocks by culture or other detection methods including polymerase chain reaction (PCR). Any death in an SPF-SAN layer flock is investigated to determine causality. Permanent records of mortality and results of layer flock testing are kept for several years (usually five). Egg users should be notified immediately when any test results indicate infection with a specified pathogen and when any deterioration in egg production or hatchability is observed in the source layer flock (47).

Layer flocks providing vaccine-quality SPF-SAN embryonated eggs for production of influenza vaccines (human, live attenuated) for intranasal administration should be evaluated frequently to detect exposure of the flock to avian pathogens, which have the potential to cause quality failure in assessments for adventitious agents (as defined in section A.1.4). The quality of SPF-SAN embryonated eggs varies according

to the extent of avian pathogen testing performed in the layer flocks (48). Avian pathogens of interest in SPF-SAN layer flocks may vary by geographical region (37, 43–45, 47) and include, as a minimum: avian adenoviruses, avian encephalomyelitis virus, avian infectious bronchitis viruses, avian infectious laryngotracheitis virus, avian leukosis viruses, avian nephritis virus, avian orthoreoviruses, avian reticuloendotheliosis virus, chicken anaemia virus, egg drop syndrome virus, fowlpox virus, infectious bursal disease viruses, influenza A viruses, Marek's disease virus, Newcastle disease virus, *Mycobacterium avium*, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Salmonella gallinarum*, *Salmonella pullorum*, *Salmonella* species, and *Haemophilus paragallinarum*.

If vaccine-quality SPF-SAN embryonated eggs are used for production of influenza vaccine (human, live attenuated) for intranasal administration, the manufacturer should ensure that layer flock health surveillance is consistent with the requirements of the relevant national animal health authority and the national regulatory authority. The SPF-SAN egg supplier should provide the manufacturer with a quality control certificate showing the testing methods used and the test results performed in accordance with the requirements of the relevant national animal health authority and the national regulatory authority.

The use of vaccine-quality embryonated eggs from SPF-SAN layer flocks for production of influenza vaccine (human, live attenuated) for intranasal administration is not a regulatory requirement in any country where such vaccine is currently manufactured.

Use of vaccine-quality non-SPF embryonated eggs

As a large number of embryonated eggs are needed for human influenza vaccine production, it may not always be feasible to use vaccine-quality embryonated eggs from SPF-SAN layer flocks. Nowadays, the largest volume of embryonated eggs used for human influenza vaccine production worldwide are vaccine-quality non-SPF (42, 49).

If vaccine-quality non-SPF embryonated eggs are used for production of influenza vaccines (human, live attenuated) for intranasal administration, the manufacturer should ensure that the layer flock health surveillance is consistent with the requirements of the relevant national animal health authority and the national regulatory authority (36, 42). The manufacturer should ensure that the non-SPF layer flocks are managed with strict attention to environmental cleanliness and control of access to the flock. The manufacturer should ensure that the vaccine-quality non-SPF embryonated eggs used for vaccine production are highly consistent in their physical and biological qualities, thereby meeting specified requirements of cleanliness and viability.

Hens and roosters in non-SPF flocks are kept under conditions similar to the parent flock for the production of day-old commercial layer chicks. The vaccination programme against common avian pathogens in non-SPF layer flocks has similarities by region and country and generally includes: Marek's disease virus, *Salmonella* species, avian coccidia, Newcastle disease viruses, avian infectious bronchitis viruses, Gumboro, avian infectious laryngotracheitis virus, avian encephalomyelitis virus, *Escherichia coli*, and chicken anaemia virus (if necessary).

Sera are collected throughout the life of the non-SPF layer flock and tested for antibodies against *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Salmonella* species, Newcastle disease viruses, avian infectious bronchitis viruses, avian reoviruses, avian adenoviruses, avian infectious laryngotracheitis virus, avian influenza viruses, avian encephalomyelitis virus, and chicken anaemia virus. Vaccine-quality non-SPF embryonated eggs can be produced free of antibodies for specific diseases by adjusting the vaccination programme of the source layer flock (35, 43).

If vaccine-quality non-SPF embryonated eggs are used for production of influenza vaccine (human, live attenuated) for intranasal administration, the manufacturer should ensure that the flock health surveillance programme is consistent with the requirements of the relevant national animal health authority and the national regulatory authority. The supplier of vaccine-quality non-SPF embryonated eggs should provide the manufacturer with a quality control certificate showing the vaccination programme and the tests carried out under their animal health surveillance programme. The control certificate should be in accordance with the requirements of the relevant national animal health authority and the national regulatory authority.

There should be much greater scrutiny of adventitious agent (as defined in section A.1.4) testing when vaccine-quality non-SPF embryonated eggs are used throughout the vaccine development and production process. If the vaccine strain is produced in vaccine-quality non-SPF embryonated eggs, the national regulatory authority and national control laboratory should specify the additional tests for the detection of adventitious agents that could be derived from the substrates used in preparation of the donor virus, seed virus strains, and vaccine virus. Knowledge of local, regional, and national zoonotic avian diseases would aid decision-making on adventitious agents testing for influenza vaccines (human, live attenuated) for intranasal administration (50, 51). Moreover, knowledge of the vaccination and antibody testing programmes of non-SPF layer flocks required by the relevant national animal health authority and the national regulatory authority should provide a foundation for sound decisions on what adventitious agents to test when vaccine-quality non-SPF embryonated eggs are used.

Only cell cultures meeting the requirements of the national regulatory authority should be used in the production of influenza vaccine (human, live attenuated) for intranasal administration. Cell cultures should also conform to the principles established in Requirements for the use of animal cells as in vitro substrates for the production of biologicals (14, 36).

A.4 Control of source materials

A.4.1 Choice of vaccine strain

The World Health Organization, with the participation of WHO collaborating centres and WHO essential regulatory laboratories, reviews the global influenza epidemiological situation twice annually and recommends influenza reference viruses for the composition of seasonal influenza vaccines for the northern and southern hemispheres in accordance with available evidence (3). With the participation of WHO collaborating centres, WHO essential regulatory laboratories and WHO H5 reference laboratories, the Organization also reviews and reports the circulation of wild-type influenza viruses of pandemic potential, as well as the development status of candidate influenza vaccine viruses to be used for pandemic preparedness (3).

Preparation of the live attenuated vaccine master seed virus is typically the responsibility of the laboratories engaged in production of a specific influenza vaccine (human, live attenuated) for intranasal administration. The preparing laboratory uses an attenuated master donor virus (which contributes the essential attenuating genes) and a wild-type influenza virus reference strain to derive a reassortant virus. The reassortant virus is fully characterized to determine whether it has the proper genetic and phenotypic properties; if it is satisfactory, it becomes a master seed virus, which may be used to produce a working seed virus and vaccine.

Although master and working seed viruses for an influenza vaccine (human, live attenuated) for intranasal administration are typically used exclusively by the preparer, it is possible in some circumstances for a seed virus to be provided to other manufacturers. In this case, it is expected that validated quality control procedures would be in place at the receiving manufacturer in order adequately to identify the genetic characteristics, as well as the antigenic and attenuating properties, of the seed virus before its use in vaccine production.

Influenza vaccines (human, live attenuated) for intranasal administration should include the surface glycoproteins (haemagglutinin and neuraminidase) of the influenza virus reference strains recommended by WHO for inclusion in vaccines (3), or from strains antigenically closely related to them, as approved by the national regulatory authority.

Influenza reference viruses for antigenic analysis and for preparation of reassortant viruses may be obtained from the WHO Collaborating Centres for Reference and Research on Influenza or other custodian laboratory (see Appendix 2).

In some years, the specific influenza reference viruses used for the preparation of influenza vaccines (human, live attenuated) and influenza vaccines (inactivated) differ, but the vaccine viruses in any case are antigenically representative of the WHO-recommended influenza reference viruses (3).

The passage history of the parental and reassortant viruses, together with full documentation of the characterization of the genetic and phenotypic properties of the master seed virus, should be submitted to the national regulatory authority for approval.

A.4.2 **Substrate for virus propagation**

A.4.2.1 **Eggs used for seed virus growth**

Vaccine seed virus is produced in vaccine-quality embryonated eggs from healthy layer flocks that are monitored by methods approved by the relevant national animal health authority and the national regulatory authority (see section A.3.2).

In some countries, vaccine-quality 9–11-day-old embryonated eggs are used.

In production of vaccine seed virus, the egg source layer flock should not have been vaccinated with live Newcastle disease virus vaccine. In addition, layer flocks should not be receiving any chemotherapeutic agents (e.g. antimicrobial agents or coccidiostats). It is also recommended that vaccine-quality embryonated eggs be obtained from young hens.

In countries where use of live Newcastle disease vaccine or any other live vaccine is mandatory, vaccination should take place during the first few weeks of the hen's life and well before the use of flocks for egg supply.

Hence, the use of vaccine-quality SPF-SAN embryonated eggs is required for growth of the seed virus for influenza vaccine (human, live attenuated) for intranasal administration (see section A.3.2).

A.4.2.2 **Eggs used for vaccine production**

Vaccine is produced in vaccine-quality embryonated eggs from healthy layer flocks that are monitored by methods approved by the relevant animal health authority and the national regulatory authority (see section A.3.2).

In some countries, vaccine-quality 9–11-day-old embryonated eggs are used.

In production of vaccine, the egg source layer flock must not have been vaccinated with live Newcastle disease virus vaccine. In addition, layer flocks should not be receiving any chemotherapeutic agents (e.g. antimicrobial agents or coccidiostats). It is also recommended that vaccine-quality embryonated eggs be obtained from young hens.

In countries where use of live Newcastle disease vaccine or any other live vaccine is mandatory, vaccination should take place during the first few weeks of the hen's life and well before the use of flocks for egg supply.

Hence, the use of vaccine-quality SPF-SAN embryonated eggs is encouraged for the manufacture of influenza vaccine (human, live attenuated) for intranasal administration (see section A.3.2).

A.4.3 **Master cell bank and manufacturer's working cell bank**

A.4.3.1 **Cell bank system**

A cell line used for the manufacture of influenza vaccine (human, live attenuated) for intranasal administration should be based on a cell bank system. The national regulatory authority should approve the master cell bank and should establish the maximum number of passages (or population doublings) by which the manufacturer's working cell bank is derived from the master cell bank and the maximum number of passages (or population doublings) of the production cultures.

WHO has established a reference cell bank of Vero cells characterized in accordance with Requirements for the use of animal cells as in vitro substrates for the production of biologicals (14, 36). Those requirements may be revised during the lifespan of this document on influenza vaccines (human, live attenuated) for intranasal administration: manufacturers and national regulatory authorities are encouraged to monitor WHO publications for corresponding updates (<http://www.who.int/immunization/en/>).

The WHO 10-87 cell bank of Vero cells is stored at the European Collection of Animal Cell Cultures (ECACC), Porton Down, England, and at the American Type Culture Collection (ATCC), Rockville, MD, USA. This cell bank should not be considered as the master cell bank for direct use in vaccine production but may be used to establish master cell banks for thorough requalification. Producers of biologicals and national regulatory authorities can obtain culture of these Vero cells (free of charge), as well as additional background information, from Quality, Safety and Standards of Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.

