

Annex 4

Recommendations to assure the quality, safety and efficacy of acellular pertussis vaccines

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Recommendations published by WHO are intended to be scientific and advisory in nature. The parts of each section printed in type of normal size have been written in such a form that, should a national regulatory authority (NRA) desire, they may be adopted as they stand as definitive national requirements or used as the basis of such requirements. Those parts of each section printed in small type are comments and additional guidance. It is recommended that modifications be made only on condition that the modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the recommendations set out below.

Introduction

Pertussis immunization is an integral part of immunization programmes in all regions of the world. It is recommended for all infants and children and in some countries it is also recommended for adults and adolescents. Whole-cell pertussis vaccines, which have been used for more than 50 years, have been shown to provide protection against pertussis and still serve as the foundation of global pertussis control. However, there is an increasing interest in acellular pertussis vaccines which have also been shown to be safe and effective and which have been successfully introduced into many national immunization programmes. A detailed comparison of acellular and whole-cell pertussis vaccines is beyond the scope of this document; however, these issues are discussed in detail in a WHO position paper on pertussis vaccines (1). As a consequence of the increasing demand for acellular pertussis vaccines, new manufacturers are entering the field. The expansion in the number and use of acellular pertussis vaccines, the development of new vaccines and advances in the standardization of quality-control methods have prompted WHO to update its current Guidelines for acellular pertussis vaccines (2).

These Guidelines were approved in 1996, with the recognition that further improvements in the production and evaluation of these vaccines would follow. Since then, stakeholders have gained additional experience with these vaccines, and limitations in the original Guidelines have been identified (3–7). Acellular pertussis vaccines are almost exclusively administered in combinations with diphtheria and tetanus toxoid vaccines. Moreover, in recent years there has been increased interest in the use of more complex combination vaccines – a trend which increases the challenges of clinical evaluation. Furthermore, the evaluation of the clinical efficacy of any new acellular pertussis vaccine formulations has become increasingly difficult due to the decrease in the prevalence of pertussis cases worldwide and for additional reasons discussed below in Part C. The goal of this revision is to address these issues concerning the Guidelines in the light of new information.

Background

The development of acellular pertussis vaccines was stimulated by scientific advances that led to the identification of components such as toxins and surface proteins of *Bordetella pertussis* that are believed to play a role in pathogenesis and induction of protective immunity. The first acellular pertussis vaccines were produced through a purification process that resulted in the enrichment of two protein components – namely pertussis toxin (PT) and filamentous haemagglutinin (FHA) – that were protective in animal models, and were introduced for routine use in Japan around 1980 as a response to increasing public concern over adverse

reactions to whole-cell pertussis vaccines. These vaccines were prepared from cell-free *B. pertussis* culture supernatants by ultracentrifugation, and all contained FHA and PT that were treated with formaldehyde to inactivate the PT. These vaccines also contained various amounts of other *B. pertussis* proteins as minor components, including pertactin (PRN or 69 kDa protein) and fimbriae (FIM). Epidemiological evaluations indicated that these vaccines effectively controlled pertussis disease when introduced for routine immunization (8–10).

An alternative approach to manufacturing employed individually purified pertussis antigens which, after detoxification, were used to formulate vaccines of defined composition. These purified component vaccines varied in the number of antigens incorporated, and in the PT detoxification procedures and antigen-purification processes used.

Between 1986 and 1996, several vaccines containing acellular pertussis, including some composed of purified antigens and some composed of co-purified antigens, were evaluated in a series of efficacy trials. These trials evaluated acellular pertussis vaccines containing up to five pertussis components and utilized different study designs, including randomized placebo-controlled cohort trials, household contact studies, and case-control studies (11, 12). This series of trials revealed that all the tested acellular pertussis vaccines provided some protection against pertussis, although the studies suggested differences between the vaccines. Additional detail is provided in Part C of these Recommendations. Unless vaccines of different types were tested in parallel within the same trial, comparing efficacy among different acellular vaccines must be done with caution as all the trials varied with respect to design, case-ascertainment methodology and case definition. Following the completion of these trials, many countries introduced acellular pertussis vaccines into their routine immunization programmes.

In addition to heterogeneity in production and composition, there are variations in the approaches used for control testing of acellular pertussis vaccines. The testing methodology developed in Japan was based on modifications of the tests used for whole-cell vaccines. This included a modification of the mouse intracerebral challenge assay for potency, along with additional requirements designed to monitor purity, content and residual toxin activities. In general, these tests and specifications are not vaccine-specific and can be used for evaluating new products with new antigen formulations. Alternative control testing approaches were adopted initially in Europe and North America for purified component vaccines based on the concept that the newly manufactured lots should be comparable to those evaluated in pivotal clinical studies. Control testing of these vaccines included in-process and final product tests for purity, composition, residual toxin activity and immunogenicity. As the products differ markedly from each other, specifications were product-specific and were based on the concept that the newly manufactured lots should closely match those evaluated in clinical studies. The immunogenicity of individual antigens

using antibody-binding assays was used as one of the markers of production consistency. The different methods used to evaluate potency and residual toxin activity are designed to assess different characteristics of the vaccines. However, acellular pertussis vaccines tested by both approaches have been used effectively. Importantly, both testing approaches have been applied to purified component vaccines and to co-purified vaccines. Further research is needed to develop alternative methods and to standardize the current potency and safety tests used for the evaluation of acellular pertussis vaccines.

