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**POINTS TO CONSIDER ON THE DEVELOPMENT OF LIVE
ATTENUATED INFLUENZA VACCINES**

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1. INTRODUCTION

Current requirements and guidelines on inactivated influenza vaccines^{1,2,3,4} do not address requirements for live attenuated influenza vaccines. It is intended to produce live attenuated reassortant influenza vaccines in embryonated eggs based on cold-adapted influenza virus master strains. Master strains having alternative attenuated phenotypes may also be used. The Ph Eur monograph for yellow fever vaccine⁵ another live viral vaccine grown in embryonated eggs, might serve as an example for live influenza vaccines. Likewise, more general Ph Eur monographs on extraneous agents⁶ and on sterility⁷ are relevant. However, issues specific to live attenuated influenza vaccines covering seasonal production, safety controls, use on official recommendation only, determination of efficacy in different target groups and possible long term protective efficacy have not been addressed in the above mentioned monographs.

2. SCOPE

The CPMP Note for Guidance on Influenza Vaccines¹ covers Quality as well as Safety and Efficacy issues that are mainly related to the yearly change of vaccine strains in inactivated influenza vaccines produced in eggs. This paper addresses Quality and Safety & Efficacy issues that are pertinent to live attenuated influenza vaccines produced in eggs. Some aspects may also be applicable to live attenuated influenza vaccines produced in cell culture. For specific requirements of cell culture influenza vaccines (inactivated), reference is made to the Annex to CPMP note for Guidance on Influenza Vaccines – Note for Guidance on Cell Culture Inactivated Influenza Vaccines¹⁸.

3. QUALITY ISSUES

Live attenuated influenza vaccines are produced in fertilised hens' eggs under appropriate conditions defined by the phenotypic characteristics of the attenuated master strain. In contrast to currently available inactivated influenza vaccines which are purified, very limited downstream processing can be performed on live vaccines. Therefore, only high quality starting materials tested or certified for compliance with all regulations pertinent to the manufacture of vaccines for human use should be used during production in order to eliminate any potential source of contamination that might affect the final vaccine. In particular, these issues relate to the egg substrate, to the attenuated parent strain, to the donor strain of the relevant haemagglutinin (HA) and neuraminidase (NA) and to the resulting attenuated reassortant. Influenza vaccines are updated periodically to include new influenza strains. Whenever this occurs, there are severe constraints on the time available for quality tests.

3.1. Live-attenuated parent strain

3.1.1 Development

Detailed documentation is requested on the history of all biological agents used, i.e. viruses and cells, the method of preparation and a complete list of all starting materials used for construction of the attenuated parent strain.

3.1.2 Characterisation

Phenotypic and genetic properties of the attenuated parent strain need intensive investigation and should be documented in detail in the core dossier.

The phenotypic characterisation includes studies on the markers for attenuation as, for example, on the cold-adapted (ca) or temperature sensitive (ts) phenotype, performed in vivo and/or in vitro under conditions allowing the detection of revertants to wild type, or reassortant strain with wild virus. The absence of any neurovirulent capacity of the attenuated parent strain should be demonstrated. These studies should be performed in appropriate animal models and cover issues raised by the potential direct neurovirulence caused by the vaccine virus strains themselves or on the potential indirect neurovirulence due to secondary infections. The relevant guideline⁸ applies. The suitability of small animal species for the test of neurovirulence should be explored¹⁷.

The genetic characterisation of the attenuated parent strain includes (i) determination of the nucleotide sequence of the complete viral genome, (ii) reports on the analysis of the molecular basis of the attenuated phenotype and (iii) demonstration of the genetic stability of the attenuated parent strain by comparison of the nucleotide sequence of the viral genome at different passage levels.

In particular, attention should be paid to the testing for extraneous agents. These tests are performed according to the relevant requirements and guidelines on extraneous agents of viral seeds⁶, on cell substrates^{9,10,11,12,13}, on tests for sterility⁷ and on tests for mycoplasmas¹⁴.