A.4.3.2 Identity test

The master cell bank should be characterized according to WHO's Requirements for the use of animal cells as *in vitro* substrates for the production of biologicals (14, 36) as they relate to continuous cell lines, or to human diploid cells, as appropriate.

The manufacturer's working cell bank should be identified by means, among others, of biochemical (e.g. isoenzyme analysis), immunological and cytogenetic marker tests, and DNA fingerprinting approved by the national regulatory authority and the national control laboratory.

A.4.4 Cell culture medium

At every stage of preparation of donor viruses and vaccines, the sera used for propagation of cells (including the donor strains, master seed lot, working seed lot, master cell bank, working cell bank and production cell cultures) should be tested for freedom from bacteria, fungi and mycoplasma according to the WHO General requirements for the sterility of biological substances (52).

Sera used in the propagation of cells should also be tested for freedom from adventitious agents according to WHO Requirements (14, 36). Manufacturers are encouraged to explore the possibilities of using serum-free media for the production of influenza vaccines (human, live attenuated) for intranasal administration.

Suitable tests for detecting bovine viruses in serum, using either primary bovine testis cells or continuous bovine kidney-cell lines known to be sensitive to bovine viruses, are given in Appendix 1 of Recommendations for the production and control of poliomyelitis vaccine (oral) (53, 54).

Where approved by the national regulatory authority and the national control laboratory, alternative tests for bovine viruses may be used. As an additional monitor of quality, sera may be examined for freedom from phage and endotoxin.

The source(s) of serum of bovine origin should be approved by the national regulatory authority. The serum should comply with the *WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products* (55).

Human serum should not be used. If human serum albumin is used, it should meet the Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives (56) and comply with the *WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products* (55).

Penicillin and other beta-lactam antimicrobial agents should not be used at any stage of manufacture.

Other antimicrobial agents may be used at any stage of manufacture, provided that the quantity present in the final product is acceptable to the national regulatory authority. Non-toxic pH indicators may be added, e.g. phenol red at a concentration of 0.002%. Only substances that have been approved by the national regulatory authority may be added.

Trypsin used for preparing the master cell bank, working cell bank and production cell cultures should be tested and demonstrated to be free from cultivable bacteria, fungi, mycoplasmas and infectious viruses, especially parvoviruses appropriate to the species of animal used. The methods used to ensure this should be approved by the national regulatory authority and the national control laboratory.

The source of trypsin of bovine origin, if used, should be approved by the national regulatory authority. Bovine trypsin, if used, should comply with current *WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products* (55).

A.4.5 **Virus strains**

A.4.5.1 **Origin and preparation of virus strains**

Strains of influenza virus used in the production of influenza vaccines (human, live attenuated) for intranasal administration should be identified by historical records, which should include information on the origin of the donor virus strains, both the attenuated virus donor strain and the wild-type virus reference strain, as well as on the process used for preparing the attenuated master seed lot and working seed lot.

Only strains that have been isolated in vaccine-quality embryonated eggs (see section A.3.2), in cells derived from eggs or in mammalian cells approved by the national regulatory authority for human vaccine production should be used (unless reverse genetics technology is being used for preparation of the seed lot, as described later in this section). The national regulatory authority should also approve the attenuated virus donor strain and wild-type virus reference strain used for preparing the master seed lot. The vaccine seed viruses should have the attenuation phenotype of the attenuated donor virus strain, and the surface antigens (haemagglutinin and neuraminidase) should correspond to the influenza reference viruses recommended by WHO for vaccine preparation (3).

Experience shows that candidate seed viruses derived by classical reassortment should be cloned at least three times by limiting dilution passage in vaccine-quality SPF-SAN embryonated eggs or plaque purified in qualified cells to ensure purity of the desired attenuated seed virus. Since antigenic changes are possible during the development of reassortant viruses, the absence of antigenic changes in the master seed lot and working seed lot should be demonstrated at least by haemagglutination inhibition tests using antibodies to the haemagglutinins of the candidate influenza vaccine virus and of the wild-type reference virus strain.

Where reassortant viruses are used, the method for producing the reassortant should be approved by the national regulatory authority. Preparation of the live attenuated vaccine master seed viruses are typically the responsibility of the laboratories engaged in production of specific influenza vaccines (human, live attenuated) for intranasal administration.

Master and working seed viruses for an influenza vaccine (human, live attenuated) for intranasal administration are typically used exclusively by the preparer. If a master or working seed virus is provided to another manufacturer, the receiving manufacturer should use validated methods to identify the genetic characteristics and the antigenic and attenuating properties of the seed virus before using it in vaccine production. The receiving manufacturer should also have current good manufacturing practices (cGMP) in place to handle the master seed.

Where reverse genetics techniques are used to generate the reassortant vaccine virus, the influenza haemagglutinin and neuraminidase genes may be derived from a variety of sources (egg isolate, mammalian cell isolate or virus in clinical specimen). With reverse genetics, the source of viral genes is less critical than for classical reassortment because the haemagglutinin and neuraminidase genes are expected to be free of adventitious agents associated with wild-type virus by virtue of the recombinant DNA technology employed (15).

The cell substrate used for transfection to generate the reassortant virus by reverse genetic techniques should be appropriate for human vaccine production and approved by the national regulatory authority. The overall process for derivation of the reassortant virus prepared by reverse genetics should be approved by the national regulatory authority. *WHO guidance on development of influenza vaccine reference viruses by reverse genetics* (15) and WHO Guidelines for assuring the quality of pharmaceutical and biological products prepared by recombinant DNA technology (57) should be considered.

For work with highly pathogenic wild-type influenza viruses and newly emerging pandemic viruses, higher levels of biocontainment are required; Biosafety risk assessment guidelines for the production and quality control of human influenza pandemic vaccines (13) should be followed. Facilities for vaccine production should provide containment features to accommodate the candidate influenza vaccines that are derived from the wild-type influenza viruses.

If any materials of animal (non-avian) origin are used in vaccine production, they should comply with the *WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products* (55) and should be approved by the national regulatory authority.

Reference strains for antigenic analysis may be obtained from WHO Collaborating Centres for Reference and Research on Influenza (see Appendix 2) or from another custodian laboratory.

A.4.5.2 Seed lot system

The production of vaccine should be based on a virus seed lot system. The influenza virus contained in each seed lot should be identified as the appropriate strain by methods acceptable to the national regulatory authority. The maximum number of passages between a master seed lot and a working seed lot should be approved by the national regulatory authority. The virus in the final vaccine should be not more than one passage removed from the working seed lot. The haemagglutinin and neuraminidase of the seed lot viruses should be identified by suitable tests.

The master seed lot may be considered for use as a working seed lot with the approval of the national regulatory authority. If the master seed lot is used as the working seed lot, the final vaccine should be not more than one passage removed from the master seed lot.

A.4.5.3 Tests on seed lots

A.4.5.3.1 Adventitious agents

The master seed lot and working seed lot should be shown to be free from relevant adventitious agents (as defined in section A.1.4) by tests or procedures approved by the national regulatory authority and the national control laboratory in accordance with the WHO General requirements for the sterility of biological substances (52, Part A, sections 5.2 and 5.3; 58).

Strategies to ensure freedom from adventitious agents (as defined in section A.1.4) in the final vaccine involve a combination of testing the seed virus and validation of the production process which depends on the substrate used for production and on the process developed for vaccine manufacture.

Validation of processes

Since the manufacturing of influenza vaccine (human, live attenuated) for intranasal administration is unlikely to include processes that are effective in inactivating potential contaminating agents, reliance is placed primarily on general precautions against microbial contamination in manufacture, on the quality of materials used for manufacture, and on testing for adventitious agents (52, 58). In some instances, removal of contaminating agents may be possible. For example, filtration steps may be used to remove bacteria or fungi derived from eggs. The production process should be validated, including steps designed for removal of potential contaminating microbial agents. Process validation may be performed with appropriate model agents. If removal of a potential contaminant cannot be demonstrated and validated, a testing strategy should be implemented to document the ability of individual steps and the overall process to prevent the introduction of potential microbial contaminating agents (52, 58).

Cells used to prepare vaccine

The susceptibility of cell cultures to various human pathogens should be taken into account and used in considering a list of potential human pathogens to be included in testing for adventitious agents in master seed lots and working seed lots passaged in the cells. Pathogens to be considered include adenovirus, parainfluenza virus, respiratory syncytial virus, coronavirus, rhinovirus, enterovirus, human herpesvirus 4 (Epstein–Barr virus), herpes simplex virus, cytomegalovirus and mycoplasmas (14, 36).

It is recognized that, when a vaccine strain changes, there may be time constraints that make testing master seed lots and working seed lots for adventitious agents problematic, and the full results of such testing may not always be available before further processing. The development and use of properly validated rapid assays such as immunoassays or PCR is therefore encouraged.

If an adventitious agent is detected in a master seed lot and/or working seed lot prepared in cell culture, those lots should not be used for vaccine production.

Embryonated eggs used to prepare vaccine

Whether vaccine-quality SPF-SAN or non-SPF embryonated eggs are used for vaccine preparation, a judicious testing strategy for specific potential adventitious agents is needed (see section A.3.2). The use of properly validated rapid assays such as immunoassays or PCR is encouraged.

The use of vaccine-quality SPF-SAN embryonated eggs does not eliminate the need for adventitious agent testing. However, there should be much greater scrutiny of adventitious agent testing when vaccine-quality non-SPF embryonated eggs are used for passage of the attenuated virus donor strain, wild-type virus reference strain, master seed lot, working seed lot or vaccine. In this case, the national regulatory authority should specify any additional tests for the detection of adventitious agents that could be derived from the substrates used in preparation of the master seed and working seed lots and in preparation of the vaccine (see section A.3.2).

If a potential contaminating agent is detected, the virus master seed lot or virus working seed lot should not be used for vaccine production.

A.4.5.3.2 Attenuation

Retention of key genetic and phenotypic characteristics related to attenuation may be demonstrated for either the master seed lot or the working seed lot of a virus strain to be introduced into clinical use.

The methods used to demonstrate attenuation may vary for each vaccine virus but should include at least one of the following:

- the full genetic sequence of the virus master seed lot or virus working seed lot,
- in vitro tests for the key phenotypic markers of the attenuated virus donor strain, and
- studies in an appropriate animal model to assess the in vivo aspects of vaccine virus attenuation.

Specific tests used to assess virus attenuation should be approved by the national regulatory authority and the national control laboratory.

The development of influenza vaccine (human, live attenuated) for intranasal administration should include an early assessment of the biosafety level required to work with the attenuated donor virus and the master seed viruses derived from the donor. Direct handling of an established attenuated donor virus strain for which much experience exists (such as the cold-adapted H2N2 strains) and of master seed viruses prepared for seasonal influenza vaccine viruses can generally be done using established biosafety level 2 (BSL-2) practices (13, 40, 59).