Scope

These Recommendations apply to co-purified and purified component acellular pertussis vaccines. The document covers only antigens produced by *B. pertussis*. While other approaches are possible (e.g. antigens produced from *B. bronchiseptica* or *Escherichia coli*) they are not considered in this document.

Although these Recommendations apply to the production and quality control of acellular pertussis vaccines, the acellular pertussis component is combined most commonly with other antigens (e.g. diphtheria and tetanus toxoids, *Haemophilus influenzae* type b conjugate vaccine, inactivated polio vaccine and hepatitis B). Therefore the tests recommended for the final bulk or final product of acellular pertussis vaccines should be performed on the final bulk or final product of the combined vaccines.

These revised Recommendations highlight the advances made in the development, manufacturing and testing of acellular pertussis vaccines and aim to provide guidance on the following issues:

- improvement of quality control of existing vaccines on the basis of new information and experience;
- evaluation of new products and new combinations through control of manufacturing;
- evaluation of the vaccines in both nonclinical and clinical studies.

The main changes made to the WHO Guidelines published in 1998 are as follows.

- The title of the document is upgraded from “Guidelines” to “Recommendations”.
- Advice on clinical and nonclinical evaluation of acellular pertussis vaccines is added to guide NRAs and vaccine manufacturers in approaches that can be used to assess the safety, efficacy and quality of vaccines.

- Details are provided for the modified intracerebral challenge assay used to evaluate the potency of the acellular pertussis vaccine (Appendix 1), and for the histamine sensitization test (HIST) based on temperature measurement to determine the residual activity of PT in the vaccine (Appendix 2).
- Information is provided for the performance of the mouse respiratory challenge method. Although the method is currently not recommended for routine potency testing, it may have an important role in nonclinical testing as discussed in Part B of these Recommendations.

General considerations

Written descriptions of detailed procedures or the standard operating procedures used for the production and testing of the acellular pertussis vaccines or combined vaccines containing acellular pertussis component(s), together with evidence of appropriate validation of each production step and relevant control tests, should be submitted for approval to the NRA as part of the licence application. Proposals for any variations in manufacturing and/or control methods should be submitted for approval to the NRA according to national regulatory requirements before they are implemented.

There is as yet no consensus on the ideal antigenic composition of acellular pertussis vaccines. Currently, various acellular pertussis vaccine products are available from diverse manufacturers, differing in the number of components, their concentrations and their degree of adsorption to different adjuvants. In addition, these individual antigens have been derived from different strains of *B. pertussis*, purified by different methods and treated with different detoxification agents. All currently available acellular pertussis vaccines contain detoxified PT (PTxd) and some vaccines formulated with PTxd alone have been shown to provide a significant degree of protection. However, clinical and laboratory studies have suggested that the protective efficacy of PT may be enhanced by other antigens (11–19). Ongoing research is essential to identify the protective mechanisms, to identify immunological markers of protection against pertussis, and to develop and improve relevant laboratory models. Additionally, because all current acellular pertussis vaccines are administered in more complex combination vaccines, research is encouraged on models that allow the concurrent testing of multiple components (e.g. diphtheria and tetanus toxoids).

Manufacturers should demonstrate consistency in manufacturing and formulation and should adhere strictly to the production process used for the manufacture of the vaccine lots used in the clinical trials supporting regulatory approval. In addition, laboratory tests should show consistency in the safety,

potency, and physicochemical and immunological characteristics of new vaccine lots compared with lots evaluated in clinical studies. Special care should be taken in the validation of those test procedures that are used for ensuring consistency of production lots for specificity, sensitivity and accuracy. Manufacturers should ensure that sufficient quantities of reference vaccine of adequate stability are available for routine in-house testing and for confirmatory tests undertaken by the NRA.

An in-house standard, when used, should be assigned a value so that trend analyses can be monitored and quality-control testing limits can be defined. When an International Standard exists, the in-house standard should be calibrated against that standard.

Use of a suitable freeze-dried vaccine preparation as an in-house reference may offer advantages of stability. A successful example of conditions which have been used for diphtheria vaccines (adsorbed) is as follows: vaccine lyophilized in the presence of 3.5% polygeline (1:1) under freeze-drying cycle at -50°C load, -50°C freeze over 2.5 hours, then primary drying at -35°C (100 μbar vacuum) and secondary drying at 30°C (30 μbar vacuum) (20).

