



ANNEX 1

**WHO GUIDELINES ON NONCLINICAL EVALUATION OF  
VACCINES**

This document provides guidance to National Regulatory Authorities (NRAs) and vaccine manufacturers on the nonclinical evaluation of vaccines by outlining the international regulatory expectations in this area. It should be read in conjunction with the guidelines on clinical evaluation of vaccines: regulatory expectations, in order to complete the understanding of the whole process of vaccine evaluation (4). Vaccines present a diverse class of biological products and their nonclinical testing programs will depend on product specific features and clinical indication. Therefore, the following text is written in the form of guidelines instead of recommendations. Guidelines allow greater flexibility than Recommendations with respect to specific issues related to particular vaccines.

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## Introduction

Recent progress in biotechnology and basic immunology is leading to the development of a broad range of novel vaccines raising exciting possibilities for the prevention of infectious diseases (1,2). In addition, improvements to already licensed vaccines are also being considered leading to new products as well as the introduction of new adjuvants. However, the complexity and novelty of these products presents scientific and regulatory challenges, as criteria for their safety, potency and quality assessments may not always exist. Because of product diversity and since new approaches, technologies and methodologies develop over time, it is emphasized that judgement based on the best science available should always form the basis for deciding on the type and extent of nonclinical evaluation for these products.

Nonclinical evaluation plays an essential part in the overall development of vaccine candidates. There is at present limited guidance regarding nonclinical evaluation programs for these products. In this guidance document, the general principles of nonclinical evaluation of vaccines are discussed, with particular attention being given to the regulatory expectations for new and novel vaccines.

Preclinical testing is a prerequisite to move a candidate vaccine from the laboratory to the clinic and includes all aspects of testing, product characterization, proof of concept/ immunogenicity studies and safety testing in animals conducted prior to introducing the product into humans. Nonclinical evaluation, within the context of this document, means all *in vivo* and *in vitro* testing performed before and during clinical development of vaccines. For example, nonclinical evaluation may be necessary when changes in the manufacturing process or product formulations are made or to further evaluate potential safety concerns that may have arisen from phase 1 and 2 trials or have been described in the literature with similar products.

### 1. General Remarks

Nonclinical studies are aimed at defining the *in vitro* and *in vivo* characteristics of candidate vaccines including safety and immunogenicity evaluations. Non clinical studies in animals are valuable tools to identify possible risks to the vaccinees and help to plan protocols for subsequent clinical studies in human subjects. However, in all cases, when safety testing in animals is performed, there should be a clear rationale for doing so and the study should be performed in compliance with the National and International laws for the protection of laboratory animals (25), biosafety requirements (27) and with Good Laboratory Practice (GLP) (13). However, there may be situations where full compliance with GLP is not possible. If the study or part of the study were not conducted in compliance with GLP, areas of noncompliance should be defined and a statement of the reason for the noncompliance should be justified and reported.

Potential safety concerns for a vaccine product include those due to inherent toxicities of the product, toxicities of impurities and contaminants, and toxicities due to interactions of the vaccine components present in the vaccine formulation. In addition, the immune response induced by the vaccine may lead to undesired toxic side effects.

Despite efforts to maximize the predictive value of nonclinical toxicity studies there are always limitations to reducing uncertainties of risk. The limitations of animal testing in reflecting clinical safety and efficacy in humans should be recognized since pathogenesis and immune responses are frequently species-specific. Moreover, potential safety concerns identified during animal testing may not necessarily indicate a problem in humans. However, any signal observed in nonclinical toxicity studies should be carefully addressed in human clinical trials and may require additional nonclinical testing. Likewise, a lack of detectable toxicity does not necessarily mean a vaccine will be safe in humans. Potential safety concerns related to specific types of vaccine candidates are considered in section 6.

The development and subsequent validation of *in vitro* tests as alternatives to nonclinical evaluation of vaccine candidates in animals is encouraged as it may lead to the improvement of nonclinical testing as well as to the reduction of animal usage.

The need and extent of nonclinical testing will depend on the product under consideration. For example, for a product for which there is no prior nonclinical and clinical experience, nonclinical testing would be expected to be more extensive than for those vaccines previously licensed and used in humans. In some cases, it may not be necessary to perform preclinical safety studies prior to the initiation of Phase 1 clinical trials. For example, in the case of transfer of technology, where the access to database of the originally developed vaccine exists, data from nonclinical bridging studies (e.g., physico-chemical characterization and abbreviated *in vivo* studies) may be acceptable to further develop the product.

Early communications between the vaccine manufacturer and the respective National Regulatory Authority to agree on the requirement and type of nonclinical testing are recommended.

## 1.1 Scope

For the purpose of this document, vaccines are considered a heterogeneous class of medicinal products containing immunogenic substances capable of inducing specific, active and protective host immunity against infectious disease.

While the majority of vaccines are being developed for pre- and post- exposure prophylaxis, in some cases, they may be indicated for therapeutic use against infectious diseases, e.g., HIV, HPV etc. Both prophylactic and therapeutic vaccines for infectious disease indications are considered in this document.

Vaccines for human use include one or more of the following: micro-organisms inactivated by chemical and/or physical means that retain appropriate immunogenic properties; living micro-organisms that have been selected for their attenuation whilst retaining immunogenic properties; antigens extracted from micro-organisms, secreted by them or produced by recombinant DNA technology; chimeric micro-organisms; antigens produced *in vivo* in the vaccinated host following administration of a live vector or nucleic acid or antigens produced by chemical synthesis *in vitro*. The antigens may be in their native state, truncated or modified following introduction of mutations, detoxified by chemical or physical means and/ or aggregated, polymerised or conjugated to a carrier to increase immunogenicity. Antigens may be presented

plain or in conjunction to an adjuvant, or in combination with other antigens, additives and other excipients.

Therapeutic vaccines for non infectious diseases (e.g., certain cancer vaccines) and monoclonal antibodies used as immunogens (e.g., anti-idiotypic antibodies) are NOT considered here.

## **2. Characterization of candidate vaccines**

### **2.1 Vaccine production**

The biological nature of the starting materials, the manufacturing process and the test methods needed to characterize batches of the product are important elements for the design and the interpretation of nonclinical testing of vaccines. Many vaccines are produced using prokaryotic or eukaryotic micro-organisms and subtle changes in these organisms may radically affect the vaccine product. Therefore, the establishment of a seed lot system is essential for vaccine production. Moreover, the quality, safety and potency of these products are usually sensitive to changes in manufacturing conditions. The quality and safety of vaccine preparations cannot be assured solely by end product testing, but depends on the strict control of the manufacturing process following principles of Good Manufacturing Practice (12). This includes demonstration of the purity and quality of the starting material (raw materials and seeds), in process control testing, testing for process additives and process intermediates and the development and establishment of lot release tests. Moreover, as the relationship between physical and chemical characteristics, and the immunogenicity and efficacy of these products is frequently not completely understood, biological characterization through the use of biological assays should always complement the physical and chemical product characterization. The development of appropriate laboratory methods to characterize a vaccine formulation with respect to its components, as well as its safety and potency, is a prerequisite to the clinical use of any new or novel bacterial, viral, or parasite vaccines.