Work with highly pathogenic wild-type influenza viruses and newly emerging pandemic viruses requires higher levels of biocontainment, and the WHO Biosafety risk assessment and guidelines for the production and quality control of human influenza pandemic vaccines (13) should be followed. Facilities for vaccine production should provide adequate containment features to accommodate the candidate influenza vaccines that are derived from wild-type influenza viruses. Guidance for safe handling of influenza viruses is subject to revision based on evolving experience, and the biosafety level to be used should be decided in consultation with the national regulatory authority.

For candidate influenza vaccine viruses prepared using highly pathogenic avian influenza viruses as the starting material, additional tests should be performed. Optimally, the strategy to demonstrate elimination of highly pathogenic characteristics includes assessment of the ability of the candidate influenza vaccine virus to produce plaques in cell culture with and without trypsin, the ability to cause chick embryo death, pathogenicity in chickens (as defined in section A.1.4), and attenuation in ferrets (13). Specific tests used to assess the removal of highly pathogenic features should be approved by the national regulatory authority and the national control laboratory.

For candidate influenza vaccine viruses prepared using non-highly pathogenic influenza viruses of animal origin (avian, swine, equine, canine, others), additional safety tests may also be needed as described in WHO published guidance (13). Specific tests used to assess the candidate influenza

vaccine viruses should be approved by the national regulatory authority and the national control laboratory.

The virus seed lot should be stored at a temperature below -60°C unless it is in the lyophilized form, in which case it should be stored at a temperature lower than -20°C .

A.5 Control of vaccine production

A.5.1 Production precautions

The general production precautions formulated in the WHO good manufacturing practices and quality assurance for biological products should be followed (39, 57, 60). Although the WHO Recommendations for the production and control of influenza vaccine (inactivated) (2) are similar in many ways, the manufacture of influenza vaccines (human, live attenuated) for intranasal administration has important differences. The following should be observed.

- For embryonated egg-derived vaccines, only allantoic and amniotic fluids may be harvested.
- Beta-lactam antimicrobial agents should not be used at any stage in the manufacture of the vaccine and should not be permitted to come into contact with any part of the production equipment.

Minimal concentrations of other suitable antimicrobial agents may be used.

Small quantities of antimicrobial agents other than beta-lactam agents may be added with the approval of the national regulatory authority. However, if an antimicrobial agent is to be added, samples for sterility testing should be taken before the antimicrobial agent is added.

A.5.2 Production of monovalent virus pool

A.5.2.1 Single harvests

For egg-derived vaccines, each strain of virus should be grown in the allantoic cavity of vaccine-quality embryonated eggs derived from healthy layer flocks (see section A.3.2). After incubation at a controlled temperature, both the allantoic and amniotic fluids may be harvested to prepare a single harvest.

In some countries, vaccine-quality 9–11-day-old embryonated eggs are used.

It is recognized that adventitious agents (as defined in section A.1.4) can be introduced at any point in the process. It is a wise precaution to pool the allantoic fluids from a limited number of eggs (e.g. 30–50) and to test these small pools for sterility and virus titre before blending

into the single harvest. The pools should be stored at a temperature of 2–8 °C. In case any contamination is detected in downstream processes, the manufacturer can test this small allantoic fluid pool for adventitious agents and virus titre to identify where the contamination or deterioration of virus titre occurred.

The use of vaccine-quality SPF-SAN embryonated eggs does not eliminate the need for adventitious agent testing. If the vaccine is prepared in vaccine-quality SPF-SAN embryonated eggs, it may be possible to obtain single harvests that are free of adventitious agents. However, in the event that this is not achievable, a bioburden limit on single harvests may be considered with the approval of the national regulatory authority.

There should be much greater scrutiny of adventitious agent testing when vaccine-quality non-SPF embryonated eggs are used to produce single harvests. If vaccine-quality non-SPF embryonated eggs are used, the national regulatory authority should specify any additional tests for the detection of the adventitious agents that could be derived from the egg substrates (see section A.3.2) used in preparation of attenuated virus donor strains, wild-type virus reference strains, virus master seed lot and virus working seed lots and in preparation of the vaccine.

For influenza vaccines (human, live attenuated) for intranasal administration prepared in cell cultures, each virus strain should be grown in cells approved by the national regulatory authority.

For both egg-derived and cell-derived vaccines, a number of single harvests of the same virus strain may be combined to give a monovalent virus pool. Cell-derived monovalent virus pools should not be mixed with egg-derived monovalent virus pools.

A.5.2.2 Tests of control eggs or cell cultures

The national regulatory authority should determine the need for control samples, the sample size to be examined, the time at which the control samples should be taken during the production process, and how the control samples are to be maintained.

When vaccine-quality embryonated eggs are used, a portion (2% or at least 20 eggs, whichever is the greater quantity) of each batch of the eggs used for vaccine virus propagation is held as uninoculated controls. These control eggs are incubated for the same time and at the same temperature and humidity as the inoculated embryonated eggs. At the time of harvesting the virus from the inoculated embryonated eggs, allantoic fluids are also taken from the uninoculated control eggs and examined for the existence of haemagglutinating agents (see section A.5.2.2.1).

To ensure freedom from adventitious agents (as defined in section A.1.4), the greatest reliance is placed on continuous health monitoring of the layer flock from which the vaccine-quality embryonated eggs are obtained (see section A.3.2).

The use of vaccine-quality SPF-SAN embryonated eggs does not eliminate the need for adventitious agent testing. There should be much greater scrutiny of adventitious agent testing when vaccine-quality non-SPF embryonated eggs are used.

The national regulatory authority and the national control laboratory should approve alternative strategies and methods for ensuring freedom from adventitious agents.

The national regulatory authority and the national control laboratory may include additional tests for microorganisms if necessary. Tests for avian leukosis virus in embryonated eggs are considered essential.

When testing is performed on control cell cultures, a sample equivalent to at least 500 ml of the cell culture suspension, kept at the same cell concentration as that used for vaccine production, is tested. Control cell cultures are incubated for at least two weeks and are examined during this observation period for evidence of cytopathic changes. For the test to be valid, not more than 20% of the control cultures may have been discarded for nonspecific reasons.

If any test described in this section yields evidence of the presence of an adventitious agent in a control cell culture or a control embryonated egg, the influenza virus grown in the corresponding inoculated cultures or eggs should not be used for vaccine production.

Samples not tested immediately should be stored at or below -60°C .

A.5.2.2.1 Tests for haemagglutinating and haemadsorbing agents

For influenza vaccine (human, live attenuated) for intranasal administration prepared in vaccine-quality embryonated eggs, a sample of 0.25 ml of allantoic fluid taken from each control egg should be tested for haemagglutinating agents by the addition of chick erythrocytes, both directly and after one passage of the control allantoic fluid through vaccine-quality SPF-SAN eggs. The details of the test should be approved by the national regulatory authority and the national control laboratory.

For influenza vaccine (human, live attenuated) for intranasal administration prepared in cell culture, testing for the presence of haemadsorbing viruses at the end of the observation period or at the time the virus is harvested from the production substrate, whichever is later, should include at least 25% of control cells. The control cells should be tested using guinea-pig red blood

cells; if the red blood cells have been stored, the duration of storage should not have exceeded 7 days, and the storage temperature should have been in the range 2–8 °C. In testing for haemadsorbing viruses, calcium and magnesium ions should be absent from the medium.

This test is usually done using a 1% suspension of guinea-pig red blood cells. In some countries, however, the national regulatory authority requires that additional tests for haemadsorbing viruses be made in other types of red blood cells, including those from humans (blood group O), monkeys and chickens (or other avian species).

The results of all tests should be read after incubation for 30 minutes at 0–4 °C and again after a further incubation for 30 minutes at 20–25 °C. For tests using monkey red blood cells, a further reading should also be taken after incubation for 30 minutes at 34–37 °C.

In some countries the sensitivity of each new batch of red blood cells is demonstrated by titration against a haemagglutinin antigen before use in the haemadsorption test.

A.5.2.2.2 Tests on supernatant fluids from cell cultures

Samples of at least 10 ml of the pooled supernatant fluid from the control cell cultures collected at the end of the observation period should be tested for the presence of adventitious agents (as defined in section A.1.4) in monolayers of three indicator cell lines:

- cultures of cells of the same species and tissue type as those used for production;
- cultures of a human diploid cell line;
- cultures of another cell line from a non-human species.

The samples should be inoculated into containers of these indicator cell cultures, in such a way that the dilution of the supernatant fluid in the nutrient medium does not exceed 1 in 4. The area of the indicator cell sheet should be at least 3 cm²/ml of supernatant fluid. At least one container of each of the cell cultures should remain uninoculated and serve as a control.

The cultures should be incubated at a temperature of 35–37 °C and observed for cytopathic effect (and other evidence of a replicating adventitious agent) in the indicator cells for a period of at least two weeks.

The use of properly validated rapid assays such as immunoassays or PCR that could be conducted within the time constraints of the procedure is encouraged.

A.5.2.2.3 Identity test for continuous cell culture

For monovalent virus pools produced in continuous cell culture, the control cells should be identified by means approved by the national regulatory authority and the national control laboratory. Biochemical (e.g. isoenzyme analysis), immunological and/or cytogenetic marker tests may be considered for confirming identity.

A.5.2.3 Clarification and purification

The monovalent virus pool should be clarified and purified by centrifugation and/or other suitable methods approved by the national regulatory authority.

The aim of clarification and purification is to remove cell debris. For vaccine prepared in vaccine-quality embryonated eggs, a sterile filtration step may be considered to reduce bioburden. It is advisable to clarify and purify the influenza virus under conditions optimized for preserving its infectivity and antigenic properties.

Sterile filtration should be validated.

A.5.3 Control of monovalent virus pools

At the time when allantoic or tissue culture fluids are pooled to prepare the monovalent virus pool and before clarification and purification, samples should be set aside for examination for adventitious agents (as defined in section A.1.4). If the samples are not tested immediately, they should be stored at or below -60°C .

For the purposes of the tests recommended in this section to verify neutralization of virus harvest, hyperimmune antibody preparations should be of an origin that will not cross-react with the antigens of cells or eggs used in production of the monovalent virus pool. The virus used for the production of the hyperimmune antibody preparations should be grown either in non-avian cell cultures or in vaccine-quality SPF-SAN embryonated eggs. If vaccine-quality embryonated eggs are used, they should be obtained from a different layer flock from that used to supply the vaccine production embryonated eggs.

For vaccines intended for use in a pandemic situation, hyperimmune sera for tests described in this section may not be readily available when they are needed for release and distribution of vaccines. As an alternative for both pandemic and non-pandemic situations, a strategy to identify potential contaminating agents by PCR methods may be considered. Alternative strategies should be approved by the national regulatory authority and the national control laboratory.

A.5.3.1 Infectivity (potency)

The influenza virus content in the clarified monovalent virus pool should be determined by titration in vaccine-quality embryonated eggs or cell cultures. The number of infectious doses of vaccine should be expressed in EID₅₀, TCID₅₀ or pfu per unit volume (see section A.7.3).

Additional equivalent methods dependent on the replication of virus in embryonated eggs or cell cultures (e.g. fluorescent focus assay expressed as fluorescent focus units) may be considered for determining potency.