There are no laboratory tests, animal models and/or human immune responses that can provide complete assurance that a newly developed acellular pertussis vaccine will be adequately safe and effective. Within these limitations, these Recommendations describe a sequential approach to the collection of supporting evidence, beginning with a comprehensive programme of nonclinical testing and followed by a progression of clinical evaluations. For the purpose of this document, a newly developed vaccine would be any vaccine that contains either a novel antigen or one of the antigens in the currently licensed products (i.e. PTxd, FHA, PRN, FIM type 2 and FIM type 3) that is produced from a new strain, new process and/or new manufacturer. As described in Part B, nonclinical characterization studies should include evaluations of purity, residual toxin activity, bioactivity, reactivity with specific antibodies, induction of binding and functional antibodies, and induction of protective activity in animal models. Whenever an additional antigen is added, studies should be undertaken to characterize its interaction with other components in the product. If the antigen is novel, more extensive characterization studies would be expected. This document assumes that only those vaccines that have already undergone extensive nonclinical testing would be considered for clinical evaluation, with agreement with the local NRA responsible for evaluating adequacy of nonclinical information. Although efficacy trials appear very difficult, if not impossible, safety and immunogenicity trials of adequate design and size are possible and should be conducted. In Part C, the Recommendations provide guidance on issues related to the design and

evaluation of the clinical studies. Most studies are expected to be comparative studies. Thus the choice of a comparator vaccine is a particularly important issue because the potential comparator vaccines differ substantially in formulation and composition. Finally, because the tools for clinical evaluation are limited, rigorous post-marketing monitoring of the vaccines will be needed in order to determine if they are achieving acceptable levels of clinical safety and efficacy.

Given these limitations, some caution is appropriate when considering a transition from whole-cell to acellular pertussis vaccines. Specifically, whole-cell pertussis vaccines are safe and effective, and offer some advantages as described in the WHO position paper (1). Thus, although these Recommendations describe an approach for approval of new acellular pertussis vaccines, the path for approval of new acellular pertussis vaccines requires considerable effort and includes significant challenges.

International Standards and Reference Preparations

Subsequent sections of this document refer to WHO reference materials that may be used in laboratory or clinical evaluations. The WHO catalogue of international biological standards should be consulted for the latest list of appropriate WHO International Standards and reference materials (see: <http://www.who.int/bloodproducts/catalogue/en/index.html>). Key standards and reference materials include:

- the First WHO International Standard for acellular pertussis vaccine for use in modified mouse intracerebral challenge assay (MICA) and other protection assays (Code no. JN1H 3 with assigned unitage per ampoule of 34 IU);
- the First WHO International Standard for pertussis toxin for standardization of assays used to monitor the residual PT activity in pertussis vaccines, e.g. histamine sensitization tests and Chinese hamster ovary (CHO) cell assay (Code no. JN1H 5 with assigned unitage per ampoule of 10 000 IU);
- the First WHO International Standard for pertussis antiserum (human) (Code no. 06/140 with assigned unitage per ampoule of 335 IU anti-PT IgG and 65 IU IgA anti-PT; 130 IU IgG anti-FHA and 65 IU IgA anti-FHA; 65 IU IgG anti-69K and 42 IU IgA anti-69K);
- the First WHO International Reference Reagent for standardization of clinical serology assays (Code no.06/142 with assigned unitage per ampoule of 106 IU anti-PT IgG and 18 IU IgA anti-PT; 122 IU IgG anti-FHA and 86 IU IgA anti-FHA; 39 IU IgG anti-69K and 38 IU IgA anti-69K);

- the First WHO International Standard for monoclonal antibody to *B. pertussis* fimbriae type 2 for the determination of serotype of *B. pertussis* strains (Code no. 06/124);
- the First WHO International Standard for monoclonal antibody to *B. pertussis* fimbriae type 3 for the determination of serotype of *B. pertussis* strains (Code no. 06/128);
- the First WHO International Reference Reagent for pertussis antiserum (mouse) (Code no. 97/642 with assigned unitage per vial of 17 units of anti-PT, 143 units of anti-FHA, 30 units of anti-PRN and 32 units of anti-FIM 2 and 3).

The above-mentioned WHO International Standards and reference materials and other reagents from the WHO International Standards Laboratory are in the custody of the National Institute for Biological Standards and Control (NIBSC), Health Protection Agency, Potters Bar, England (see: <http://www.nibsc.ac.uk>).

These Reference Preparations are available for the calibration and establishment of regional, national or in-house reference materials. Samples are distributed free of charge, on request, to NCLs.

Terminology

The definitions given below apply to some common terminology used throughout this document. The terms may have different meanings in other contexts.

Master seed lot: a quantity of bacterial suspension that is derived from a single strain, has been processed as a single lot and has a uniform composition. It is used for inoculating media for the preparation of working seed lot.

Working seed lot: a quantity of bacterial suspension of a single substrain derived from the master seed lot by growing the organisms and maintaining them in aliquots in the frozen form or in the lyophilized form, stored at -20°C or below (in the liquid form stored at -80°C or below). The working seed lot should be prepared from the master seed lot by as few cultural passages as possible, having the same characteristics as the master seed lot and intended for inoculating media for the preparation of single harvests.

Single harvest: the culture filtrate or the suspension of bacteria obtained from one batch of cultures that have been inoculated with the working seed lot (or with the inoculums derived from it), harvested and processed together.