Consistency of production is of paramount importance, and the demonstration that the product does not differ from vaccine lots that have been shown to be safe and adequately immunogenic and protective in clinical studies is a crucial component of vaccine evaluation, licensing and batch release. For that reason, manufacturers should make all effort to characterize these clinical lots and try to keep some of these lots for future reference if possible.

Where no appropriate animal model exists for testing potency or where direct serological or immunological correlates of clinical protection are not available, the challenge is to ensure that each production batch has the same protective efficacy as those batches shown to be protective in clinical trials. In such cases, emphasis is increasingly being placed on assuring the consistency of production using modern physical, chemical and immunological methods that enable characterization of some products to a degree of precision not previously possible.

The vaccine lots used in preclinical studies should be adequately representative of the formulation intended for the clinical investigation and, ideally, preclinical testing should be done on the same lot as proposed for the clinical trials. If this is not

feasible, then these lots should be comparable with respect to physico-chemical data, stability, formulation etc.

At a minimum, candidate vaccines for clinical trials should be prepared under conditions of Good Manufacturing Practice for clinical trial material (20). However full GMP will be required at the later stages of clinical development (12,19).

Any change in the manufacturing process during vaccine development should be considered carefully to evaluate the impact on the quality, safety and efficacy of the vaccine and the possible need for additional nonclinical and clinical investigations.

Subsequent change in production methods or scale-up following product licensure will necessitate further product characterisation to demonstrate comparability with the original lot(s) used to demonstrate safety and efficacy of the product. The extent of comparability testing needed depends on the nature of the changes implemented (37). These should be documented and the National Regulatory Authority consulted regarding all changes. Regulatory authorities should clearly define and implement into regulations what changes require only a notification and what changes require a formal approval before implementation (28).

The procedures used in the characterization and control of existing licensed traditional vaccines are likely not applicable to newer products developed using state of the art technology to protect against the same infection. For example, specific guidelines have been developed for the production and control of acellular pertussis vaccines that differ from those applied to whole cell pertussis vaccine (5). Likewise, the tests applied to the characterization and control of traditional inactivated cholera vaccine for parenteral use are not necessarily applicable to the new inactivated whole cell cholera vaccine intended for oral administration, and an appropriate potency test for the oral vaccine needs to be developed.

## 2.2 Potency

Potency tests measure biological activity of a vaccine but do not necessarily reflect the mechanism of protection in humans. Potency measurement is often used to verify the consistency of the manufacturing process. The initial concept of potency testing for vaccines was to quantify the biological activity of the vaccine in comparison with reference preparation of known bioactivity, where the antigenic component(s) were not well defined.

Classical challenge studies in animals immunized with a vaccine under consideration have been developed into routine potency assays (e.g., for Diphtheria and Tetanus toxoids). In the case of the whole cell pertussis potency assay, which consists of intracerebral challenge of immunized and non-immunized animals, a correlation was established with clinical protection in humans (5). Where no suitable animal challenge model exists, potency is often based on measurement of immune responses, usually serological (e.g., influenza and Hepatitis B vaccines).

More recently, recombinant DNA methodology and modern physico-chemical techniques have resulted in the manufacture of highly purified products that can be

better characterized than classic biologicals. However, the ability to measure the “relevant” biological activity for such products may still be lacking. For these products, physico-chemical characterization, such as amount of antigen, size of the antigen, protein content or other physico-chemical parameters can be used as a measure of consistency, but not necessarily of the potency of a vaccine.

For live attenuated vaccines, the approach to potency measurement is generally different. The potency of live viral vaccines is usually based on titration of the minimum infective dose in cell culture or chicken embryos, which in fact, may be considered as a surrogate marker of potency, but not potency itself. A similar approach is taken to the potency measurement of live attenuated bacterial vaccines, BCG, and typhoid vaccine (live Ty21A oral), where the number of live organisms present is the measure of potency.

For vectored vaccines that express inserts encoding heterologous vaccine antigens, it is not sufficient to determine the “biological activity” of the entire construct by measuring Colony Forming Units (CFU) or infectious titre. In these cases, considerations should be given to other alternative methods such as the quantitation of the expression of the insert, or the evaluation of the effective dose (ED<sub>50</sub>) of the vectored vaccine.

### **2.3 Stability**

The stability evaluation of vaccines is complex, as they are very susceptible to inactivation by environmental factors. Potency as defined in the glossary, should be measured as a part of the stability testing, except in those cases where potency testing based on biological activity is not available. Physical and chemical product characterization should be included in the stability evaluation. For a product entering human clinical trials, sufficient data should be generated to support the stability of the product for the duration of the preclinical and clinical trial. In certain cases, accelerated stability data may be used to support preliminary data generated at the normal storage temperature. Stability data to support licensure should be carried out under the proposed storage conditions and should be based on long-term, real time stability studies. Finally, the stability of standards and reference materials also need to be considered in order to ensure that procedures used to measure relevant parameters are reliably standardized.

### **2.4 International and National guidelines**

The World Health Organization (WHO), through considerable international consultation, develops Recommendations and Guidelines on the production and control of vaccines and other biologicals of significance (6), and these form the basis for assuring the acceptability of products globally. These documents specify the need for appropriate starting materials, including seed lot system and cell banks; strict adherence to established protocols; tests for purity, potency, and safety at specific steps during production; and the keeping of proper records. Guidelines allow greater flexibility than Recommendations with respect to specific issues related to particular vaccines.

WHO also provides guidance on the establishments in which vaccines are manufactured. Recommendations can be found in the WHO document on good manufacturing practice for biologicals (12). Particular attention should be given to developing documented standard operating procedures for both production processes and testing procedures. These should be introduced as early as possible during the development of a vaccine and be well established by the time Phase III clinical studies are undertaken and an application for marketing authorization is filed. The basic principles for the production and control of vaccines can be found in WHO Technical Report Series (TRS) (14,3,15,16,17). Specific WHO guidelines and recommendations for particular vaccines are also available (6) and should be consulted where appropriate.