3.1.3 Seed lots

For production of live attenuated influenza vaccine, a seed lot system of the attenuated parent strain should be established using eggs from specific pathogen free (SPF) flocks¹⁵. The method of preparation should be described in detail and storage conditions of seed lots should be validated with respect to infectivity, genetic stability and sterility.

Genetic stability parameters comprise a demonstration of the retention of the defined phenotypic properties and the genetic structure of the attenuated parent strain throughout seed lot production as evidenced according to the requirements described under point 3.1.2. A stability program for the seed lot system should be established.

Extraneous agent testing of seed lots of the attenuated parent strain should be performed according to relevant Ph Eur monographs on extraneous agents of viral seeds⁶, on tests on sterility⁷ and on tests on mycoplasmas as indicated above (see 3.1.2).

3.2 Wild type Influenza HA & NA donor strain

3.2.1 Isolate and passage history

The antigenic identity of the influenza HA & NA donor virus should be in accordance with the seasonal recommendations of the WHO and the origin and history of the donor strain should be documented.

3.2.2 Seed Lots

Seed lots of the donor strain should be derived from the primary isolate by propagation on eggs or in controlled and certified cell substrates. Eggs used for propagation of the donor strain should be from hens certified to be free from specified pathogens (SPF)¹⁵.

3.2.3 Testing of Seed Lots

The following tests should be performed on the final seed lot of an influenza HA & NA donor strain that will be used for reassortment with the live attenuated parental influenza strain. If agreed with the competent authority, these tests can also be performed at the level of the reassortant Working Virus Seed.

Tests should demonstrate the absence of extraneous agents in the donor strain seed lot according to current Ph Eur monographs^{6,7}. Specific screening for human respiratory agents able to replicate in eggs should also be performed. In addition to tests already described in relevant guidelines, a multiplex PCR could be developed that is validated to detect genomes of human respiratory agents which may replicate in the egg substrate used for production of the seeds.

Since removal or inactivation of microbial contaminants is unlikely to be possible at any level of the production process of live attenuated influenza vaccine, the presence of any microbial agent in the seed lots of the attenuated parent strain and of the donor strain is not acceptable.

3.3 Preparation of the live attenuated reassortant virus

Reassortment between the live attenuated parent strain and the influenza HA & NA donor strain using classical techniques or reverse genetics methodology should be performed with biological reagents, for example antisera, enzymes, etc., certified to be free from infectious agents. With the use of reverse genetics methodology, the principles and recommendations of the Note for Guidance on Gene Transfer Medicinal Products¹⁹ should be adhered to. The presence of the correct HA and NA derived from the influenza donor strain should be demonstrated; this can be achieved by using specific antisera and certified reference reagents and/or other methods.

3.3.1 Generation and development of seed lots from live attenuated reassortant virus

A characterised clone of live attenuated reassortant virus is used for propagation in specific pathogen free embryonated eggs. A Master Virus Seed Lot and, optionally, a Working Virus Seed Lot derived from the Master Seed Lot should be established.

3.3.2 Characterization of Master and Working Virus Seeds Lots

Controls applied for identification and characterization of live attenuated reassortant virus seeds include those described under 3.1.2. The presence of genes associated with attenuation as well as the identity of HA and NA must be demonstrated. Lack of neurovirulence of the live attenuated reassortant virus should be demonstrated. Justification should be given if the test is not performed or replaced by an alternative test.

3.4 Production

In general, special attention should be given to aseptic production conditions strictly avoiding contamination of the production system with extraneous agents. Since it may be difficult to perform a test for extraneous agents on single virus harvests, uninoculated control eggs are incubated in parallel to production eggs and tested for the absence of extraneous agents. Alternatively, single harvests may be tested for extraneous agents on suitable cell substrates in the presence of influenza haemagglutinin neutralizing antibodies.

3.4.1 Egg substrate used for production

Flocks of hens used for egg production should be stringently controlled for the presence and maintenance of the SPF status at regular time intervals. Only controlled eggs from such flocks should be used for production of the live attenuated influenza vaccine.