Purified antigen(s) bulk material: the processed purified material prepared using pertussis antigen preparations processed either in a single run or a pool of those prepared in multiple runs. In some cases, purified antigen bulk

material may be adsorbed to or mixed with adjuvant and a preservative may be added. It is the parent material from which the final bulk is prepared.

Final bulk: the homogeneous finished vaccine present in a single container from which the final containers are filled either directly or through one or more intermediate containers.

Final lot or final product: a collection of sealed final containers that are homogeneous with respect to the risk of contamination during filling. A final lot must therefore have been filled from a single container in one continuous working session.

Individually purified antigen: each of the pertussis antigens that are individually isolated and purified using combinations of several physicochemical separation methods.

Co-purified antigen: two or more pertussis antigens that have been isolated and purified using combinations of several physicochemical separation methods (e.g. ammonium sulfate precipitation and density gradient centrifugation).

Comparator vaccine: an approved vaccine with established efficacy or with traceability to a vaccine with established efficacy that is tested in parallel with an experimental vaccine and serves as an active control in nonclinical or clinical testing.

Part A. Manufacturing recommendations

A.1 Definitions

A.1.1 International name and proper name

The international name should be acellular pertussis vaccine. The proper name should be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the recommendations formulated below.

A.1.2 Descriptive definition

Acellular pertussis vaccine is a preparation of purified or co-purified antigenic component(s) of *Bordetella pertussis* that have been appropriately treated by chemical means or obtained by genetic manipulation to minimize toxicity and retain potency. The preparations for human use should satisfy all the recommendations formulated below.

Currently licensed vaccines contain either PTxd alone or PTxd in combination with one or more other antigens (FHA, PRN, fimbriae type 2 [FIM-2] and fimbriae type 3 [FIM-3]).

A.2 General manufacturing recommendations

The general manufacturing requirements contained in good manufacturing practices (GMP) for pharmaceutical (21) and biological products (22) apply to the production of the acellular pertussis vaccines.

A.3 Production control

These Recommendations pertain to antigen production and purification from *B. pertussis*.

A.3.1 Control of source materials

A.3.1.1 Strains of *Bordetella pertussis*

Strains of *B. pertussis* used in preparing vaccine should be identified by a full record of their history, including origin and characteristics on isolation, and particulars of all tests made periodically to verify strain characteristics. If genetically modified *B. pertussis* is used, all relevant modified DNA sequences should be clearly delineated and fully characterized. The strains of *B. pertussis* used should be approved by the NRA.

A.3.1.2 Seed lot system

The production of the acellular pertussis component of monovalent or combined vaccines should be based on a well-established seed lot system. Cultures from the working seed lot should have the same characteristics as cultures from the master seed lot. If genetically modified *B. pertussis* is used, the relevant modified DNA sequences should be reconfirmed for each new working seed lot. The strains should be maintained by a method approved by the NRA and able to preserve the ability of the seed to yield potent vaccine in terms of the quality of the antigens produced.

Freeze-drying or storage in liquid nitrogen are satisfactory methods of maintaining strains, subject to suitable validation. In some countries, glycerol stocks are also used for this purpose, but this method would require extensive validation and approval of the NRA.

A.3.1.3 Culture media for production

B. pertussis should be cultured in culture media suitable to support its growth and the production of relevant antigens with consistent yields. Media used should be free from adventitious agents. Moreover, medium components that are known to cause allergic reactions should be avoided. Human blood or blood products should not be used in culture media either for seed lots or for vaccine production. The acceptability of the source(s) of any components of bovine, sheep or goat origin used in culture media should be approved by the NRA.

If any materials of animal origin are used in seed preparation or preservation, or in production, they should comply with the *WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products* (23) and national regulatory requirements. When animal blood or blood products are used, they should be removed in the process of production by appropriate methods.

Any change of media should be submitted for approval to the NRA.

A.3.2 **Control of manufacturing process**

A.3.2.1 **Control of production cultures**

Production cultures should be shown to be consistent with respect to growth rate, rate of change of pH, rate of production of the desired antigen(s), and additional parameters as agreed with the NRA. Acceptance specifications should be established and agreed with the NRA.

A.3.2.2 **Control of bacterial purity**

Samples of individual cultures should be tested for microbial purity by microscopic examination of stained smears, by inoculation of appropriate culture media, or by any other suitable procedure. For microscopic examination, several fields should be examined at high magnification. If a contaminant is found, the culture and any product derived from it should be discarded.

In some countries the individual cultures are tested for microbial purity by a minimum of two suitable and approved procedures.

A.3.2.3 **Control of antigen purification**

Cultures should be processed for further antigen purification in a way that minimizes contamination of crude materials with undesirable molecules, such as lipooligosaccharide (LOS), dermonecrotic toxin, adenylate cyclase toxin (ACT), and tracheal cytotoxin (TCT). Cells of *B. pertussis* may be separated from fermentation fluid by filtration or centrifugation, and should be suitably inactivated before their further processing or disposal. Absence of cells of *B. pertussis* from crude antigen solutions should be confirmed at this stage using appropriate methods approved by the NRA.