WHO Recommendations and Guidelines are intended to be scientific and advisory in nature and to provide guidance for national regulatory authorities and for vaccine manufacturers. These documents may be adopted by national health authorities as definitive national regulations or used as the basis of such regulations. They are also used as the basis for deciding the acceptability of vaccines for purchase by United Nations agencies such as the United Nations Children's Fund (UNICEF) for use in global immunization programmes. Regulatory requirements for vaccines and other biologicals are also developed by other bodies, such as the European Agency for the Evaluation of Medicinal Products (EMA) and the Center for Biologics Evaluation and Research (CBER), Food and Drug Administration (FDA) (18) and these documents can be found on the appropriate web sites ([www.emea.eu.int](http://www.emea.eu.int) and [www.fda.gov/cber](http://www.fda.gov/cber)). In addition, pharmacopoeial requirements, such as those of the European Pharmacopoeia, are also established for vaccines and are available at the following web site [www.phEur.org](http://www.phEur.org).

For newly developed products, specific WHO, national, or pharmacopoeial requirements may not be available and a national regulatory authority will need to agree on specifications with the manufacturer on a case-by-case basis during the evaluation of products for clinical trials and for licensing. For some of these novel products general Guidance from WHO for production and control can be found in relevant documents, such as those describing DNA and peptide vaccines (15,17), as well as Recommendations on animal cell substrates used for production (14).

In addition, information on how to assure the quality of biologicals in general and on procedures for approving manufacture and for setting up a national control laboratory, can be found in appropriate WHO guidelines (9,10). For vaccines for global marketing, the development of which also involves much international collaboration, it will be essential to ensure consistency of a regulatory approach for novel products such as HIV preventive vaccines (11).

## **2.5 Batch release and Independent Laboratory evaluation**

The potential variability of biological production methods has led to the establishment of national and international requirements to define procedures for assuring the quality of vaccines and for assessing consistency both amongst manufacturers and over long time periods. Licensed vaccines are subject to independent batch release by a National Regulatory Authority/ National Control Laboratory, before release onto the market. Independent evaluation entails at least an

evaluation of manufacturer's batch release data (protocol review) but in many instances it also includes independent laboratory testing in addition to that carried out by the manufacturer.

Batch or lot release tests are those tests chosen during full product characterization to demonstrate the purity, safety and potency of the product. Lot release testing provides one measure of assurance that a lot can be manufactured consistently. Validation and establishment of lot release tests and specifications is a process that continues throughout product development and should be finalized prior to licensure.

In some countries, samples of vaccine for clinical trials are required by the National Regulatory Authority, as a part of clinical trial approval. Vaccine developers are encouraged to consult the appropriate regulatory agency early during the development of a vaccine.

## **2.6 Standards and Reference Materials**

Standards and reference materials play a vital part in the licensing and quality control process, their role ranging from use in specific antigen recognition tests to assays of vaccine toxicity, immunogenicity, and potency. The standardization of the methods used to evaluate vaccines, as well as to evaluate immune responses to vaccine antigens, is also vital so that results may be compared directly between laboratories both within and between countries and between clinical trials.

WHO International Biological Standards and Reference Reagents form the primary standards globally. In addition, individual national regulatory authorities and manufacturers establish their own national or working standards for establishing the quality of each batch, where appropriate, calibrated against the International Standard. Where appropriate the WHO International Standard is referenced. Of concern is that multiple standard preparations, may result in “drifting” from the International Standard. Therefore regional working standards are being produced on large scale in an attempt to further harmonize vaccine quality. For example, the European Department for the Quality of Medicines of the Council of Europe, has been active in establishing working standards for vaccines that are calibrated against the WHO international standards, where appropriate. The complete listing of WHO International Standards and Reference Reagents can be found on the WHO web site [www.who.int/biologicals](http://www.who.int/biologicals).

## **3. Immunogenicity and other pharmacodynamic studies**

A pharmacodynamic study for a vaccine product essentially means evaluation of the immunogenicity. However, pharmacodynamic study may also extend to actual drug pharmacology of an adjuvant.

Immunization studies in animal models should be conducted since they may provide valuable “proof of concept” information to support a clinical development plan. In addition, immunogenicity data derived from appropriate animal models are useful in establishing the immunological characteristics of the product and may be of help to select the doses, schedules and routes of administration to be evaluated in

clinical trials. Nonclinical immunogenicity studies should assess the relevant immune response, e.g., humoral and/or cell mediated immune response, induced in the vaccinated animals. Depending on the immune response induced, such studies may include an evaluation of seroconversion rates, geometric mean antibody titres, or cell-mediated immunity in vaccinated animals. Nonclinical studies should, where possible, be designed to assess relevant immune responses, including functional immune response (e.g., neutralizing antibodies, opsonophagocytic activity, etc.) leading to protection. These studies may also be designed to address interference between antigens and/or live viruses. If a vaccine consists of more than one defined antigen, the response to each antigen should be evaluated (e.g. acellular pertussis vaccine consisting of 3-5 protein products). Where appropriate, challenge/protection studies with the corresponding infectious agent may be conducted to confirm the relevance of the animal models. Of primary concern in interpreting the data obtained from such studies should be how closely the animal model resembles the human disease and human immune response. It should be recognized that frequently, animal models may not predict immunogenicity and efficacy in humans.

#### **4. Toxicity assessment**

##### **4.1. Basic toxicity assessment**

The non-clinical safety assessment of vaccines needs to be viewed in the context of the evolving field of vaccine development. Thus, judgement based on the best science available should always form the basis for any decisions regarding the need for nonclinical safety studies, type of study(ies) as well as study designs. Similarly, scientific judgement should be applied to the interpretation of pre-clinical data, whereby the risk/benefit ratio, animal model, dosing etc. should be considered. For example, hypersensitivity reactions in an animal model may not necessarily prevent proceeding to clinical trials, but may indicate the necessity for careful monitoring of a particular clinical parameter.

- Section 4.1 provides a general framework for designing a pre-clinical toxicity study for a vaccine. The parameters set out in this section are considered a minimum safety assessment prior to the initiation of clinical trials in humans, in situations where preclinical safety studies are deemed necessary. As the design of any toxicity study is product specific and indications based, modifications to the framework outlined below may be necessary depending on product features, availability of animal models, methodologies, etc.
- Section 4.2 provides additional considerations for performing special toxicity assessments that may be required on a case-by-case basis.