3.4.2 Virus harvest

As for any live viral vaccine, the addition of preservatives such as thiomersal is contraindicated. Only virus harvests that comply with the tests for HA identity, and which are within an acceptable specification of bioburden should be used for further propagation. If agreed with the competent authority, tests for sterility and mycoplasmas can be performed at an appropriate stage following downstream processing of the single virus harvest. A specification should be provided for potency, e.g. determination of the egg infectious dose (EID₅₀) of live attenuated virus/ml of chorio-allantoic fluid.

3.4.3 Monovalent bulk vaccine

Tests on the monovalent bulk vaccine include tests on the retention of phenotypic and genetic markers of the live attenuated virus described under point 3.1.2.

3.4.4 Trivalent, final bulk vaccine

Trivalent bulks fall within tight specifications for potency (EID₅₀ or TCID₅₀ (tissue culture infectious dose)). For determination of the potency, a method using suitable reference reagents for each subtype should be used. Potency, ovalbumin content and bacterial endotoxin concentration should fall within tight specifications. Lack of pyrogenicity of the trivalent live attenuated influenza vaccine following intranasal applications should be demonstrated in a suitable animal species on a limited number of final bulks. Thermal stability of the final vaccine should be adequately documented in real time stability studies and in studies at elevated temperatures. An end of shelf life specification should be defined and adequately justified.

4. CLINICAL ISSUES

Since live attenuated influenza vaccines are new vaccines, the principles described in the CPMP Note for Guidance on clinical evaluation of new vaccines apply¹⁶.

Influenza vaccines are combination vaccines. This means that the efficacy of all components as well as the safety of the combination needs to be established. Specifically, interference between the influenza strains present in the vaccine needs to be addressed.

In practice, this means that either sufficient evidence of an immunological correlate for protection for those strains where insufficient clinical data can be obtained, should be provided, or clinical confirmation of protection from field studies is needed.

4.1 General issues, specific for live attenuated influenza vaccines

Apart from the principles described in the CPMP Note for Guidance on clinical evaluation of new vaccines¹⁶, some issues are specific for (live attenuated) influenza vaccines. These include, for example, persistence of protection and revaccination schedules in relation to antigenic drift and also the risk of reassorting as a source of antigenic shift.

The extent of the required clinical trials, the study designs chosen and the relevant clinical endpoints are defined by the strategy foreseen for the vaccine. The strategy to be followed for a pre-exposure intervention with the live attenuated vaccine, i.e. targeted at risk groups (as for the inactivated influenza vaccines) is different from that when the focus is on community intervention, i.e. healthy adult populations and healthy children.

The first strategy targets only direct protection of the vaccinees and may focus solely on annual efficacy data; the second additionally depends upon the indirect protection created by the intervention that persists over longer periods and is beneficial for a larger proportion of the population. For LAIVs this implies that the vaccine may be claimed to reduce disease burden in the (non-vaccinated) population, and/or may show heterologous protection. It is likely that a trial programme will be initiated with pre-exposure trials, however in case of broader claims of community protection these intentions have to be clarified at the start of the clinical trial programme. It should be taken into consideration that differences in study design, clinical endpoints, duration of follow up and population-related infection risks may preclude extrapolation of data from pre-exposure trials to community trials. The same holds for extrapolation from healthy individuals to risk group populations.

In any case, the use of live attenuated influenza vaccines should be limited until adequate standards for their safe and effective use are developed.