Two approaches have been followed for the purification of pertussis antigens for vaccine manufacturing. In the first approach, antigens have been co-purified by repeated cycles of ammonium sulfate precipitation and density gradient centrifugation to yield preparations enriched in certain proteins – mainly PT, FHA and PRN – but depleted of endotoxin (LOS). The number and proportion of each antigen in a given vaccine type may vary widely depending on the process followed, but should be reproducible for each specific product.

In the second approach each antigen is individually purified using combinations of several physicochemical separation methods.

The tests used for determination of consistency of yield and purity and their performance characteristics should be approved by the NRA.

It is advisable to sterilize purified antigens by membrane filtration or other suitable sterilizing grade filtration before further processing.

A.3.2.3.1 *Tests undertaken prior to detoxification/chemical treatment*

Characterization of antigens: rigorous characterization of the antigens by physicochemical, immunological or functional (biological) assays, as appropriate, is essential before any step is undertaken that is capable of modifying their original characteristics. Particular attention should be given to employing a range of analytical techniques based on different principles. Immunoblot analysis with specific monoclonal or polyclonal antibodies may be used to characterize antigens or antigen subunits. The specific properties of each antigen component should be determined in comparison with Reference Preparations. Specifications should be established for each individual antigen. Suitable assays include sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), single radial immunodiffusion, immunoblotting, the CHO cell test for detection of active PT, haemagglutination and high-performance liquid chromatography (HPLC). The specific activity of PT (IU/ng) should be determined.

Antigen purity: the purity of the individual or co-purified antigens claimed to contribute to vaccine efficacy should be determined by SDS-PAGE, HPLC or other appropriate analyses before detoxification. It is important that the techniques used are based on as wide a range of properties of the vaccine components as possible. Limits should be specified for all impurities detected.

The purity of the individual or co-purified antigens should be within the range of values established for each product as found for vaccine lots shown to be safe and effective in clinical trials or other lots used in support of licensing. Specifications should be set during the process of product development and validation and should be established by agreement with the NRA.

Residual levels of endotoxin: the antigens should be tested for residual endotoxin content by means of the *Limulus* amoebocyte lysate (LAL) test or other appropriate assay. This test may be carried out at a later stage. Endotoxin content should be consistent with levels found in vaccine lots shown to be safe and effective in clinical trials or for lots used in support of licensing. Specifications should be established in agreement with the NRA.

Antigen content: if it is necessary at this stage, the amount of individually purified antigens that have been characterized and purified, as appropriate, should be estimated by means of a validated quantitative assay of sufficient sensitivity,

such as an assay for protein content and, where available, a suitable quantitative immunoassay. Antigen content can be determined by ELISA; active PT content can be determined by CHO cell assay.

In cases where two or more antigens are co-purified, the proportion of each antigen claimed to contribute to vaccine efficacy should be measured by a suitable method (e.g. SDS-PAGE, HPLC, electrophoresis on non-denaturing gels, or densitometry) and should be shown to be within the range of values found for vaccine lots shown to be safe and effective in clinical trials or other lots used in support of licensing. Specifications should be set during the validation and established in agreement with the NRA.

Sterility test: bacterial and mycotic sterility for each antigen lot should be determined in accordance with the requirements of Part A, section 5, of the revised Requirements for biological substances, No. 6 (General requirements for the sterility of biological substances) (24), or by a method approved by the NRA. If appropriate, this test may be carried out at a later stage.

If a preservative is added, appropriate measures should be taken to prevent interference with the sterility test.

A.3.2.4 Detoxification

The purified PT, if it is not genetically detoxified, or co-purified antigens that contain this toxin should be subjected to appropriate detoxification methods. Other antigens may also be treated with agents to detoxify any residual PT in the preparation. The residual detoxifying agents should be removed by an appropriate method. Different chemicals are used to detoxify PT. These include, but are not limited to, formaldehyde, glutaraldehyde, a combination of both, or hydrogen peroxide. Different detoxification processes yield distinct products.

The detoxification method/process should be validated for the ability to consistently produce antigens that have acceptably low levels of biologically active PT and retain acceptable levels of immunogenicity, as measured on final formulation. In addition, the detoxification method should be validated for the ability to produce detoxified PT that does not revert to biologically active PT upon storage. If any aggregation of antigens has occurred following detoxification, the aggregates should be homogenized by an appropriate procedure such as sonication followed by filtration to remove the larger clumps.

A.3.3 Control of pertussis antigen bulk materials

Bulk materials should be prepared using antigen preparations processed either in a single run or in a pool of those prepared in multiple runs. With the approval of the NRA, the bulk materials may be adsorbed to/mixed with adjuvant and a preservative may be added.

Antigen content: the amount of each individual antigen or of co-purified antigens should be estimated by means of a validated quantitative assay, such as an assay for protein content and, where available, suitable quantitative immunoassays for individual antigens. For co-purified antigens, the ratio of antigens should be defined. When no adequate procedure is available for assessing individual antigens following chemical detoxification, a validated suitable procedure may be used to estimate the amount of individual antigens based on the amounts measured before detoxification. Specifications for antigen content should be set during the process of product development and validation and should be approved by the NRA.