##### **4.1.1 Study design**

The preclinical toxicity study should be adequate to identify and characterize potential toxic effects of a vaccine in order to conclude that it is reasonably safe to proceed to clinical investigation. Parameters to be considered in designing animal toxicology studies are the relevant animal species/strain, dosing schedule and method of vaccine administration, as well as timing of evaluation of endpoints (e.g., sampling for clinical chemistry, antibody evaluation, necropsy, etc.). The route of

administration should correspond to that intended for use in the clinical trials. When the vaccine is to be administered in human clinical trials using a particular device, the same device should be used in the animal study, where feasible (e.g., measles aerosol vaccine in the monkey model). Potential toxic effects of the product should be evaluated with regard to target organs, dose, route(s) of exposure, duration and frequency of exposure, and potential reversibility of observed toxic effects. The toxicity assessment of the vaccine formulation can be done either in a) dedicated-stand alone toxicity studies or b) combination safety/activity studies with toxicity endpoints incorporated into the design of the study. This should also include an assessment of local tolerance.

#### **4.1.2 Animal species, sex, age, size of groups**

Data regarding animals used for toxicity testing should include information on the source, species and animal husbandry procedures (e.g. housing, feeding, handling and care of animals). In general, the use of outbred animals is recommended. The health status of the animal will need to be evaluated in accordance with acceptable veterinary medical practice to assure that animals are free of any condition that might interfere with the conduct of the study. For instance, single housing of laboratory animals can be required to minimize the risk of cross-infection.

Where possible, the safety profile of a product should be characterized in a species sensitive to the biological effects of the vaccine. Ideally, the species should be sensitive to the pathogenic organism or toxin. The animal species used should develop an immune response to the vaccine antigen. In general, one relevant animal species is sufficient for use in toxicity studies to support initiation of clinical trials. However, there may be situations where two or more species may be necessary to characterize the product, for example where the mechanism of protection induced by the vaccine is not well understood (for example, intranasal influenza vaccine and intranasal measles vaccine).

In addition, when species-specific or strain-specific differences with regard to the pharmacodynamic of the product are observed, it may be necessary to address the non-clinical safety of the product in more than one safety study and in more than one animal model.

The size of the treatment group depends on the animal model chosen, i.e., the number of animals included in studies using non-human primates would be expected to be less than in studies including rodents. For small animal models, e.g., rats and mice, it is recommended that approximately 10 animals/sex/group be studied.

In general, the approximate age for rodents is six to eight weeks, and for rabbits, 3 to 4 months, at the start of the study.

#### **4.1.3 Dose, route of administration, controls**

The toxicity study should be performed with a dose maximizing exposure of the animal to the candidate vaccine and the immune response induced, such as peak antibody response. In general, dose response evaluation is not required, as part of the

basic toxicity assessment and the lethal dose does not have to be determined. However, pilot dose response studies may be conducted to determine which dose induces the highest antibody production in animal model. If feasible, the highest dose (in absolute terms) to be used in the proposed clinical trial should be evaluated in the animal model. However, sometimes the dose is limited by the total volume that can be administered in a single injection and therefore guidelines for animal welfare should be followed. In such cases, the total volume may be administered at more than one sites using the same route of administration. Alternatively, a dose that exceeds the human dose on mg/kg bases and that induces an immune response in the animal model may be used. In such cases, the factor between human and animal dose should be justified.

The number of doses administered in the animal model should be equal to or exceed the number of doses proposed in humans. To better simulate the proposed clinical usage, vaccine doses should be given as episodic doses, rather than daily doses, the dosing interval used in the toxicity study may be reduced (e.g., 2 to 3 weeks interval) compared to the proposed clinical dosing interval. The nonclinical dosing interval may be based on the kinetics of the primary and secondary antibody response observed in the animal model. A single dose study may be performed in situations where vaccine induced antibodies are expected to neutralize a live viral vector, thus limiting the expression of the gene of interest (e.g. anti-adenovirus immune response), or when immune responses induced in animals are expected to react with species-specific proteins present in the vaccine formulation (e.g., human recombinant cytokines used as adjuvants).

The route of administration should correspond to that intended for use in the clinic. If toxic effects are observed in safety studies using a particular route of administration (e.g., intranasal), separate toxicity studies using a different route of administration (e.g., intravenous) may be helpful in understanding the full spectrum of toxicity of the product.

The study design should include a negative control group(s) to evaluate a baseline level of treatment. If appropriate, active control groups (e.g., vaccine formulation without antigen) may be added. The study should include an additional treatment group to be sacrificed and evaluated as described below at later time points after treatment, to evaluate reversibility of adverse effects observed during the treatment period and to screen for the potential delayed adverse effects.

#### **4.1.4 Parameters monitored**

Toxicity studies should address the potential for local inflammatory reactions, including effects on the draining lymph nodes, systemic toxicity, and effects on the immune system. A broad spectrum of information should be obtained from the toxicity studies. In-life parameters to be monitored should include daily clinical observations, weekly body weights and weekly food consumption. During the first week of administration it is recommended to do frequent measurement of body weight and food consumption, if feasible, as these are sensitive parameters indicating “illness”. Interim analysis of haematology and serum chemistries should be considered within approximately 1-3 days following the first and last dose administration and at the end of the recovery period. Haematology and serum

chemistry analyses include at the minimum, an evaluation of relative and absolute differential white blood cell counts (lymphocytes, monocytes, granulocytes, abnormal cells) and albumin/globulin ratio, enzymes, electrolytes, respectively. In some cases, it may be of value to also evaluate coagulation parameters, urine samples, serum immunoglobulin classes etc. Data should be collected not only during treatment, but also following the treatment free phase (e.g., 2 weeks or more following the last dose) to determine persistence, exacerbation and/or reversibility of potential adverse effects.

At study termination, final body weights (fasted) should be obtained. Terminal blood samples should be collected and serum chemistry, hematology and immunological investigations should be done as described above, under 4.1.4 (parameters monitored). Immune response induced by the vaccine candidate should be evaluated in order to confirm the animal exposure that is also a confirmation of the choice of the relevant animal model. A complete gross necropsy and tissue collection and preservation including gross lesions and organ weights, should be conducted (annex 1, 32). Histopathological evaluations on tissues should be performed whereby special attention should be paid to the immune organs, i.e. lymph nodes (local and distant to application site), thymus, spleen, bone marrow and Peyer's patches or bronchus-associated lymphoid tissue, as well as organs that may be primarily affected due to the particular route of administration. Histopathological examinations should always include pivotal organs (brain, kidneys, liver, reproductive organs) and the site of vaccine administration. The extent of the list of tissues to be examined (i.e., from a reduced list limited to immune and pivotal organs to full list as provided in annex 1) will depend of the vaccine in question, and the knowledge and experience obtained by previous nonclinical and clinical testing of the vaccine components. For example, full tissue examination will be required in the case of novel vaccines with no prior nonclinical and clinical experience. Therefore, the list of tissues to be tested should be defined on a case by case basis, following consultation with the relevant Regulatory Authority. Data should be reported in full as the original collection of values, and summarized.