A.5.3.2 Attenuation

Retention of key genetic and phenotypic characteristics related to attenuation of the candidate influenza vaccine virus should be demonstrated for the monovalent virus pool of a strain to be introduced into clinical use. The methods used to demonstrate attenuation of the monovalent virus pool may vary (60) and may include at least one of the following:

- the full genetic sequence of the virus of the monovalent virus pool,
- in vitro tests of the monovalent virus pool for the key phenotypic markers of the attenuated virus donor strain, and
- studies in an appropriate animal model (e.g. ferrets) to assess the in vivo aspects of attenuation of the vaccine preparation (13).

Attenuating features of the vaccine virus in the monovalent pool are expected to be identical with those of the seed virus (see section A.4.5.3.2).

A.5.3.3 Identity

Each monovalent virus pool lot should be identified as containing live attenuated influenza virus of the appropriate strain by methods acceptable to the national regulatory authority.

A.5.3.4 Adventitious agent tests

The tests described in this section form the traditional basis for identifying adventitious agents (as defined in section A.1.4). For vaccines intended for use in a pandemic situation, hyperimmune sera needed for tests in vaccine-quality embryonated eggs or cell cultures described in this section may not be readily available when they are needed for release and distribution of vaccines. More recent developments involving PCR methods therefore need to be considered, both to improve the specificity and sensitivity of adventitious agent testing and to anticipate situations in which delays in vaccine availability would be detrimental. The national regulatory authority and the national control laboratory should approve the specific methods used to fulfil adventitious agent testing.

The use of vaccine quality SPF-SAN embryonated eggs does not eliminate the need for adventitious agent testing. There should be much greater scrutiny of adventitious agent testing when vaccine-quality non-SPF embryonated eggs are used.

The monovalent virus pool passes if there is no evidence of any adventitious agent attributable to the virus pool.

A.5.3.4.1 Tests in vaccine-quality embryonated eggs

For vaccines produced in vaccine-quality embryonated eggs, a sample of at least 10 ml of each monovalent virus pool should be tested for the presence of adventitious agents (as defined in section A.1.4) by inoculation of eggs. After neutralization of the influenza virus by hyperimmune antibody preparation, the monovalent virus pool should be inoculated in vaccine-quality embryonated eggs. At least 0.25 ml of the virus/antibody mixture per egg should be used for inoculation of one group of eggs by the allantoic route and a separate group of eggs by the yolk sac route. The national regulatory authority should approve the method of incubation of the embryonated eggs and the observation time. None of the embryonated eggs should show evidence of the presence of any adventitious agents.

A.5.3.4.2 Tests in cell cultures

For vaccines produced in embryonated eggs or cell cultures, a sample of at least 5 ml of the monovalent virus pool should be tested for freedom from adventitious agents (as defined in section A.1.4) using cell cultures as described below. After neutralization of the influenza virus by hyperimmune antibody preparation, the monovalent virus pool should be inoculated on cell cultures of human cells, simian cells, chicken cells, or cells of the species used for vaccine production. The national regulatory authority should approve the cell cultures, the method of incubation and the period of observation. None of the cell cultures should show evidence of the presence of any adventitious agents. When chicken cells are used for vaccine production, the absence of avian leukosis viruses should be ascertained by testing.

A.5.3.4.3 Tests for bacteria, fungi and mycoplasma

The monovalent virus pool should be tested for freedom from bacteria, fungi and mycoplasma according to the WHO General requirements for the sterility of biological substances (52, 58).

A.5.3.4.4 Test for mycobacteria

Each monovalent virus pool should be tested for the presence of mycobacteria by methods appropriate for the detection of the organisms most likely to be found in the embryonated eggs or cell cultures used.

It is common practice to concentrate the virus harvest by centrifugation and to inoculate the pellet into guinea-pigs or onto solid media shown to be suitable for the detection of mycobacteria.

A.5.3.5 Residual cell substrate DNA in cell-derived vaccines

For viruses grown in continuous cell culture, the purified monovalent virus pool should be tested for residual cellular DNA. The purification process should be shown to consistently reduce the level and molecular size of cellular DNA (14). This test should be appropriate for the cell culture used and should be approved by the national regulatory authority; it may be omitted with the agreement of the national regulatory authority if the manufacturing process is validated for residual DNA.

A.5.3.6 Tests for chemicals used in production

The concentration of each chemical added during production should be determined in the monovalent virus pool vaccine using methods approved by the national regulatory authority. The concentrations should not exceed the limits specified by the national regulatory authority. For preservatives, the national regulatory authority should approve the method of testing and the concentration.

Alternatively, tests for chemicals may be performed on the final bulk.

A.5.4 Control of final bulk

Final bulks are prepared by mixing and diluting monovalent virus pools of the relevant strains. In the preparation of the final bulk, only preservatives, stabilizers or other substances, including diluents, approved by the national regulatory authority should be added. Such substances should have been shown by appropriate tests not to impair the safety or effectiveness of the product in the concentrations used and should not be added before samples have been taken for any tests that would be affected by their presence. The operations necessary for preparing the final bulk should be conducted in such a manner as to avoid contamination of the product.

A.5.4.1 Infectivity (potency)

The influenza virus content of the clarified final bulk suspension should be determined by infectivity titration in vaccine-quality embryonated eggs or cell cultures. The number of infectious doses of vaccine should be determined (total and for each component virus) and the results expressed as EID₅₀, TCID₅₀ or pfu per human dose (see section A.7.3).

Vaccines for use during pandemics are likely to contain only one strain. Additional methods dependent on the replication of virus in

vaccine-quality embryonated eggs or cell cultures (e.g. fluorescent focus assay expressed as fluorescent focus units) may be considered for determining potency.

This test may be omitted if such a test is performed on each final lot.

A.5.4.2 Sterility

Each final bulk should be tested for sterility by a method approved by the national regulatory authority and national control laboratory.

Many countries have regulations governing sterility testing. Where these do not exist, the WHO General requirements for the sterility of biological substances (52, 58) should be satisfied.

If a preservative, stabilizer or other substance has been added to the vaccine, appropriate measures should be taken to prevent it from interfering with the sterility test.

A.5.4.3 Protein and residual DNA content

Measurements of protein and residual DNA may be specified, depending on the nature of the vaccine being produced (14, 61, 62). For example, a vaccine could be prepared from allantoic fluid and not be further purified before preparation of the final vaccine; this preparation would have an extremely high protein and ovalbumin content. Alternatively, a vaccine could be prepared from the harvested medium used to propagate cell cultures and purified to remove residual DNA and protein. The aim should be to establish parameters suitable for the production and intended use of a vaccine to ensure consistency of the composition and clinical performance of the vaccine. The parameters specified should be approved by the national regulatory authority.

A.6 Filling and containers

The requirements concerning filling and containers given in WHO good manufacturing practices for biological products (39) should apply. Single-dose containers may be preferred. If multidose containers are used, a suitable method to protect the integrity of the product before administration should be approved by the national regulatory authority.

Care should be taken to ensure that the materials of which the components of the container system are made do not adversely affect the virus content of the vaccine under the recommended conditions of storage.

A.7 Control tests on final product

Samples should be taken from each filled final lot for the tests mentioned in this section and its subsections.

A.7.1 Identity

An identity test should be performed on at least one container from each final lot by a method approved by the national regulatory authority and national control laboratory.

The virus strains in the final containers should be identified by methods appropriate for the haemagglutinin and neuraminidase antigens. Phenotypic and genetic information may be useful in full specific identification of the vaccine viruses.

A.7.2 Sterility

Vaccine (reconstituted if lyophilized) should be tested for sterility as described in section A.5.4.2.

A.7.3 Infectivity (potency)

The virus content of each of at least three containers selected at random from each filled final lot should be determined individually by methods fully approved by the national regulatory authority and national control laboratory.

The determination of the infectivity per dose should be based on inoculation of at least five vaccine-quality embryonated eggs or cultures per dilution, using 10-fold dilutions.

The number of infectious doses per human dose of vaccine should be determined (total and for each component virus), and the results should be expressed in EID₅₀, TCID₅₀ or pfu per dose.

Additional methods dependent on the replication of virus in vaccine-quality embryonated eggs or cell cultures (e.g. fluorescent focus assay expressed as fluorescent focus units) may be considered for determining potency.

The requirements for virus content per human dose should be based on clinical trials in humans that demonstrate the dose range needed to ensure the safety and effectiveness of the influenza vaccines (human, live attenuated) for intranasal administration as approved by the national regulatory authority. Re-characterization of the relation of an infectious dose to a human dose may become necessary and should be undertaken with the approval of the national regulatory authority.

A.7.4 Endotoxin

A test for endotoxin should be included (e.g. the *Limulus* amoebocyte lysate (LAL) test) as a test of safety and manufacturing consistency of the final lot.

A.7.5 Residual moisture of lyophilized vaccines

The residual moisture in a representative sample of each lyophilized lot should be determined by a method approved by the national regulatory authority and the national control laboratory.

A.7.6 Inspection of final containers

Each container in each final lot should be inspected visually; any container showing abnormalities, such as lack of integrity, should be discarded.

A.8 Records

The requirements in section 8 of WHO good manufacturing practices for biological products (39) should apply.

A.9 Retained samples

The requirements in section 9 of WHO good manufacturing practices for biological products (39) should apply.

A.10 Labelling

The requirements in section 7 of WHO good manufacturing practices for biological products (39) should apply. The label on the carton, the container or the leaflet accompanying the container should state:

- that the vaccine has been prepared from virus propagated in vaccine-quality embryonated eggs (as per the description in section A.3.2) or in cell cultures;
- the type of cell line, i.e. monkey, dog, others (if appropriate);
- the strain or strains of influenza virus present in the preparation;
- the infectivity titre per human dose of the vaccine expressed as EID₅₀, TCID₅₀ or pfu (or other similar means of determining infectivity, e.g. fluorescent focus assay expressed as fluorescent focus units);
- the volume per dose and the nominal volume of vaccine in the container available for recovery and administration;
- the seasonal influenza vaccine composition for which the vaccine is intended (3);
- the name and quantity of any antimicrobial agent in the vaccine;
- the name and concentration of any preservative added;
- the temperature recommended during storage and transport;
- the expiry date;
- any special dosing schedules (e.g. two doses for a pandemic vaccine).

A.11 **Distribution and transport**

The requirements in section 8 of WHO good manufacturing practices for biological products (39) should apply.

A.12 **Stability**

A.12.1 **Stability testing**

Adequate stability studies form an essential part of the development of influenza vaccines (human, live attenuated) for intranasal administration. Current guidance on evaluation of vaccine stability is provided in Guidelines on stability evaluation of vaccines (38).

The stability of the influenza vaccine (human, live attenuated) for intranasal administration, including each of the active components in the vaccine in final form and at the recommended storage temperatures for the vaccine in final form, should be demonstrated to the satisfaction of the national regulatory authority on final containers from at least three final lots.

In some countries, vaccine infectivity titre should comply with the final product specifications at the expiry date (see section A.7).

Since vaccine may be stored in monovalent form for a significant period before preparation of the final bulk vaccine, stability studies may be performed on the single harvests or on the monovalent pools as well as on the final vaccine.