Residual activity of pertussis toxin: the amount of residual biologically active PT in the individually or co-purified antigens should be estimated after detoxification by means of a sufficiently sensitive test such as the HIST or the CHO cell assay. Adjuvants and other vaccine components may interfere with the adequate performance of the CHO cell assay (25) and special care must be taken to ensure that they do not interfere with the tests (e.g. by adequate dilution of test solutions). At the concentration of vaccine final formulation, the total amount of residual bioactive PT from all pertussis antigens should not exceed that found in vaccine lots shown to be safe in clinical trials or other lots used in support of licensing. Specification should be established in agreement with the NRA.

Residual levels of endotoxin: the bulk material or antigens should be tested for residual endotoxin content by means of the LAL test or other appropriate assay. At the concentration of vaccine final formulation, the total amount of residual endotoxin should not exceed that found in vaccine lots shown to be safe in clinical trials or other lots used in support of licensing. The limits applied to the vaccine concentration of individual components should be agreed with the NRA.

Sterility test: each purified antigen bulk should be tested for bacterial and mycotic sterility in accordance with the requirements of Part A, section 5, of the revised Requirements for biological substances, No. 6 (General requirements for the sterility of biological substances) (24), or by a method approved by the NRA. If a preservative is added, appropriate measures should be taken to prevent interference with the sterility test.

A.3.4 Control of final bulk

Currently there is no stand-alone acellular pertussis vaccine, so the tests described below are undertaken at the final bulk stage of combined vaccines.

If a stand-alone acellular pertussis vaccine were to be developed, the procedures described here could be adapted for such a product.

A.3.4.1 Preparation

The antigen bulk materials should be pooled to prepare the pertussis bulk. Current preparations may contain PTxd alone or together with FHA, with or without PRN and FIM 2 and 3, to produce one, two, three, four or five component acellular pertussis vaccines. The bulk of acellular pertussis vaccine may be adsorbed to/mixed with aluminium hydroxide or phosphate gel or another appropriate adjuvant prior to or after blending with other components (e.g. diphtheria toxoid, tetanus toxoid, inactivated polio vaccine (IPV)) to produce the final formulation (final bulk). A suitable antimicrobial preservative may be added.

A.3.4.2 Control tests

The following control tests on final bulk may be performed on the final product in agreement with the NRA.

A.3.4.2.1 Detoxifying agents

The content of residual detoxifying agent in the final bulk should be determined. The method and limits should be approved by the NRA.

If formaldehyde has been used, the residual content should not exceed 0.2 g/l. The residual content of glutaraldehyde should not exceed 0.1 g/l.

A.3.4.2.2 Preservative

Consideration should be given to the effect of the preservative on the stability of the vaccine formulation and possible interactions between the vaccine components and the preservative. The content of preservative should be determined by a method approved by the NRA. The amount of preservative in the vaccine dose should be shown to have no deleterious effect on the antigen(s) and should not impair the safety of the product for humans. The preservative, its use at different stages of the manufacturing process, and its concentration or residual amount should be approved by the NRA. If any modification of preservative content in already licensed vaccine is made, general principles for vaccine evaluation described in the WHO Guidelines on regulatory expectations related to the elimination, reduction or replacement of thiomersal in vaccines should be followed (26).

Phenol should not be used as a preservative.

A.3.4.2.3 Adjuvant

The nature, purity and concentration of the adjuvant or adjuvants added to the vaccine should be determined by a method approved by the NRA. When aluminium compounds are used as adjuvant the concentration of aluminium should not exceed 1.25 mg per single human dose. When calcium adjuvants

are used, calcium should not exceed 1.3 mg per single human dose. If other substances are used as adjuvants, specifications should be set and agreed with the NRA. The formulation should be such that the suspension appears homogeneous after shaking and remains as such for a defined period (e.g. the time needed for vaccine administration). Adsorption of antigens to the adjuvant should be investigated, when possible, by tests designed to determine which, and how much of each, are adsorbed. Consistency of adsorption is important, and the adsorption of the antigen in production lots should be demonstrated to be within the range of values found for vaccine lots which have been shown to be clinically safe and effective, or for lots used in support of licensing.

A.3.4.2.4 *Sterility*

Each final bulk should be tested for bacterial and fungal sterility in accordance with the requirements given in Part A, section 5, of the revised Requirements for biological substances, No. 6 (General requirements for the sterility of biological substances) (24) or by a method approved by the NRA.

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

A.3.4.2.5 *Residual activity of pertussis toxin*

Each final bulk of vaccine should be tested for active PT using a HIST or another test that is sufficiently sensitive to detect the level of toxin activity agreed with the NRA. For products containing genetically detoxified PT, this test may not be necessary if agreed with the NRA. Currently the HIST method used for the determination of residual bioactive PT in licensed acellular pertussis vaccines is based on the response to a histamine challenge of mice injected with the test vaccine. Two possible test outcomes (end-points) are in use. One is based on the lethal effect of the histamine challenge dose and the other on the decrease in body temperature following the histamine challenge. If an alternative assay is used, it should be at least as sensitive and specific as a validated HIST assay (either of the two tests mentioned above). The alternative method(s) should be approved by the NRA.