#### **4.1.5. Local tolerance**

Local tolerance evaluation should be conducted either as a part of the repeated dose toxicity study or as a stand-alone study. Tolerance should be determined at those sites, which come into contact with the vaccine antigen as a result of the method of administration, and also at those sites inadvertently exposed (eye exposure) to the vaccine. More details have been published elsewhere (24).

If abnormalities are observed in the basic toxicity study outlined in section 4.1., further studies may be necessary in order to evaluate the mechanism of the toxic effect.

### **4.2. Additional toxicity assessments**

#### **4.2.1. Special immunologic investigations**

In certain cases results from immune response evaluations derived from nonclinical and clinical studies, or from natural disease data, may indicate immunological aspects of toxicity, e.g., precipitation of immune complexes, humoral

or cell-mediated immune response against antigenic determinants of the host itself as a consequence of molecular mimicry (Verdier 2002; Wraith, Goldman & Lambert, 2003) or exacerbation of the disease (e.g., inactivated measles vaccine). In such cases, additional studies to investigate the mechanism of the effect observed might be necessary.

Great similarity of vaccine determinants and host molecules could cause autoimmune reactions induced by molecular mimicry (26). Therefore, any vaccine antigen which might present mimicry with a host antigen should be considered with caution, even though it is recognized that molecular mimicry does not necessarily predispose to auto-immunity.

Since considerable efforts may need to be undertaken in selecting/developing relevant animal models to address the above issues, caution should be exercised and a strong rationale provided when developing vaccines for diseases associated with autoimmune pathology.

If data suggest that the pathogen against which the vaccine is directed may cause autoimmune pathology, studies may need to be conducted to address this concern on a case-by-case basis, if an appropriate animal model exist.

It should be noted that observations of biological markers for autoimmune reactions are not necessarily linked to pathogenic consequences. For instance, the presence of autoimmune antibodies does not necessarily indicate the induction of autoimmune disease (36).

When hypersensitivity reactions induced by the antigen(s), adjuvants, excipients and preservatives are of concern, additional investigations may be warranted.

#### **4.2.2. Developmental toxicity studies**

Developmental toxicity studies are usually not necessary for vaccines indicated for immunization during childhood. However, if the target population of the vaccine includes pregnant women and women of childbearing potential, developmental toxicity studies should be considered, unless a scientific and clinically sound argument is made by the manufacturer that conducting such studies is not necessary. For a preventive vaccine, reproductive toxicity assessments are generally restricted to pre- and postnatal developmental studies, since the primary concern is any potential untoward effect on the developing embryo/foetus/newborn. The need to conduct fertility and post-weaning assessments would need to be considered on a case-by-case basis. The animal model chosen should develop an immune response to the vaccine, which is usually determined by serum antibody measurements. In addition, it is important to evaluate maternal antibody transfer by measuring vaccine induced antibody in cord or foetal blood in order to verify exposure of the embryo/foetus to maternal antibody. The route of administration should mimic the clinical route of administration. Ideally, the maximal human dose should be administered. If it is not possible to administer the full human dose, e.g., due to limitations in total volume administration or if local toxicity is observed that may result in maternal stress, a dose that exceeds the human dose on a mg/kg bases and able to induce an immune response in the animal should be used.

To assess any potential adverse effects of the vaccine during the period of organogenesis, the pregnant animal is usually exposed to the vaccine during the period from implantation through closure of the hard palate and end of pregnancy defined as stages C, D and E in the ICH S5a document (29). Because of the relative short gestation period of most animal models used, pre-mating treatment is frequently required in order to assure maximal exposure of the embryo/foetus to the vaccine induced immune response. For a preventive vaccine, the number of doses administered depends on the response onset and duration of the response. Booster immunizations at certain times during the period of gestation may be necessary to maintain high level of antibody throughout the gestation period and to expose the developing embryo to the actual components of the vaccine formulation. Endpoints include, but are not limited to, viability, resorptions, abortions, foetal body weight, and morphology. The reader is referred to the other publications for guidance on endpoints used to evaluate potential toxic effects of the product on embryo/foetal development (29). It is also recommended that post natal follow up of pups from birth to weaning be incorporated in the study design to assess normal growth, body weight gain, nursing activity and viability. Therefore, studies should be designed to divide test groups into subgroups whereby half of the animals are subjected to C-sectioning and the other half is allowed to deliver their pups.

#### **4.2.3. Genotoxicity and carcinogenicity studies**

Genotoxicity studies are normally not needed for the final vaccine formulation. However, they may be required for particular vaccine components such as novel adjuvants and additives. If needed, the *in vitro* tests for the evaluation of mutations and chromosomal damage should be done prior to first human exposure. The full battery of tests for genotoxicity may be performed in parallel with clinical trials (30).

Carcinogenicity studies are not required for vaccine antigens. However, they may be required for particular vaccine components such as novel adjuvants and additives.

#### **4.2.4. Safety pharmacology**

The purpose of safety pharmacology is to investigate the effects of the candidate vaccine on vital functions. If data from nonclinical and/or human clinical studies suggest that the vaccine (e.g. one based on specific toxoids) may affect physiological functions (CNS, respiratory, cardiovascular, renal functions) other than the immune system, safety pharmacology studies should be incorporated into the toxicity assessment. Useful information on this topic can be found in the Note for Guidance on safety pharmacology studies for human pharmaceuticals (31).

#### **4.2.6 Pharmacokinetic studies**

Pharmacokinetic studies (e.g. determining serum or tissue concentrations of vaccine components) are normally not needed. The need for specific studies should be considered on a case by case basis (e.g. novel adjuvants or alternative routes of administration) and may include local deposition studies which would assess the retention of the vaccine component at the site of injection and its further distribution

(e.g. to the draining lymph nodes). Distribution studies should be considered in case of new formulations, novel adjuvants or when alternative routes of administration are intended to be used (e.g., oral or intranasal).

## 5. Special Considerations

### 5.1 Adjuvants

Adjuvants may be included in vaccine formulations or co-administered with vaccines to enhance the immune responses to particular antigen(s), or to target a particular immune response. It is important that the adjuvants used comply with pharmacopoeial requirements where they exist, and that they do not cause unacceptable toxicity.

Adjuvant activity is a result of multiple factors and the immune response obtained with one particular antigen/adjuvant formulation cannot be, as a rule, extrapolated to another antigen. Individual antigens vary in their physical and biological properties and antigens may differ with regard to help from an adjuvant. Adjuvants must be chosen based on what type of immune response is desired and adjuvants must be formulated with the antigen in such a way that distribution of both is optimised to ensure presentation to the relevant lymphatic tissues. The vaccine administration route is also an important factor influencing the efficacy and safety of an adjuvant.