- Within the scope of the current influenza vaccination recommendations, clinical efficacy can be studied in pre-exposure trials in the usual risk group populations for annual vaccination with inactivated influenza vaccine, defined by national policies. The clinical endpoints chosen should include the clinically relevant conditions to be prevented (influenza infections as well as secondary morbidity or mortality). Efficacy is measured in randomised trials versus inactivated influenza vaccines of the current year or a placebo. Follow-up of the study may be for one year, with a revaccination recommendation for subsequent years. Follow up over several years is necessary to determine the persistence of protection for vaccine strains of the current year and previous and/or subsequent years following repeated (annual) administration of the live attenuated influenza vaccine. For claims of heterologous protection longer-term active controlled comparative studies over at least two years (with documented divergent antigenic strains) are necessary. This long-term follow up is required to determine the level of heterologous protection and persistence of protection for non-vaccine strains. Outcomes are relevant to define the usefulness of LAIV for (annual) revaccination and/or or to redefine the intervals to re-vaccination.
- For broader claims extending the pre-exposure use of LAIVs it is likely that populations for whom influenza vaccination is presently not a specific recommendation, such as healthy children, are studied. Before embarking on such studies the disease burden in these populations needs to be defined, in terms of personal risks and the relevance (as source of infection) for risk groups. The clinical endpoint chosen should reflect the clinical condition to be prevented, i.e. influenza (like) illness and influenza infection as well as reduction of virus spread. However, secondary morbidity parameters should also be predefined and specific for the studied target population. Efficacy is measured in randomised, placebo controlled trials. The vaccination schedule and follow up should be based upon the proposed vaccination indication. When incorporation into a standard vaccination programme is envisaged, special consideration should be given to the primary vaccination schedule and the revaccination strategy. Long-term follow-up is necessary for claims of heterologous protection and to define the intervals to booster or re-vaccination.

A second important general issue for live attenuated vaccines is the different immunological profile compared to inactivated vaccines. Whereas for inactivated vaccines the anticipated immune response will predominantly be humoral (i.e. IgG responses), live attenuated influenza vaccines are expected to induce both a humoral (IgA and IgG) and a cellular immune response. There are also important differences with respect to the method of administration (oral or intranasal for the live attenuated vaccines vs. intramuscular or subcutaneously for inactivated vaccines). The immunological profile of the live attenuated influenza vaccine and the correlates with protection need to be defined and validated. In addition questions need to be addressed such as how to evaluate the yearly updates in pre-exposure vaccination (current influenza vaccination recommendation) if this is necessary.

4.2. Pharmacology / Immunology

Pharmacodynamic studies are needed to establish the immunological profile of the vaccine and define and validate the correlates for clinical protection. Humoral immunity should focus on local mucosal immunoglobulin production (IgA) as well as the serum anti-haemagglutinin response.

If at all necessary, recommendations for yearly updates should be defined and validated. The validation process may include a detailed evaluation of vaccine failures. In addition, the immunogenicity of other structural and non-structural influenza proteins expressed by the vaccine should preferably be investigated. A strong and sustained immune response to these components may affect the effectiveness of repeat vaccination.

Claims of heterologous protection need to be studied in clinical settings over a sufficient number of years. This implies studying memory responses, development of cellular immunity and its qualitative and quantitative characteristics, in relation to heterologous protection. A clinical trial program on heterologous protection should be supported by investigations of cross-protection in relevant *in vitro* assays and preclinical animal models. These studies are also necessary in order to define a rational (re)vaccination strategy.

Single or repeated application required for protective efficacy:

Pharmacodynamic studies should be performed to define the effective dose and number of applications in the primary vaccination schedule.

Evidence of interference between influenza subtypes

Possible interference between virus components should be studied in a suitable animal model measuring immunological correlates, if available. Should correlates for protection not exist, immunised animals need to be challenged.

Challenge studies in humans may be considered, when absolutely necessary and considered ethically justified.

Interference with other vaccines

Possible interference with other vaccines should be studied when simultaneous administration or separate administration within a short, predefined time frame, is anticipated (e.g. childhood vaccinations, or pneumococcal vaccination). Special attention should be given to possible interference with other vaccines that may be mucosally delivered.

4.3 Efficacy

➤ Target population

The target population chosen will depend upon the proposed vaccination indication (see general issues). The choice of the envisaged vaccination indication determines:

1. The study design
2. choice of control: placebo or active control (inactivated influenza vaccine)
3. clinical endpoints: disease incidence as well as reduction of virus spread; or disease incidence as well as reduction of secondary complications and mortality
4. The primary vaccination schedule and the age at primary vaccination
5. The duration of follow up, i.e. annual updates or re-vaccination strategy (see below).