The susceptibility to histamine sensitization of the mice used in each test should be established using a suitable reference or control preparation of PT. The specific activity of the standard or positive control should be calibrated using the WHO International Standard (currently JN1H-5) and should be expressed in IU because the nominal protein masses of the toxin preparations do not necessarily predict their biological activity in HIST. The detection limit for the PT of the test should be defined and accepted by the NRA.

The acceptance criteria for content of residual bioactive PT should be consistent with the results for lots shown to be safe and effective in clinical trials

or other lots used in support of licensing. Specifications should be established in agreement with the NRA.

There is at present limited information about the relation between the level of residual active PT in an acellular pertussis vaccine and its clinical safety. Therefore the residual toxicity should be reduced as far as is feasible without undue compromise of immunogenicity. There is no internationally agreed upper limit for active PT in acellular pertussis vaccines. In some countries, upper limits of PT per single vaccine dose are a requirement for DTaP vaccines (27, 28). A recent collaborative study has provided preliminary data on the content of bioactive PT in DTaP-based combination vaccines using the HIST lethal end-point method (29).

The HIST lethal end-point method (see Appendix 3) measures the proportion of animals that die upon histamine challenge due to sensitization with residual PT in the vaccine. Assay sensitivity is verified by titration of a PT standard (calibrated in IU). Once linearity has been established by repeated experiments, the assay may be simplified to include in each test only a single dose of PT standard to ensure assay sensitivity.

Some laboratories include a standard toxin at a concentration near the acceptance limit to verify assay sensitivity.

The HIST based on measurement of the reduction in temperature (either rectal or dermal) produced by histamine challenge (see Appendix 2) has been successfully used in some countries. It has also been optimized to provide a quantitative estimate of the activity of a test vaccine relative to the activity of a PT standard.

Although the CHO cell assay is highly sensitive for detection of PT activity, the test may not be suitable for the final bulk vaccine because of possible interference (e.g. presence of adjuvant or inactivating agents).

Development of an alternative to HIST is encouraged. An in vitro assay system comprising an enzymatic HPLC (E-HPLC) assay and a carbohydrate binding assay is under evaluation as a potential alternative to the HIST (30). Any alternative assay to the current HIST to determine the residual PT activity should be validated and approved by the NRA.

A.3.4.2.6 *Reversion to toxicity*

Accelerated reversion testing, consisting of HIST performance on final bulk or the final lot incubated for at least four weeks at 37 °C, may be used to demonstrate that it is unlikely that the chemically inactivated PT will regain some of its toxicity before the vaccine expiry date. Some NRAs may not require this test for the release of each new lot but only as part of process validation. For products

containing genetically detoxified PT, this test may not be necessary as agreed with the NRA.

A.3.4.2.7 *Immunological activity*

Two methods are currently used in the lot release procedure to assess the immunogenicity or potency in mice after vaccination with acellular pertussis vaccine: the mouse immunogenicity test (MIT) and the MICA. Importantly, none of these assays can be considered as an index of clinical efficacy. Use of any of these tests requires validation and agreement with the NRA. Active PT in the vaccine (if any) may enhance the potency of vaccines obtained in these assays, with the degree of enhancement depending on the immunizing antigens, mouse strain, test method and assay conditions (31, 32).

The MIT is a non-lethal animal model designed to evaluate antibody responses in immunized mice to all the antigens claimed to contribute to vaccine efficacy. Currently, ELISA is used to measure the binding activity rather than the functional activity of antibody for each of the antigens. An international mouse reference serum containing antibodies to five antigens is available to monitor consistency of the ELISA stage.

The MIT is designed to assess consistency of manufacture by evaluating whether the results of the MIT for lots manufactured post-licensure are consistent with the results of lots with acceptable performance in clinical trials. Due to the different composition of different vaccine products, specifications are product-specific. Additionally, to ensure adequate performance, MIT requires the use of a product-specific reference or control vaccine analogous in composition to the test vaccine.

In the absence of an international reference vaccine for MIT, each manufacturer is responsible for the development of a reference vaccine that can allow for meaningful assessment of the immunogenic activity of the test vaccine with respect to the established specifications. Although a clinical lot with established efficacy may be considered as the reference vaccine, this is usually impractical due to considerations such as availability and long-term stability. However, the vaccine lot selected as reference should be sufficiently similar to the clinical lots in composition, manufacturing process, immunogenicity and/or protective effect. Stabilization of the reference vaccine is recommended, but careful attention should be given to any effects the stabilizing procedure may have on its activity.

The manufacturer is responsible for monitoring the stability of the reference and for replacement as needed. When monitoring stability of the reference or testing a candidate replacement for the reference, testing approaches that allow for higher precision (e.g. more tests, more animals per test, or more dilutions per test) are encouraged in order to improve the ability to detect changes in activity.

Establishment of the specifications for each product should be based on the response observed in the test for vaccine clinical lots and other lots used in support of licensing. The specifications for the antibody response to each antigen claimed to contribute to efficacy should be established and approved by the NRA. Additional details on the method are provided in Appendix 4.