The effect of the adjuvant should be demonstrated in preclinical immunogenicity studies. If no toxicological data exist for a new adjuvant, toxicity studies of the adjuvant alone should first be performed. In general, assessment of new or novel adjuvants should be undertaken as required for new chemical entity (33,34,35). These data may be generated by the vaccine manufacturer or by the producer of the adjuvants. In addition to assessing the safety of the adjuvant by itself it is also important to assess whether the antigen/ adjuvant combination exerts a synergistic adverse effect in the animal model compared to the individual components (21, 22). In cases where species-specific proteins are used as novel adjuvants (e.g., cytokines), the issue of species specific response should be considered.

When evaluating the safety profile of the adjuvant/ vaccine combination, the formulation proposed to be used clinically, should be used.

Compatibility of the adjuvant(s) with all antigenic components present in the vaccine should be evaluated (e.g., lack of immune interference).

If applicable, adsorption of all antigenic components present in the vaccine should be shown to be consistent on a lot to lot basis. Potential desorption of antigen during the shelf life of the product should be performed as a part of stability studies, reported and specifications set, as this may affect not only immunogenicity but also the toxicity profile of the product.

It should be noted that no adjuvant is licensed in its own right but only as a component of a particular vaccine.

## **5.2. Additives (Excipients and preservatives)**

Where a new additive is to be used, for which no toxicological data exist, toxicity studies of the additive alone should first be performed and documented according to the guidelines for new chemical entities (34). The compatibility of a new additive with all vaccine antigens should be documented as well as the toxicological profile of the particular final vaccine formulation in animal models as outlined in section 4.

## **5.3 Vaccine formulation and delivery device**

The vaccine formulation, e.g., liquid form, capsules or powder, as well as the delivery device, are factors that may have an impact on the uptake of the vaccine, its effectiveness and safety. Ideally, the delivery device and vaccine formulation tested in an animal safety study should be identical to the one intended to be used clinically. However, animal models in which clinically intended delivery devices can be used may not be available. In these instances, in order to develop an appropriate animal model, it may be necessary to conduct pilot studies to define and optimize the conditions for drug delivery in the animal model so that it can be used to assess the preclinical safety of the product.

## **5.4. Alternate routes of administration**

When using vaccine formulations given by alternate routes of administration (e.g., intranasal, oral, intradermal, rectal and intravaginal routes), it can be assumed that their potency, relevant immunogenicity, tolerability, toxicity, and long-term safety may be different to products delivered by the parenteral route. Thus, when different routes of administration are proposed, non-clinical safety studies may have to be conducted using vaccine formulation and/ or adjuvant alone in a suitable animal model addressing specific safety concerns associated with vaccine administration via these routes. The following will discuss issues for vaccines administered using alternate routes of administrations that may need to be considered in addition to those described elsewhere in this document.

### **5.4.1. Animal models**

Of special consideration for vaccines administered via alternate routes should be the anatomy and physiology of the site of vaccine administration of the particular animal model chosen and its accessibility to the test article administration. For example, for intranasal administered products, the species chosen should ideally be receptive to spray administration of the product. In general, rabbits and dogs are useful test models for use of spray devices, however their olfactory bulbs are highly protected and special techniques would be required to ensure that the test article reaches this organ. Mice and rats are useful models for intranasal administration studies, however administration of the test article is limited to droplets. Nonhuman primates may also be considered, especially if they are susceptible to infection by the micro-organism in question, after nasal administration (e.g., intranasal measles vaccine).

Depending on the level of concern regarding a particular route of administration

or when species specific differences with regard to the sensitivity of the animal to the vaccine candidate exist, it may be necessary to address the preclinical safety of the product in more than one safety study and in more than one animal model.

#### **5.4.2. Dose**

As the optimal dose derived from studies using the parental route of administration may differ from the dose used for alternate route(s) of administration, dose finding studies may need to be conducted for a particular route of administration. Also, considerations should be given to the total volume of the administered test article as it may affect the outcome of the safety study. For example, intranasal administration of more than 5  $\mu$ l of test volume per nostril to a mouse would result in the test article being swallowed, rather than being adsorbed by the nasal mucosa.

#### **5.4.3. Endpoints**

The toxicity endpoints would include those described in section 4 of this document and may include additional outcome measures that will depend on the route of administration, specific concerns associated with the particular route and target organ. For example, if concerns exist for the potential passage of vaccine components to the brain following intranasal administration, immunohistology and “in situ” methods and/or neurologic assays and examinations may need to be performed. For vaccines administered by inhalation, outcome measures may include pulmonary function tests and data on histopathology of the lungs. Considerable efforts may need to be undertaken to develop appropriate methods to address potential safety concerns associated with the use of new routes of administration.

#### **5.4.4. Immunogenicity assessment**

The development of appropriate assays measuring mucosal immune responses is critical for vaccines that are expected to function as mucosal immunogens because serologic assays alone may not reflect the relevant immune response for a mucosal vaccine. Thus, in addition to measuring serological responses, it may be necessary to evaluate T cell responses, antibody secreting cells and cytokine production. In addition, assays may need to be developed to assess the induction of local and systemic responses at sites distant from administration of the vaccine antigen.

## 6. Specific considerations for particular types of vaccines

In addition to the testing strategies outlined in sections 3, 4 and 5, studies may be necessary to address specific safety concerns associated with particular product types using adequate in vitro and in vivo test methods. The following will discuss specific testing requirements for live attenuated and combination vaccines. For other product categories, detailed information regarding the production and control of vaccines is available in the WHO guidance documents for production and control (6), and should be consulted. For example, in recently developed guidelines for DNA (15) and synthetic peptide vaccines (17), as well as for particular vaccines such as Hib conjugated vaccine (38), the issues relevant for nonclinical testing are discussed and should be considered in the development of an appropriate design for the nonclinical study of the vaccine in question.

### 6.1 Live attenuated vaccines

An assessment of the degree of attenuation, and the stability of the attenuation phenotype, is an important consideration for the nonclinical testing programme of a live attenuated vaccine. Laboratory markers of attenuation are invaluable for this purpose. These markers should be capable of distinguishing the attenuated vaccine from fully virulent wild type strains and, ideally, of detecting partial reversion to full virulence. To assess the stability of the attenuation phenotype, the vaccine may be passaged under production conditions beyond the maximum passage number to be used for production. Stability of attenuation may also be assessed by passage under conditions that are outside the conditions to be used for vaccine production. For example, higher or lower temperature may provide a selection pressure for reversion to virulence. The marker(s) of attenuation may be subsequently used to qualify new vaccine seed preparations and to monitor the effect of any significant changes in production conditions of the attenuated phenotype.