“The clinical program should include sufficient numbers of patients representing the specific risk groups if a clinical indication is sought in any of the existing high risk categories ”

In paediatric studies adequate numbers of patients should be included from the predefined age specific risk categories. The treatment groups should be stratified for these specific risk categories in order to draw sensible conclusions regarding safety and efficacy of the live attenuated influenza virus vaccine in these special populations.

In agreement with the Note for Guidance on clinical evaluation of new vaccines¹⁶ baseline characteristics of the study population should be adequately defined, including a description of pre-vaccination protection status against any of the strains included in the vaccine.

➤ **Study design and choice of control**

The trials using clinical endpoints should be designed as prospective randomised controlled studies. Secondary contact studies may provide adequate supportive evidence for protective efficacy. Uncontrolled studies should be avoided. The community studies should preferably be designed as placebo controlled studies, although an active controlled study might be considered whenever feasible and appropriately justified. Randomisation may be based on individuals or clusters. Pre-exposure studies may be designed as active controlled studies, with an inactivated influenza vaccine of the current year as control. However, placebo controlled studies may be preferred in certain settings (i.e. healthy population studies), since they allow the best estimate of absolute vaccine efficacy. But active controlled studies should be designed also as a superiority trial. The immunological correlate of protection for inactivated influenza vaccines is not a validated comparator for live attenuated influenza vaccines. The immunological responses and correlates of protection for live attenuated influenza vaccine will differ from inactivated influenza vaccines. In addition, the defined seroprotective level of inactivated influenza vaccine is not unequivocally correlated to an established clinical protection rate. Finally, for most of the target groups correlates for protection have not been defined (i.e. risk groups and children).

When strategies of a dual or sequential vaccination regimen using live and conventional inactivated vaccines, which might lead to an enhancement of the immune response to inactivated influenza vaccine, are explored, these are the subject of a separate clinical program. Such studies can serve at most as supportive evidence for efficacy of the live attenuated influenza vaccine.

➤ **Clinical endpoints**

For any influenza vaccine trial, the primary endpoint is defined by the trial objectives and its target population. In most cases the primary endpoint will be prevention of clinical disease (influenza or flu-like disease). However, for specific claims (e.g. reduction in complication risk) or to assess the clinical benefit in special patient populations, the primary clinical endpoint should reflect this, e.g. reduction of secondary complications and/or mortality in elderly and subjects at increased risk of influenza related morbidity and mortality and reduction of virus spread as well as secondary complications (e.g. otitis media, pneumonia) in infants and children and / or healthy adults.

Endpoints relating to a daily lifestyle (absenteeism, use of health care resources, costs) are not valid primary endpoints to establish the efficacy of the vaccine. They may be considered as part of an effectiveness evaluation of the intervention in a certain population.

For annual updates, clinical endpoints cannot yet be defined. Efficacy studies in these cases are dependent upon definition of correlates of protection (see below).

➤ **Primary vaccination schedule and duration of follow up**

For any of the (co-) primary clinical endpoints the study should have a sufficiently long follow-up to allow reliable conclusions with respect to persistence of protection following the primary vaccination and relevance for the target population.

- **Annual update**

Since the CPMP Guideline on Harmonisation of Requirements for Influenza Vaccines¹ is most likely not applicable, available parameters as defined above, if necessary, should be used for the evaluation of annual updates. Since the time scale for the annual updates is very limited, defining correlates for protection is of major importance. These correlates allow for relatively small scale, short term immunogenicity studies. Sample sizes for the annual updates will thus be defined by the correlate(s) for protection and the established safety profile. Safety information for seasonal updates should be relevant for the intended target groups.

- **Heterologous protection and revaccination**

Claims of cross protection against influenza strains not present in the vaccine require long term follow up over at least 2 subsequent seasons following the primary season in which the primary vaccination was given. However, the actual number of seasons required depends upon the characteristics of annual epidemic(s) and the subsequent antigenic drift(s) (or shift). The predefined clinical endpoints should be appropriately documented by follow-up data.