Formulation of the influenza vaccine (human, live attenuated) for intranasal administration must be stable throughout its shelf-life. Acceptable limits for stability should be agreed with the national regulatory authority.

Following licensure, continued monitoring of vaccine stability is recommended to support shelf-life specifications and to refine the stability profile. Data should be provided to the national regulatory authority on an annual basis.

For influenza vaccines (human, live attenuated) for intranasal administration, thermal stability for lot release should be explored to determine whether the testing provides any value in the overall understanding of vaccine quality (safety and efficacy) and the effect of production variables. If there is no added value, thermal stability should not be required as a lot release assay (38).

When any changes are made in the production process that may affect stability of the product, the influenza vaccine (human, live attenuated) for intranasal administration produced by the new method should be shown to be stable. The national regulatory authority should be informed of and approve any changes that may affect stability of the product (38).

A.12.2 Storage conditions

Storage conditions for influenza vaccines (human, live attenuated) for intranasal administration should be fully validated and approved by the national regulatory authority (38).

A.12.3 Expiry date

The expiry date should be fixed with the approval of the national regulatory authority and should take into account experimental and clinical data on the stability of the influenza vaccine (human, live attenuated) for intranasal administration (38).

In general, the expiry date should not exceed one year from the date of issue by the manufacturer because the strains used in one year's vaccine may not be appropriate the next year (3). The national regulatory authority may approve an expiry date for a pandemic vaccine that differs from that of a seasonal influenza vaccine.

Part B. Nonclinical evaluation of new influenza vaccines (human, live attenuated)

B.1 General remarks

Nonclinical evaluation refers to all in vivo and in vitro testing performed before and during the clinical development of vaccines (20). Preclinical testing, as a subcategory of nonclinical testing, is a prerequisite for moving a candidate vaccine from the laboratory to the clinic and includes all aspects of product characterization, proof of concept/immunogenicity studies, and safety testing in animals conducted before introducing the product into humans.

A non-exhaustive summary of typical preclinical evaluations used to prepare a new influenza vaccine (human, live attenuated) for entry into clinical studies is provided in Table 1. Some of the in vitro assessments will also form the basis for and be incorporated into the ongoing nonclinical activities of the quality control and quality assurance oversight of product release.

The yearly changes made to keep vaccines current with influenza epidemiology are not expected to require repeated nonclinical studies. Some in vitro and in vivo studies, however, may be useful for assessing significant changes in manufacturing processes or alterations of critical product characteristics. Moreover, the continuation of some nonclinical activities would be expected to be appropriate for maintaining current good manufacturing practices for influenza vaccine (human, live attenuated) for intranasal administration. Current WHO Guidelines on nonclinical evaluation of vaccines: regulatory expectations (20) describe broadly applicable principles of preclinical and nonclinical assessments

in greater detail. Guidance that may apply to the preclinical and nonclinical assessments of influenza vaccines (human, live attenuated) for intranasal administration to be used as pandemic vaccines is also provided in Regulatory preparedness for human pandemic influenza vaccines (12).

Each candidate attenuated virus donor strain to be developed for the preparation of candidate influenza vaccines (human, live attenuated) for intranasal administration should be characterized as completely as possible to identify critical genetic and phenotypic markers, to assess viral attenuation, to assess potential virus toxicity, and to determine whether the genetic basis of attenuation prevents reversion to partial or total virulence. Early laboratory studies should determine the genetic elements responsible for the virus attenuation, singly and in the combination to be present in the distributed vaccine. It is advisable to select attenuated virus donor strains with stable markers that are not dependent on retention of the markers of attenuation. The presence of additional stable markers can be used to differentiate vaccine strains from wild-type virus reference strains or other vaccine viruses in epidemiological surveillance.

The delivery device may have an impact on the uptake of a vaccine, its safety and its effectiveness (20). Influenza vaccines (human, live attenuated) may be administered intranasally using proprietary or nonproprietary delivery devices including syringes, sprayers and other liquid delivery devices. The preclinical evaluation of influenza vaccines (human, live attenuated) should include a rigorous assessment to establish the suitability of the intranasal delivery device to support the safety and effectiveness of the vaccine.

Table 1
Nonclinical evaluation^a of new influenza vaccines (human, live attenuated) for intranasal administration

Area of nonclinical evaluation	Primary concern	Scope of nonclinical evaluation
In vitro assessments		
Process development, quality control and quality assurance	Process is expected to meet cGMP standards	Process control and laboratory studies related to all steps in process to prepare vaccine
Product characterization	Product quality is appropriate for use in preclinical and clinical studies	Genetic, biochemical, and biological characteristics of vaccine including features of attenuation

continues

Table 1 *continued*

Area of nonclinical evaluation	Primary concern	Scope of nonclinical evaluation
In vivo assessments		
Toxicity and safety testing	Product risks are appropriate for anticipated uses	Safety indicators such as: range of safe dose; single and repeated safe doses; safety parameters for clinical monitoring; potential for reversion to virulence
Immunogenicity and efficacy	Product effects are appropriate for anticipated uses	Effect indicators such as: type of immune responses for clinical evaluation; frequency and duration of immune responses; priming and boosting parameters; protective effects in animal challenge studies

^a Sources of guidance on nonclinical evaluation of vaccines: WHO; International Conference on Harmonisation; European Medicines Agency; United States Food and Drug Administration; Medical Research Council.

B.2 Process development and product characterization

Before clinical trials are initiated, it is generally advisable for production processes and the product itself to be in the expected final form. However, the information needed to proceed to clinical trials may vary with the nature of the vaccine. The preclinical and nonclinical development plans should therefore be discussed with and approved by the national regulatory authority and the national control laboratory.

Although all preclinical and most nonclinical studies will be performed before licensure for an influenza vaccine (human, live attenuated) for intranasal administration, the suitability of an attenuated virus donor strain needs continuous careful review (see section A.4.5.3.2). This review ensures that the candidate influenza vaccine viruses containing prevalent antigens from new wild-type virus reference strains do not substantially alter the established parameters of safety and effectiveness confirmed by clinical studies. The development and use of influenza vaccines (human, live attenuated) for intranasal administration therefore require long-term commitment by manufacturers and national regulatory authorities to conduct laboratory studies as part of the nonclinical evaluation of the vaccines.

Attenuation of influenza vaccines (human, live attenuated) for intranasal administration is based on the attenuated virus donor strain used for vaccine

preparation (see section A.4.5.3.2). A complete genetic sequence of the attenuated virus donor strain should be obtained in order to map as carefully as possible where attenuating mutations have been introduced. Since each passage of an influenza virus may introduce new mutations, the possibility exists for reversion to a less attenuated or fully virulent form of virus. Studies should therefore be carried out to determine whether reverting mutations appear at any point in the planned production and use of vaccine strains. These studies should also determine, to the extent possible, whether the haemagglutinins and neuraminidases of the wild-type influenza viruses affect the stability of attenuating mutations.

For example, when a highly pathogenic avian influenza virus is used, close attention should be paid to ensuring that the haemagglutinin retains the modifications that eliminate the highly pathogenic phenotype.

Phenotypic markers should be mapped genetically to the extent possible with *in vitro* systems. Key phenotypic features and adaptations of the attenuated virus donor strain should be demonstrated. For example, the ability to replicate efficiently at relatively low temperature should be demonstrated consistently for a cold-adapted attenuated virus donor strain. For temperature-sensitive attenuated virus donor strains, it should be possible to demonstrate reproducibly the temperature at which viral replication is inhibited. *In vitro* studies should be confirmed for the candidate influenza vaccine (human, live attenuated) seed viruses in order to ensure that attenuating features are fully retained throughout the vaccine process steps.

B.3 Nonclinical toxicity and safety testing

B.3.1 Preclinical toxicity

Preclinical toxicity studies are designed primarily to demonstrate the safety and tolerability of a candidate influenza vaccine (human, live attenuated) for intranasal administration. The design of a preclinical toxicity study should meet the criteria outlined in a specific, written protocol in order to support an intended clinical trial. The protocol should state the background, rationale and objectives of the nonclinical studies and describe the design, methodology and organization, including statistical considerations, and the conditions under which studies are to be performed and managed. WHO has published Guidelines on nonclinical evaluation of vaccines: regulatory expectations (20).

Toxicity tests for influenza vaccines (human, live attenuated) for intranasal administration should include:

- an evaluation of the initial safe dose and of subsequent dose escalation schemes relevant to the clinical dose;
- an evaluation of single and repeated doses as appropriate;

- a determination of a set of relevant safety parameters for clinical monitoring;
- a demonstration of potential reversibility of virulence of attenuated vaccine strains; and
- local tolerability studies, which are typically included as part of the general toxicity evaluation.

The toxicity assessment of formulations of influenza vaccines (human, live attenuated) for intranasal administration can be done either in stand-alone animal toxicity studies or in combination with studies of safety and activity that include toxicity end-points in their design (20). The parameters to be considered in designing animal toxicity studies with influenza vaccines (human, live attenuated) for intranasal administration include:

- relevant animal species, e.g. ferrets, mice, non-human primates;
- ability to infect the animal, including sero-susceptibility status;
- virus strain, i.e. seasonal influenza strains, novel human influenza viruses, pandemic viruses;
- dosing schedule, i.e. one, two or three doses;
- method of vaccine administration, e.g. nasal spray or nasal drops;
- timing of evaluation of end-points; and
- clinical chemistry, antibody responses, histological examination of target organs, and necropsy.

Despite efforts to maximize the predictive value of nonclinical toxicity studies, animal models have limited applicability to human experiences and it is possible that the animal studies will not accurately reflect the risks of using influenza vaccines (human, live attenuated) for intranasal administration in humans.

The design and value of repeated-dose toxicity tests should be considered on a case-by-case basis. If an influenza vaccine (human, live attenuated) for intranasal administration is intended to be clinically tested in women of childbearing age, the need for reproductive toxicity studies and studies of embryo-fetal and perinatal toxicity should be considered on a case-by-case basis. Reproductive toxicity studies, where appropriate, will need to be undertaken before licensing (20).

Any changes in the composition of the human influenza vaccine (live, attenuated) for intranasal administration should be carefully considered both by the manufacturer and by the national regulatory authority. In general, there should be no need to repeat the toxicity assessments for an annual strain

change as long as attenuation has been adequately documented. However, it may be necessary to repeat some or all of the toxicity studies when a novel human influenza virus subtype emerges (e.g. influenza A virus H5N1) and a new influenza vaccine (human, live attenuated) for intranasal administration is prepared. Special considerations on the preclinical and nonclinical evaluation of influenza vaccines (human, live attenuated) for intranasal administration for pandemic use are provided in Regulatory preparedness for pandemic influenza vaccines (12).

Any changes in formulation, ingredients or excipients in the human influenza vaccine (live, attenuated) for intranasal administration may require some or all of the toxicity studies to be repeated. The manufacturer should discuss these changes with and secure the approval of the national regulatory authority.