If the wild type organism is neurotropic, or if passages through neural tissue have been used in the attenuation of a virus vaccine, then a test for neurovirulence should be performed at least at the level of the vaccine seed. A neurovirulence test is not necessarily required for all live attenuated vaccines. Specifications for an appropriate neurovirulence test depend on the organism under test and should be capable of distinguishing the attenuated vaccine from fully virulent wild type strains and, ideally, of detecting partial reversion to full virulence. Specific reference preparations may be needed for this purpose. Neurovirulence tests in small animal models may be acceptable.

If the live attenuated vaccine is based on a genetically modified organism, then an environmental risk assessment may be required as part of the pre-clinical evaluation. An investigation into the possible shedding of vaccine organisms following administration contributes to the environmental risk assessment. For all live attenuated vaccines, information on the likelihood of exchange of genetic information with non-vaccine strains may be required and suitable nonclinical tests may be designed to provide data for this purpose.

## 6.2. Combined vaccines

New combinations produced either by formulation or at the time of reconstitution of antigens or serotypes should be studied for appropriate immunogenicity in an animal model, if available, before initiation of human clinical trials (7,8). Combined antigens should be examined by appropriate physico-chemical means to evaluate possible changes to antigen properties on combination, such as degree of adsorption to aluminium adjuvants, as well as a stability of the combination.

The immune response to each of the antigens in the vaccine should be assessed, including the quality of response, the potential interference and incompatibilities between combined antigens. It is preferable to study a new combination in comparison with the individual antigens in animals to determine whether augmentation or diminution of response occurs.

The safety of the new combination should be evaluated in an animal model on a case by case basis and especially if there is a concern that combining antigens and/or adjuvants may lead to toxicity problems (e.g., novel adjuvant).

Similar consideration for nonclinical testing will also apply to cases where a new candidate monocomponent vaccine is developed from an already licensed combined vaccine (e.g., monovalent OPV vs. trivalent OPV).

### Authors

The first draft of these Guidelines on preclinical evaluation of vaccines was prepared by the WHO drafting group following its meeting held at RIVM, 14-15 March 2002: Dr M. Gruber, Scientific Reviewer, Division of Vaccines and Related Products Application, Center for Biologics Evaluation and Research, Food & Drug Administration, Rockville, MD, USA;

Dr A. Homma, Bio-Manguinhos Oswaldo Cruz Foundation, Rio de Janeiro, Brazil;

Dr J.G. Kreeftenberg, Bureau for International Cooperation, National Institute of Public Health and the Environment (RIVM), Bilthoven, the Netherlands;

Dr J.W. van der Laan, National Institute of Public Health and the Environment (RIVM), Bilthoven, the Netherlands;

Dr E. Griffiths, Coordinator, Quality Assurance and Safety of Biologicals Team, WHO;

Dr I. Knezevic, Quality Assurance and Safety of Biologicals Team, WHO.

A second draft was prepared following 1) discussion on special immunological considerations held at AFSSAPS, Lyon, 17 June 2002 with the following participants: Dr Florence Fuchs, Dr Dominique Masset, Dr Christelle Raignier and Dr Marc Pallardy (AFSSAPS) and 2) meeting of drafting group held in Geneva, 1-2 July 2002.

A third draft was prepared by the drafting group after an informal WHO Consultation on Preclinical Evaluation of Vaccines: Regulatory Expectations, held in Geneva, 12-13 December 2002, with the following participants:

Dr T. Bektimirov, Deputy Director, Tarasevic State Research Institute for Standardization and Control of Medical Biological Preparations, Moscow, Russian Federation; Dr E. Chaves Leal, Vice Director, Instituto Nacional de Control de Calidad Saude, Rio de Janeiro, Brazil; Dr W. Egan, Acting Director, Office of Vaccines Research and Review, Center for Biologics Evaluation and Research, Food & Drug Administration, Rockville, MD, USA; Dr E. Griffiths, Associate Director General, Biologics and Genetic Therapies, Health Canada, Ottawa, Canada; Dr M. Gruber, Scientific Reviewer, Division of Vaccines and Related Products Application, Center for Biologics Evaluation and Research, Food & Drug Administration, Rockville, MD, USA; Dr M. Haase, Paul Ehrlich Institut, Langen, Germany; Dr A. Homma, Developing Country Vaccine Manufacturer's Network., c/o Bio-Manguinhos Oswaldo Cruz Foundation, Rio de Janeiro, Brazil; Dr J.G. Kreeftenberg, Bureau for International Cooperation, National Institute of Public Health and the Environment (RIVM), Bilthoven, the Netherlands; Dr J.W. van der Laan, National Institute of Public Health and the Environment (RIVM), Bilthoven, the Netherlands; Dr R. Leke, Department of Immunology and Microbiology, Faculty of Medicine, University of Yaounde, Yaounde Cameroon; Dr Lei Dianliang, Deputy Director, National Institute for the Control of Pharmaceutical and Biological Products, Beijing, People's Republic of China; Dr Lazara Martínez Muñoz, Centro para el Control Estatal de la Calidad de los Medicamentos, Havana, Cuba; Dr D. Masset, Agence française de Sécurité Sanitaire des Produits de Santé, Saint-Denis Cedex, France; Dr R. Mignolet, RLM Consulting, Wavre, Belgium; Dr P. Minor, Head, Division of Virology, National Institute for Biological Standards and Control, Potters Bar, UK; Dr G. Oreffici, Istituto Superiore di Sanita, Rome, Italy; Dr M. Pallardy, AFSSAPS Veille Toxicologique/Vigilance, St Denis Cedex, France; Dr J. Petriciani, Carlsbad, USA; Dr P. Pitisuttithum, Vaccine Trial Centre, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; Dr F. Reigel, Vice Director, Swissmedic, Biological Medicines and Laboratories, Bern, Switzerland; Dr J. Robertson, National Institute for Biological Standards and Control, Potters Bar, UK; Dr L. Slamet, Deputy for Therapeutic Products, Narcotic, Psychotropic and Addictive Substance Control, Directorate General of Food and Drug Control, Ministry of Health, Jakarta, Indonesia; Dr A.K. Tahlan, Central Drugs Laboratory, Central Research Institute, Kasauli, India; Ms C. Chamberlin, Scientific Secretary of European Pharmacopoeia Group of Experts on Vaccines, Strasbourg, France; Dr B. Meignier, IABS c/o Director, External R&D, Aventis Pasteur SA, Marcy l'Etoile, France; Dr B.J. Ledwith, Director, Biologics Safety Assessment, Merck Research Labs., West Point, PA, USA; Dr F. Verdier, Head, Product Safety Assessment, Aventis Pasteur, Marcy l'Etoile, France; Dr G. del Giudice, Head, Animal Models and Serology, Research Center, Chiron SpA, Siena, Italy; Dr M.P. Kieny, Director, IVR, WHO; Dr L. Rago, Coordinator, QSM, WHO; Dr D. Wood, Acting Coordinator, QSB, WHO; Mr L. Belgharbi, ATT, WHO; Dr N. Dellepiane, ATT, WHO; Dr P. Duclos, VAM, WHO; Dr D. Kioy, TDR, WHO; Dr I. Knezevic, QSB, WHO; Dr E. Uramis Diaz, ATT, WHO; Dr S. Osmanov, VIR, WHO.