The efficacy of the live attenuated influenza vaccine after repeated administration and usefulness in seronegative vs. seropositive and previous influenza vaccine naïve vs. influenza vaccine experienced individuals needs to be addressed in these comparative clinical trials. The studied populations need to be stratified accordingly. Appropriate in- and exclusion criteria (age category, serological status, influenza vaccination history, co-morbidity, co-medication, etc.) and predefined clinical endpoints should be described in the protocol. The studies should address the strategy for revaccination or interval to booster vaccination.

4.4 Preclinical and clinical safety issues

As a new vaccine, the dossier should include a sufficient number of vaccinees in the target categories for safety evaluation. The assessment of the safety profile should be carried out in accordance with the recommendations described in the Note for Guidance on the clinical evaluation of new vaccines¹⁶. This includes an assessment of interaction with other inhaled or intranasally administered medicinal products if relevant. Safety issues to be closely monitored during clinical trials include:

- a Possible transmission of shed vaccine virus to non-vaccinated individuals. Monitoring should include studies of genetic stability of the vaccine construct with genotypic and phenotypic characterisation (virulence) of all strains isolated in a transmission situation.
- b Clinical signs and symptoms of neurotoxicity.
- c Local adverse effects on the nasal mucosa: the incidence of runny nose, sore throat, sinusitis and bacterial superinfections. In infants and very young children this point might be of particular importance since adverse effects on the nasal mucosa, such as congestion, might lead to breathing difficulties. Special attention should also be paid to subjects with pre-existing respiratory dysfunction (e.g. COPD, BPD).
- d Exacerbations of asthma and a decline of respiratory function in the asthmatic population.
- e Ovalbumin allergy: the live vaccine will contain ovalbumin and/or other egg proteins. When the vaccine is intended for annual re-immunisation, the development of sensitisation and allergic reactions to egg proteins may become a safety problem.

Other issues include:

- Deleterious effect on the nasal mucosa induced by the vaccine viruses, excipients or by superinfecting micro-organisms such as *Haemophilus influenzae* or *Staphylococcus aureus* should be investigated in appropriate animal models such as ferrets.
- In the case of pandemic influenza particular care should be taken when using a live attenuated influenza virus vaccine-containing novel HA and NA of a potential or emerging pandemic influenza virus. In the human population there would be no immunity to this virus. Such a vaccine should only be used upon WHO recommendation to avoid unnecessary transfer of these antigens into circulating wild-type influenza viruses before the pandemic occurs.
- In general, the propensity for the development of reassortants during double infections with wild and vaccine viruses needs to be investigated in animal models. The possibility of emergence of novel influenza subtypes must be investigated.
- Possible harm to non-human hosts should be excluded for the live attenuated master strain and for the annual updates.
- Immunogenicity of the live attenuated master strains as well as the annual updates should be investigated in an appropriate animal model using pre-defined parameters.
- Practical problems of delivery; for example unintended spraying into the eyes should be addressed in preclinical studies using appropriate animal models.
- Titres of shed vaccine virus obtained from both animal models and from the target groups selected for clinical trials should be measured.
- Possible transmission of shed vaccine virus to non-vaccinated individuals should be explored.
- Possible haematogenous spread of the vaccine should be explored.
- Particular attention should be given to the possible risk of transmission of vaccine virus to immunocompromised individuals.
- Long term follow up studies in postmarketing should be committed to in which special attention is paid to the benefits and risks in special populations, such as young children and individuals with co-morbidity at increased risk of influenza primary and secondary morbidity.

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¹⁸ CPMP/BWP/2490/00 Note for Guidance on Cell Culture Inactivated Influenza Vaccines - Annex to Note for Guidance on Harmonisation of requirements for Influenza Vaccines CPMP/BWP/214/96 - (revision released for consultation January 2001).

¹⁹ Note for Guidance on the Quality, Preclinical and Clinical Aspects of Gene Transfer Medicinal Products (CPMP/BWP/3088/99).