B.3.2 Preclinical safety testing

Every effort should be made in the preclinical studies to identify markers of attenuation (genetic sequences) that can be used to monitor the results during clinical evaluation phases. Primary investigations of attenuation in animals may be done in a number of influenza-responsive species such as ferrets, mice, and non-human primates to establish the relationship of specific genetic features to the attenuation phenotype. Studies in animals may be designed to confirm the location and degree of replication of candidate influenza vaccine (human, live attenuated) in the respiratory tract of the host.

Results from animals inoculated with the attenuated virus donor strain (or its derivative candidate vaccine virus) compared with results from animals inoculated with the wild-type reference virus strain should indicate whether the attenuated virus donor strain (or the candidate vaccine virus) is adequately safe to allow limited clinical trials to be undertaken.

The potential of influenza vaccines (human, live attenuated) for intranasal administration for neuro-invasiveness and neuro-virulence should be considered if the wild-type virus used for seed virus preparation demonstrates neuro-invasiveness. The national regulatory authority should be consulted on the need for preclinical evaluation of the potential for neurological effects in suitable animal models.

Animal studies cannot completely predict what will happen in humans because of differences in anatomy, host temperature, and other variables. Features that appear to be attenuating in animals may not fully predict the genotype or phenotype of attenuation in humans. In addition, the vaccine dose tolerated by animals may differ greatly from that tolerated by humans. Results of studies in animals therefore do not obviate the need for confirmation in clinical trials in humans.

B.4 Nonclinical immunogenicity and efficacy

B.4.1 Nonclinical immunogenicity

Assessment of immune responses in animals can provide some assurance that an influenza vaccine (human, live attenuated) has replicated in the host. Influenza virus infection triggers a multitude of immune responses in the innate and adaptive immune systems, which can be assessed in an animal model. Although there is no specific correlate of protection defined for influenza vaccine (human, live attenuated) for intranasal administration, antibodies directed against viral haemagglutinins in the blood and mucosal secretions have been shown to have protective effects in animal models and in humans. Antibody responses to neuraminidase and other viral proteins, as well as cellular responses involving T helper and cytotoxic cells, have also been identified during recovery from influenza infection. Despite the lack of specific correlates of protection, immune responses in animals should be evaluated for their potential to provide an *in vivo* correlation with prevention of infection and/or reduction of disease signs and pathogenicity.

The specific immune parameters to assess in animals depend, in part, on the nature of the influenza vaccine (human, live attenuated) for intranasal administration. Parameters should at least include antibodies directed against haemagglutinins (e.g. serum neutralizing, haemagglutination inhibition, or single radial haemolysis antibodies).

While immune responses in animals do not necessarily predict immune responses in humans, it is nevertheless possible to derive useful information from preclinical immunogenicity studies.

B.4.2 Nonclinical efficacy

Protective efficacy can be assessed directly after immunization in animals by infectious challenge with wild-type or selected laboratory strains of influenza viruses. Preclinical studies in animals also allow assessment of the protective effects of a candidate influenza vaccine (human, live attenuated) for intranasal administration against potentially lethal influenza virus infections, which may become important in preparing vaccines for highly pathogenic influenza viruses with pandemic potential. Furthermore, animal studies can be designed to assess the breadth of immune and clinical protection of a given influenza vaccine (human, live attenuated) for intranasal administration against different influenza A subtypes and drift variants that may indicate the need for new antigens in the vaccine.

While efficacy identified in an animal model does not necessarily predict the protective effect in humans, it is nevertheless possible to derive useful information from preclinical efficacy studies in animals.

Part C. Clinical evaluation of new influenza vaccines (human, live attenuated)

C.1 General remarks

The clinical evaluation of new influenza vaccines (human, live attenuated) for intranasal administration includes studies undertaken as part of the developmental process, the licensure procedure, and/or studies performed in the post-marketing period. Extensive guidance for manufacturers and regulatory authorities on the clinical development of vaccines is found in the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (18) and Guidelines for good clinical practices (GCP) for trials on pharmaceutical products (63). Guidance that may apply to the clinical evaluation of influenza vaccines (human, live attenuated) for intranasal administration to be used as pandemic vaccines is provided in Regulatory preparedness for human pandemic influenza vaccines (12).

There are a number of issues to be considered in the clinical development and use of safe and effective influenza vaccines (human, live attenuated) for intranasal administration. Since influenza vaccines generally have been multivalent to provide protection against influenza A and influenza B viruses, each component of influenza vaccines (human, live attenuated) for intranasal administration must be proved safe and protective in the context of a formulated multivalent vaccine (64). Since wild-type influenza viruses constantly mutate, influenza vaccines (human, live attenuated) for intranasal administration will require frequent re-evaluations of composition and change, as recommended by WHO, on the influenza viruses to use in vaccines in countries of the northern and southern hemispheres (3).

There is no established immune correlate of protection for influenza vaccines (human, live attenuated) for intranasal administration and it is possible that immune correlates may be specific for individual influenza vaccines (human, live attenuated) (63). Despite potential difficulties, large field trials and routine immunization campaigns have shown that satisfactory influenza vaccines (human, live attenuated) for intranasal administration can be prepared, distributed and administered (8, 27, 64).

Possible variation in the attenuating features of each influenza vaccine (human, live attenuated) for intranasal administration make it important to carefully characterize the vaccine viruses derived from a new attenuated donor virus before licensure. Although animal studies may not perfectly predict human clinical experiences, nonclinical and preclinical studies with an influenza vaccine (human, live attenuated) for intranasal administration should help in determining the genetic elements of attenuation in humans, evaluating the possibility of reversion to partial or full virulence post-vaccine administration, and in determining potential targets for safety data collection as the clinical trials begin.

The delivery device may have an impact on the uptake of a vaccine, its safety and its effectiveness (20). Influenza vaccines (human, live attenuated) may be administered intranasally using proprietary or nonproprietary delivery devices – syringes, sprayers and other liquid delivery devices. The clinical evaluation of influenza vaccines (human, live attenuated) should include a rigorous assessment of the suitability of the intranasal delivery device to support the safety and effectiveness of the vaccine.

C.2 Clinical evaluation strategy

The clinical development of a new influenza vaccine (human, live attenuated) for intranasal administration will resemble that of other vaccine products; a typical strategy is provided in Table 2.

C.2.1 Clinical studies for licensure

Early (Phase I) studies focus on safety aspects, including replication, shedding and transmission potential of the vaccine virus, relevant pharmacokinetics, and neurotoxic and immunological effects identified in the nonclinical evaluation of the vaccine virus. Early clinical studies (Phase I and Phase II) should be designed to confirm the dose range that is well tolerated. These studies are recommended because replication of influenza vaccine (human, live attenuated) may be affected by previous host exposure to influenza viruses or vaccines as well as by underlying host factors.

As studies progress (Phase II), more information is acquired on immunological and protective effects, and trials to confirm efficacy (usually Phase III) are also undertaken. Later clinical studies (Phase II and Phase III) should be done to expand the number of subjects to be evaluated by groups targeted for vaccine use and to define the types and frequency of adverse events that have already been identified. Later clinical studies should also make it possible to identify rarer events that can only be detected when a sufficiently large population has been examined.

Table 2
Clinical evaluation^a of new influenza vaccines (human, live attenuated) for intranasal administration

Phases of clinical evaluation	Primary concern	Scope of clinical evaluation
Phase I	Safety (for common events)	Small-scale trials to determine whether significant risks exist

continues

Table 2 *continued*

Phases of clinical evaluation	Primary concern	Scope of clinical evaluation
Phase II	Preliminary effect (immunogenicity) and general safety (for typical and less common events)	Larger-scale trials to further define risks and potential benefits in populations expected to use the vaccine
Phase III	Protective efficacy (pivotal) and safety (for uncommon events)	Trial size defined to provide statistical certainty for specific end-points related to safety and efficacy
Post-marketing clinical trials	Safety (for rare events) and effectiveness (to expand on original observations)	Trials specifically designed to further refine information on safety and efficacy in larger populations and/or in new populations for vaccine use
Post-marketing surveillance	Safety (for unexpected and rare events and/or signals)	Potentially includes all vaccine recipients to identify safety signals arising from routine use and expanded population studies

^a Sources of guidance on clinical evaluation of vaccines: WHO; International Conference on Harmonisation; European Medicines Agency; United States Food and Drug Administration.

Later clinical studies also assess the potential for variation among people of different ages. People with pulmonary, cardiac or immune dysfunction should be addressed as part of expanding vaccine studies. It is also desirable to establish immune correlates of protection in human populations, since this may aid future improvements in the production and evaluation of influenza vaccine (human, live attenuated) for intranasal administration.

The information needed for the successful completion of clinical trials may vary somewhat depending on the nature of the vaccine; clinical development plans should therefore be discussed with and approved by the national regulatory authority and the national control laboratory. Current WHO guidelines on clinical evaluation of vaccines describe broadly applicable principles in greater detail (18). Guidance that may apply to the clinical assessments of influenza

vaccines (human, live attenuated) for intranasal administration to be used as pandemic vaccines is provided in Regulatory preparedness for human pandemic influenza vaccines (12) and Guidelines on clinical evaluation of vaccines: regulatory expectations (18).

C.2.2 Post-marketing studies

Although most of the clinical development will precede licensure of influenza vaccines (human, live attenuated) for intranasal administration, post-marketing trials and surveillance will provide significant information about the safety and effectiveness of vaccines that cannot be predicted or detected in the pre-licensing period, particularly with regard to rare adverse events. Manufacturers and national regulatory authorities should therefore make a substantial commitment to pharmacovigilance and to clinical studies as part of a continuing, post-marketing review of influenza vaccines (human, live attenuated) for intranasal administration. In the case of a significant change in the manufacturing process, the national regulatory authority may also require clinical studies.

While clinical studies with novel influenza virus subtypes or lineages may be considered, once adequate experience with multiple candidate influenza vaccine viruses within a subtype or lineage has accumulated, post-marketing clinical trials may no longer be justified for WHO-recommended changes to the influenza viruses to be used in vaccines in countries of the northern and southern hemispheres (3).

Some national regulatory authorities may require a limited clinical evaluation to assess safety parameters for licensing purposes whenever a new candidate influenza vaccine virus is introduced.

C.3 Clinical safety

C.3.1 Initial safety assessment

An influenza vaccine (human, live attenuated) is usually given intranasally so that virus replicates in the respiratory tract tissues, predominantly the nasopharyngeal mucosa. As a result of replication of influenza vaccines (human, live attenuated) viruses in the respiratory tract, mild upper respiratory symptoms and occasional benign systemic reactions have been observed in some individuals, but with much lower frequency and severity than with wild-type influenza viruses. Initial vaccine safety trials should include a careful assessment of symptoms related to influenza virus infection.

Since vaccinated individuals may shed influenza vaccine (human, live attenuated) viruses via respiratory secretions, virus excretion should be assessed for quantity and duration, and, if possible, compared with shedding during infection with relevant virulent wild-type influenza viruses. The excreted attenuated

virus strains should maintain the characteristic phenotypes of influenza vaccine (human, live attenuated) viruses, which may be partially predicted during the nonclinical and preclinical vaccine evaluation.