The final draft was prepared by Dr E. Griffiths, Dr M. Gruber, Dr D. Masset, Dr F. Verdier, Dr D. Wood and Dr I. Knezevic, following the meeting held in Geneva, 9-10 June 2003, taking into account comments made by the Expert Committee on Biological Standardization at its meeting held in February 2003 as well as comments made by the reviewers of the document.

## Annex 1

List of tissues to be collected in a repeated dose toxicity study:

adrenal glands  
aorta  
bone (femur) and articulation  
bone (sternum) with bone marrow  
bone marrow smears (1)  
brain  
bronchi (mainstem)  
caecum  
colon  
duodenum  
epididymides  
eyes  
heart  
ileum  
injection site(s) (a sample will be taken from the area injected)  
jejunum  
kidneys and ureters  
larynx  
liver  
lungs  
lymph node (mandibular)  
lymph node (mesenteric)  
mammary gland  
oesophagus  
optic nerves  
ovaries and oviducts  
pancreas  
parathyroid glands  
Peyer's patches  
pituitary gland  
prostate  
rectum  
salivary glands (mandibular, parotid, sublingual)  
sciatic nerves  
seminal vesicles  
skeletal muscle  
skin  
spinal cord (cervical, thoracic, lumbar)  
spleen  
stomach  
testes

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(1) Bone marrow smears should be prepared at the scheduled necropsy for all animals including any moribund animals killed during the study. The smears should be fixed in methanol and then stained by the May Grunwald-Giemsa method.

thymus  
thyroid glands  
tongue  
trachea  
ureters  
urinary bladder  
uterus (horns + cervix)  
vagina  
all gross lesions.

## Annex 2

### *Glossary*

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

**Adjuvants:** are substances that are intended to enhance relevant immune response and subsequent clinical efficacy of the vaccine.

**Booster vaccination:** Vaccination given at a certain time interval after primary vaccination in order to enhance immune responses and induce long term protection.

**Combination vaccine:** A vaccine that consists of two or more antigens, combined either by the manufacturer or mixed immediately before administration and intended to protect against: 1) multiple diseases or 2) one disease caused by different strains or serotypes of the same organism.

**Dissemination:** Evaluation of the release of live vaccines in the environment (e.g. viral shedding).

**Genetically modified organism (GMO):** an organism or a micro-organism in which the genetic material has been altered in a way that does not occur naturally by mating and/ or natural recombination. This definition covers micro-organisms including viruses, viroids, cell cultures including those from animals but does not cover naked recombinant DNA and naked recombinant plasmids.

**Good Clinical Practice (GCP):** A standard for clinical studies which encompasses the design, conduct, monitoring, terminations, audit, analyses, reporting and documentation of the studies and which ensures that the studies are scientifically and ethically sound and that the clinical properties of the pharmaceutical product (diagnostic, therapeutic or prophylactic) under investigation are properly documented.

**Good Laboratory Practice (GLP):** A quality system concerned with the organizational process and the conditions under which non-clinical health and environmental safety studies are planned, performed, monitored, recorded, archived and reported. GLP principles may be considered as a set of criteria to be satisfied as a basis for ensuring the quality, reliability and integrity of studies, the reporting of verifiable conclusions and the traceability of data.

**Good Manufacturing Practice (GMP):** A part of the pharmaceutical quality assurance which ensures that products are consistently produced and controlled to the quality standards appropriate to their intended use and a required by the marketing authorization. In these guidelines, GMP refers to the current GMP guidelines published by WHO.

**Immunogenicity:** Capacity of a vaccine to induce antibody mediated and/or cell-mediated immunity and /or immunological memory.

**Nonclinical evaluation of vaccines:** All *in vivo* and *in vitro* testing performed before and during clinical development of vaccines. The potential toxicity of a vaccine should be defined not only prior to initiation of human trials, but throughout clinical development.

**Plasmid:** Double-stranded circular DNA molecules capable of replicating in bacterial cells.

**Potency:** The measure of biological activity, using a suitably quantitative biological assay, based on the attribute of the product that is linked to the relevant biological properties.

**Preclinical evaluation of vaccine:** All *in vivo* and *in vitro* testing prior to first testing of vaccines in humans. This is prerequisite to the initiation of clinical trials and includes product characterization, proof of concept/ immunogenicity studies and animal safety testing conducted prior to introducing the product into the humans.

**Preclinical toxicity study:** A study designed with the primary purpose of demonstrating the safety and tolerability of a candidate vaccine product. The preclinical toxicity study design should meet the criteria outlined in the section “Study design” to be considered supportive of the intended clinical trial.

**Primary vaccination:** First vaccination or series of vaccinations given within a predefined period, with an interval of less than 6 months between doses, to induce clinical protection.

**Product characterization:** Full battery of physical, chemical and biological tests conducted for a particular product. These tests include but are not limited to in-process control testing, testing for adventitious agents, testing process additives and process intermediates, and lot release.

**Protocol or Study Plan:** A document that states the background, rationale and objectives of the nonclinical studies and describes its designs, methodology and organization, including statistical considerations, and the conditions under which it is to be performed and managed.

**Relevant animal model:** is an animal which develops an immune response similar to the expected human response after vaccination. It is acknowledged that species specific differences in immune responses will likely exist. Ideally, the animal species used should be sensitive to the pathogenic organism or toxin.

**Route of administration:** The means by which the candidate vaccine product is introduced to the host. Routes of administration may include the intravenous, intramuscular, subcutaneous, transcutaneous, intradermal, transdermal, oral, intranasal, intranodal, intravaginal and intrarectal routes.

**Seroconversion:** Predefined increase in antibody concentration, considered to correlate with the transition from seronegative to seropositive, providing information on the immunogenicity of a vaccine. If there are pre-existing antibodies, seroconversion is defined by a transition from a predefined low level to a significantly

higher defined level, such as four fold increase in geometric mean antibody concentration.

**Validation:** The action of proving in accordance with the principles of Good Manufacturing Practice, that any procedure, process, equipment (including the software or hardware used), material, activity or system actually leads to the expected results.

### Annex 3

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