Initial clinical studies (Phase I), particularly with newly developed influenza A subtypes, are best performed in an environment offering the maximum guarantee of isolation and discipline. Some guidance on this may be found in Biosafety risk assessment and guidelines for the production and quality control of human influenza pandemic vaccines (13). These clinical trials are meant to ensure that the virus is not excreted at an unacceptably high titre or for an excessively long period. Clinical trials should show that the frequency of virus transmission is consistent with public health goals.

In addition to detailed assessment of safety, early clinical trials should attempt to show that the benefit–risk ratio of vaccine use is acceptable.

C.3.2 Expanded safety assessment

When determining the acceptability of an influenza vaccine (human, live attenuated) for intranasal administration in larger-scale clinical trials, limited studies in which the vaccine is administered to immunologically primed individuals from different age groups may be undertaken before the vaccine is administered to immunologically naïve individuals. The follow-up period for the assessment of safety should take into account effects that may occur many days or weeks post-administration. It should also include purposeful review of events for at least six months after final vaccine administration for a trial protocol for both early and later trials examining vaccine safety. Guidance on age progression (from adult to younger age groups) for an influenza vaccine (human, live attenuated) for intranasal administration in children has been published by WHO (12, 63).

Phase II and Phase III clinical trials should delineate the type, frequency and severity of local and systemic respiratory infection-related events such as sneezing, nasal discharge, cough, sore throat, fever and myalgia, as well as other respiratory and non-respiratory events that have been observed in preclinical studies and Phase I clinical trials. As numbers expand during Phase II and Phase III studies, and post-marketing studies, rarer adverse events may be identified for further attention.

Each influenza virus passage may introduce new mutations. Studies should therefore be pursued to determine whether reverting mutations appear at any point in the planned production and use of new attenuated donor viruses.

The possibility exists of influenza vaccine (human, live attenuated) virus transmission through contact of susceptible individuals with virus-containing secretions. However, the probability of such transmission should be small compared with that of wild-type influenza viruses, even in immunologically naïve people.

When novel wild-type reference virus strains (e.g. in the case of a pandemic vaccine) are used in the preparation of influenza vaccines (human,

live attenuated), clinical studies may be considered as a part of the assessment to determine that the newly introduced haemagglutinins and neuraminidases have not altered the safety profile of the influenza vaccine (human, live attenuated) for intranasal administration in any adverse manner.

C.4 Clinical efficacy and immunogenicity

With current methods and capabilities, clinical end-point studies provide the definitive assessment of efficacy of influenza vaccines (human, live attenuated) for intranasal administration (64). Several antibody responses, including neutralizing antibodies in blood and respiratory secretions, are associated with prevention of influenza virus infection as well as reduction of symptoms and overall pathological effects in humans. However, broadly applicable immunological correlates of protection have not been identified and relevant immune parameters may vary for influenza vaccines (human, live attenuated) for intranasal administration (63). Nonetheless, opportunities to correlate specific immune parameters with prevention of infection or illness should not be missed during clinical studies.

C.4.1 Clinical efficacy studies

Vaccine efficacy is the reduction in the odds of developing clinical disease post-vaccination relative to the odds when unvaccinated. Efficacy of influenza vaccines (human, live attenuated) for intranasal administration in a specified population should be demonstrated in adequately-powered, well-controlled clinical trials (Phase III). Since this is usually a complex and expensive undertaking and requires an infected population, the design and feasibility of efficacy clinical trials (e.g. of pandemic vaccines) should be thoroughly explored with the competent authority (12, 18).

In general, efficacy studies should demonstrate that influenza vaccines (human, live attenuated) for intranasal administration prevent laboratory (e.g. culture) confirmed influenza illnesses. The ability to document vaccine efficacy depends upon an adequate attack rate of influenza (proportion of population exposed to influenza viruses who become clinically ill) in relation to the ability to prevent infection in a study with an appropriate sample size. Since attack rates for influenza vary from year to year and different influenza types or subtypes predominate in different years, efficacy studies will generally require a large enrolment and sometimes a multi-year plan for thorough evaluation of each component strain, type or subtype contained in influenza vaccines (human, live attenuated) for intranasal administration.

C.4.2 Clinical correlates of protection

If possible, clinical trials should establish a correlate of protection that can be used to guide development of influenza vaccines (human, live attenuated) for intranasal

administration. Since influenza vaccines (human, live attenuated) are administered by the respiratory route, it should be anticipated that immune parameters other than antibodies in blood could serve as correlates of protection (63). Novel methods to measure antibody or cellular responses should be validated.

C.4.3 Clinical serological parameters

Anti-haemagglutinin antibodies in blood are the most widely studied immune mechanisms in humans. However, antibody responses to neuraminidase and other influenza viral proteins may also contribute to protection. The specific serological parameters to assess in clinical trials (Phases I–III) depend in part on the nature of the influenza vaccine (human, live attenuated) for intranasal administration but a reasonable starting point is determination of neutralizing, haemagglutination inhibition or single-radial haemolysis antibodies (63).

C.4.4 Other clinical measures of immunity

Assessment of innate, mucosal and cellular immune mechanisms may be helpful in further developing a rationale for protection resulting from influenza vaccines (human, live attenuated) for intranasal administration.

Influenza vaccines (human, live attenuated) for intranasal administration are expected to induce local innate immune responses, including interferon and other cytokines, as well as adaptive immune responses such as secretory mucosal antibodies, i.e. IgA (8, 63).

Cellular immune mechanisms are less well understood but it is expected that influenza vaccines (human, live attenuated) for intranasal administration may also mimic the T-cell responses identified during naturally occurring infections in humans.

C.5 Evaluation in special populations

In general, clinical trials should be performed first in the least vulnerable population (i.e. healthy adults). Placebo groups are essential to evaluating the incidence and intensity of adverse events following immunization.

If the initial clinical trials in healthy adults demonstrate vaccine safety at specific doses, additional studies may be undertaken to delineate the uses of influenza vaccines (human, live attenuated) for intranasal administration in different age and risk groups. Because it may be inappropriate to expose some vulnerable populations to an influenza vaccine (human, live attenuated) for intranasal administration, early clinical studies should also provide an understanding of the frequency of transmission and the potential for adverse consequences of transmission to non-vaccinated individuals.

C.5.1 Children

If clinical trials in adults demonstrate a safety profile that is suitably benign, studies in children may be undertaken to evaluate safety and efficacy of influenza vaccines (human, live attenuated) for intranasal administration (12, 63). It may be prudent to study older children first and then progressively younger children: younger children would be expected to be more prone to develop local and systemic adverse events that may not be apparent in older individuals who have been exposed to wild-type influenza viruses and vaccines.

C.5.2 The elderly

If clinical trials in healthy adults demonstrate a safety profile that is suitably benign, studies in elderly populations, particularly those with chronic diseases, may be undertaken to evaluate the safety and efficacy of influenza vaccines (human, live attenuated) for intranasal administration. Although there may be similarities between healthy adults and the elderly, different responses to influenza vaccines (human, live attenuated) for intranasal administration may result from lifetime exposure to influenza and from the accumulation of chronic, particularly cardiac and pulmonary, illnesses in the elderly.

C.5.3 Risk groups

A solid understanding of the typical post-vaccination clinical course in the healthy host is imperative before any study is initiated in a population with special risk status. All studies should be conducted with strict ethical considerations for the welfare of participants and no one should be exposed to unreasonable risk (18, 63).

Special studies may be considered for populations for whom influenza vaccination is especially important (such as subjects with chronic bronchitis and asthma) but in whom potential adverse events might be more severe.

People with immune defects that do not contraindicate exposure to a live virus vaccine should be evaluated cautiously to determine whether an influenza vaccine (human, live attenuated) for intranasal administration is beneficial without any unusual consequences such as prolonged shedding of virus.

C.6 Strain change considerations for seasonal vaccines

After an initial licensure, the vaccine is reformulated according to the circulating strains predicted for the upcoming season (3). For inactivated influenza vaccines, small-scale immunogenicity studies are requested in some parts of the world. However, without immunological correlates of protection, such studies with influenza vaccines (human, live attenuated) for intranasal administration are of limited value. Moreover, an annual clinical efficacy trial is generally not feasible because of methodological constraints and timing.

Some national regulatory authorities may recommend small-scale safety studies for influenza vaccines (human, live attenuated) for intranasal administration. The value of such studies for reassessing the benefit–risk balance is unknown. In general, when the vaccine production process remains unchanged, it is unlikely that the benefit–risk balance as assessed at the time of licensure will change. The need for additional clinical evaluations before annual re-licensure may depend upon factors that include major changes in subtypes (i.e. antigenic drifts or shifts), previously identified risks and/or reported safety signals, and impaired efficacy during vaccine use in the previous season. In such cases, a clinical study to reconfirm the benefit–risk balance may be deemed necessary.

It follows that a quality assessment of vaccine formulation for the upcoming year may be recommended and may be supplemented with a structured annual post-marketing surveillance programme in countries where vaccine is used in wide-scale vaccination programmes. Such post-marketing surveillance programmes may change over time, depending on experience. In the initial years following licensure, post-marketing surveillance studies may be requested and be replaced later by a programme of continuous surveillance. In any case, marketing authorization holders are recommended to consult with national regulatory authorities where vaccine is to be marketed and discuss details of the post-marketing surveillance programme.

C.7 **Vaccines intended for pandemic influenza**

It is expected that attenuated virus donor strains developed for seasonal influenza vaccines (human, live attenuated) for intranasal administration may be evaluated for use in pandemic situations. New attenuated virus donor strains may be prepared specifically for pandemic use. If a new attenuated virus donor strain is planned for pandemic use, the quality, safety and efficacy of pandemic influenza vaccines (human, live attenuated) for intranasal administration should be ensured by conformity with the recommendations in the present document. In addition, special regulatory considerations for pandemic influenza vaccines should be followed (12).

For an attenuated donor strain specifically prepared for pandemic use, studies should demonstrate an attenuated phenotype and stability of the attenuated donor strain genome. Clinical trials of influenza vaccines (human, live attenuated) for intranasal administration containing the haemagglutinin and neuraminidase of novel influenza viruses should be conducted in isolation units while the novel virus with pandemic potential is not spreading efficiently from human to human (i.e. WHO pandemic phases 1–5). Isolation procedures should be maintained for recipients of vaccines against novel influenza viruses for the duration of vaccine virus shedding. WHO will provide advice to national health authorities on declaration of pandemic phases. Assessment of efficacy

in the absence of widespread disease is especially challenging. Given the potential efficacy of influenza vaccines (human, live attenuated) for intranasal administration in paediatric populations, approval for the conduct of studies in children will be needed from national regulatory authorities.

Comprehensive guidance on influenza vaccines (human, live attenuated) for intranasal administration to be used as pandemic vaccines is provided in Regulatory preparedness for human pandemic influenza vaccines (12).

Part D. Recommendations for national regulatory authorities

D.1 General remarks

The general recommendations for national regulatory authorities contained in WHO good manufacturing practices for biological products (39) should apply. An extensive list of WHO guidelines with additional specific recommendations for the quality assurance of biological products to be followed by national regulatory authorities is provided in the reference section of this document.

The national regulatory authority should direct manufacturers on the influenza virus strains to be used and the recommended human doses.

The national regulatory authority should take into consideration all information available on strains before deciding on those permitted for vaccine production (3).

In addition, the national regulatory authority should provide a reference preparation of influenza vaccines (human, live attenuated) for intranasal administration to check the normal susceptibility of the titration system. The national regulatory authority should also specify the virus content requirements to be fulfilled in order to achieve adequate immunization with the recommended human dose.

As a practical matter, the national regulatory authority will need to collaborate with the manufacturer to develop an acceptable reference preparation.

D.2 Release and certification

A vaccine lot should be released only if it fulfils national requirements and/or Part A of these recommendations. A protocol based on the model in Appendix 1, signed by the responsible official of the manufacturing establishment, should be prepared and submitted to the national regulatory authority in support of the request for release of vaccine for use. A statement signed by the authorized

official of the national regulatory authority should be provided at the request of the manufacturing establishment, certifying that the lot of vaccine in question meets all national requirements as well as the manufacturing recommendations provided in Part A of these recommendations.

The release certificate should state the number under which the lot was released by the national regulatory authority and the number appearing on the labels of the containers. Importers of influenza vaccines (human, live attenuated) for intranasal administration should be given a copy of the official national release document of the country of production. The purpose of the certificate is to facilitate the exchange between countries of influenza vaccines (human, live attenuated) for intranasal administration. An example of a suitable certificate is given in Appendix 3.

D.3 **Manufacturing changes**

In the case of a new vaccine, the national regulatory authority should assess vaccine safety and efficacy by arranging for studies in human volunteers of one or more of the lots of influenza vaccine (human, live attenuated) for intranasal administration that have satisfied the above-mentioned recommendations. Such studies should include assessment of the immune responses and adverse reactions in various age groups.

In the case of significant change in the manufacturing process, preclinical, nonclinical and clinical studies may also be required by the national regulatory authority.

Some national regulatory authorities require a limited clinical evaluation for licensing purposes whenever a new vaccine strain is introduced.

Authors

The first draft of these revised WHO Recommendations for the production and control of influenza vaccine (human, live attenuated) was prepared in May 2008 by Dr C.P. Alfonso, WHO, Geneva, Switzerland, and Dr R. Levandowski, Bethesda, MD, USA.

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A draft incorporating these comments was produced in December 2008 and further revised to generate a document that was reviewed at the informal consultation on WHO Recommendations for the production and

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A further revision was produced in the light of comments received and was proposed for endorsement by the Expert Committee on Biological Standardization at its meeting in October 2009 in Geneva, Switzerland.

The present recommendations reflect the discussions of the Expert Committee on Biological Standardization; with requests for certain modifications, the Expert Committee endorsed the document.

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Appendix 1

Summary protocol for influenza vaccines (human, live attenuated) for intranasal administration (master/working seed lot Type A or Type B)

The model summary protocol that follows is provided as general guidance to manufacturers. It is not intended to constrain them in the presentation of data relevant to the complete review of the quality control tests performed on the vaccine. It is important to note that satisfactory test results do not necessarily imply that the vaccine is safe or effective, since many other factors must be taken into account, including the characteristics of the manufacturing facilities.

Name and address of manufacturer _____
 Laboratory reference no. of lot _____
 No. of lyophilized lot (if applicable) _____
 Date when the processing was completed _____

Information on manufacture

Substrate for manufacture

Vaccine-quality embryonated eggs or cell culture _____

For vaccine-quality embryonated eggs, qualification of eggs:

Layer flock status, e.g. SPF-SAN _____

Supplier name and address _____

Remarks _____

For cell culture, qualification of cells:

Cell bank reference no. _____

Species and tissue origin _____

Passage history _____

Remarks _____

Virus used to inoculate vaccine-quality embryonated eggs or cells for the manufacture of the lot:

Strain and sub-strain _____

Passage level _____

Source and reference no. _____

Remarks _____

Results of sterility test _____

Results of tests for adventitious agents _____

Results of tests for attenuation _____

Conditions of storage _____

Monovalent virus pool Type A or Type B

Name and address of manufacturer _____

Laboratory reference no. of the virus pool _____

Virus used to inoculate vaccine-quality embryonated eggs or cells

Master seed strain and source _____

Passage level of master seed _____

Working seed lot, reference no. and source _____

Date of inoculation

Date of harvesting allantoic or amniotic fluid or
cell-culture fluids _____

Storage conditions before use in final bulk _____

Clarification/purification procedure _____

Antibiotics (if any) used during preparation _____

Identification of preservatives/stabilizers
(if any) _____

Tests on monovalent pool

Determination of infectivity (potency)

Method _____

No. of vaccine-quality embryonated eggs or cell cultures
inoculated _____

Date of determination _____

Results _____

Determination of attenuation

Method _____

Date of determination _____

Results _____

Determination of identity of virus

Method _____

Date of determination _____

Results _____

Test for adventitious virus agents

Method _____

Date of test _____

Results _____

Test in vaccine-quality embryonated eggs (for egg-based vaccine)

Method _____

Date of test _____

Results _____

Test in cell cultures

Method _____

Date of test _____

Results _____

Test for adventitious bacterial agents

Method _____

Date of test _____

Results _____

Test for adventitious fungal agents

Method _____

Date of test _____

Results _____

Test for adventitious mycoplasma agents

Method _____

Date of test _____

Results _____

Test for adventitious mycobacterial agents

Method _____

Date of test _____

Results _____

Test for chemicals used in production (may be performed on final bulk)

Method _____

Date of test _____

Results _____

Test for residual DNA (for cell-culture vaccine)

Method _____
 Date of test _____
 Results _____

Final bulk

Name and address of manufacturer _____
 Identification of final bulk _____
 Identification of monovalent virus pool(s) used to prepare
 final bulk _____
 Date of manufacture _____

Control of final bulk*Test for infectivity (potency) (may be performed on final bulk)*

Method _____
 Date of test _____
 Results _____

Test for sterility

Method _____
 Date of test _____
 Results _____

Test for total protein

Method _____
 Date of test _____
 Results _____

Test for ovalbumin (for egg-based vaccine)

Method _____
 Date of test _____
 Results _____

Test for residual DNA (for cell-culture vaccine)

Method _____
 Date of test _____
 Results _____

Test for chemical used in production

Method _____
 Date of test _____
 Results _____

Control of final product

Test for infectivity (potency)

Method _____

Date of test _____

Results _____

Test for identity

Method _____

Date of test _____

Results _____

Test for sterility

Method _____

Date of test _____

Results _____

Test for endotoxin

Method _____

Date of test _____

Results _____

Test for residual moisture (if applicable)

Method _____

Date of test _____

Results _____

Inspection of final containers

Results _____

Other tests _____

Additional comments (if any) _____

A sample of a completed final container label and package insert should be attached.

Certification by producer

Name of head of production (of final product) _____

Certification by head of the quality assurance department taking overall responsibility for production and control of the final product:

I certify that Lot No. _____ of Influenza Vaccine (Human, Live Attenuated) for Intranasal Administration, whose number appears on the label of the final container, meets all national requirements¹ and satisfies Part A of the Requirements for Biological Substances No. 17, revised 1990.

Signature _____

Name typed _____

Date _____

Certification by the national regulatory authority

If the vaccine is to be exported, provide a copy of the certificate from the national regulatory authority as described in section D.2, a label of a final container, and a leaflet of instructions to users.

¹ If any national requirement(s) is(are) not met, specify which one(s) and indicate why release of the lot has nevertheless been authorized.

Appendix 2

Reference laboratories

WHO Collaborating Centres for Reference and Research on Influenza

- WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia
- WHO Collaborating Centre for Reference and Research on Influenza, National Institute of Infectious Disease, Tokyo, Japan
- WHO Collaborating Centre for Reference and Research on Influenza, National Institute for Medical Research, London, England
- WHO Collaborating Centre for the Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention, Atlanta, GA, USA
- WHO Collaborating Centre for Studies on the Ecology of Influenza in Animals, St Jude Children's Research Hospital, Memphis, TN, USA

Essential regulatory laboratories

- Therapeutic Goods Administration, Immunology and Vaccines, Canberra, Australia
- National Institute of Infectious Disease, Tokyo, Japan
- National Institute for Biological Standards and Control, Potters Bar, England
- Center for Biologics Evaluation and Research, Division of Viral Products, Food and Drug Administration, Rockville, MD, USA

WHO reference laboratories for diagnosis of influenza A/H5 infection

- WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia
- Department of Microbiology, Faculty of Medicine, University of Hong Kong, China, Hong Kong Special Administrative Region
- National Influenza Centre, Centre for Health Protection, China, Hong Kong Special Administrative Region
- Virology and Zoonotic Disease Research Program, US Naval Medical Research Unit 3 (NAMRU-3), Cairo, Egypt

- WHO Collaborating Centre for Reference and Research on Influenza, National Institute for Medical Research, London, England
- Institute Pasteur, Unité de Génétique Moléculaire des Virus Respiratoires, Paris, France
- National Institute of Virology, Pune, India
- WHO Collaborating Centre for Reference and Research on Influenza, National Institute of Infectious Disease, Tokyo, Japan
- Federal State Research Institution, State Research Centre for Virology and Biotechnology VECTOR, Novosibirsk Region, Russian Federation
- WHO Collaborating Centre for the Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention, Atlanta, GA, USA
- WHO Collaborating Centre for Studies on the Ecology of Influenza in Animals, St Jude Children's Research Hospital, Memphis, TN, USA

Appendix 3

Model certificate for the release of influenza vaccine (human, live attenuated) for intranasal administration¹

The following lots of influenza vaccine (human, live attenuated) for intranasal administration

called [trade name and/or common name of the product] _____
produced by [name and address of manufacturer] _____²
in [list of manufacturing sites] _____³

whose marketing authorization number is _____ [insert marketing authorization number] and whose numbers appear on the labels of the final containers, meet all national requirements,⁴ the manufacturing recommendations in part A of the WHO Recommendations to assure the quality, safety and efficacy of influenza vaccines (human, live attenuated) for intranasal administration (revised 2009)⁵ and Recommendations for good manufacturing practice and quality assurance for biological products,⁶ and has been approved for release.

Lot number [including sub-lot number and packing lot numbers if relevant]	Container type and number of doses per container	Storage conditions	Number of containers/ batch size	Date of start of period of validity (e.g. manufacturing date) and/or expiry date

As a minimum, this certificate is based on examination of the manufacturing protocol.

¹ To be provided by the national regulatory authority of the country where the vaccines have been manufactured.
² Name and address of manufacturer.
³ Country.
⁴ If any national requirement(s) is (are) not met, specify which one(s) and indicate why release of the lot(s) has nevertheless been authorized by the national regulatory authority.
⁵ To be published in WHO Technical Report Series, No. 977, 2013, Annex 4; with the exception of the provisions on shipping, which the national regulatory authority may not be in a position to control.
⁶ WHO Technical Report Series, No. 822, 1992, Annexes 1 and 2.

The number of this certificate is: _____
The date of issue of this certificate is: _____
Title of authorizing official⁷ (typed): _____
Name of official (typed): _____
Signature of official: _____
Date: _____

⁷ Or her or his representative.