Tests which show only that the test product does not differ significantly from the reference vaccine are not recommended. Specifications must be carefully justified and should take into account the precision of the test and the maximum allowable deviation from the reference vaccine.

The MICA: is a lethal challenge model in mice which detects mouse protective activity provided by the vaccine. The potency of each final bulk is expressed as a relative potency to a reference vaccine. That reference vaccine should be calibrated against the WHO International Standard for acellular pertussis vaccines (currently JN1H-3) (33) and the protective activity should be expressed in IU.

The assay method and the specifications should be approved by the NRA. Additional details on the method are provided in Appendix 1.

The specifications, where used currently, are that the vaccine passes the potency test if the result of a statistically valid assay shows that the estimated potency of the vaccine is not less than 4.0 IU in the volume recommended for a single human dose and if the lower fiducial limit ($p = 0.95$) of the estimated potency is not less than 2.0 IU for primary immunization vaccine.

Active PT has been shown to enhance the relative potency of vaccines obtained in this assay, with the degree of enhancement depending on mouse strain, assay conditions etc. (32).

Other assays

The development of alternative assays to MIT and MICA is encouraged.

An alternative assay – the guinea-pig immunogenicity test – has been adopted in some countries (34, 35). The assay allows immunogenicity testing of the acellular pertussis components and the diphtheria and tetanus toxoid components using the same group of animals. Adoption of this or another alternative method for routine lot release would require validation and approval by the NRA.

Respiratory challenge method(s) such as the mouse intranasal challenge assay (INCA) (see Appendix 5) have been evaluated in WHO international collaborative studies. They have discriminated between vaccines with different protective capacity in mice. The current respiratory challenge assays are not optimized or designed for use as a routine test for determining vaccine potency. Nevertheless, they can be used to assess potential impact of changing the manufacturing process and/or

formulation, the activity and stability of new antigens and formulations, or the potential interactions in new combinations. This model is an important tool in new product development as described in Part B of this document.

A.4 **Filling and containers**

The requirements concerning filling and containers given in Good manufacturing practices for biological products (22) should apply to vaccine filled in the final form.

Single dose and multiple dose containers may be used. Vaccine in multidose containers should contain a suitable antimicrobial preservative.

A.5 **Control of final product**

Quality control procedures and tests should be validated and approved by the NRA to ensure that the final containers contain the appropriate amounts of each of the vaccine antigens, as designed for the acellular vaccine formulation.

Unless otherwise justified and authorized, the following tests should be performed on labelled containers from each final lot by means of validated methods approved by the NRA.

A.5.1 **Identity**

An identity test should be performed on at least one container from each final lot by means of a validated method approved by the NRA.

A.5.2 **Sterility**

Final containers should be tested for sterility by a method approved by the NRA. Many countries have regulations governing the sterility testing of the final product. Where these do not exist, the Requirements published by WHO should be met (24). If a preservative has been added to the vaccine, appropriate measures should be taken to prevent it from interfering with the test.

A.5.3 **Adjuvant content**

The content of adjuvant should be determined by a method approved by the NRA. When aluminium compounds are used as adjuvants, the concentration of aluminium should not exceed 1.25 mg per single human dose. If a calcium adjuvant is used, the concentration of calcium should not exceed 1.3 mg per single human dose. If other substances are used as adjuvants, appropriate specifications should be set for the substance with adjuvant effect.

A.5.4 **Preservative content**

The content of preservative(s) should be determined by methods approved by the NRA. The amount of preservative per dose should be shown not to have

any deleterious effect on the antigen(s) nor cause untoward adverse reactions in humans. The preservative and its concentration or residual amount should be approved by the NRA. If any modification of thiomersal content in an already licensed vaccine is made, general principles for vaccine evaluation described in the WHO Guidelines on regulatory expectations related to the elimination, reduction or replacement of thiomersal in vaccines should be followed (26).

A.5.5 pH

The pH of each final lot should be within the range of values found for vaccine lots shown to be clinically safe and effective.

In some countries, determinations for osmolality and withdrawable content are also required.

A.5.6 Endotoxin

In some countries, determination of endotoxin content may be required with specifications approved by the NRA.

A.5.7 Innocuity test

Each final lot should be tested for unexpected toxicity (sometimes called abnormal toxicity or innocuity) by a method approved by the NRA.

This test may be omitted for routine lot release once consistency of production has been well established to the satisfaction of the NRA and when GMP is in place. Each lot, if tested, should pass a test for unexpected toxicity.

A.5.8 Immunological activity

An immunological activity test (MIT, MICA, or approved alternative) should be carried out as described in section A.3.4.2.7, on each final lot, if such a test has not been conducted on the final bulk.

A.5.9 Inspection of final containers

Each container in each final lot should be inspected visually or mechanically, and those showing abnormalities should be discarded.

A.6 Records

The recommendations given in Good manufacturing practices for biological products (22) should apply.

A model of a suitable summary protocol to be used for pertussis vaccines is given in Appendix 6.

A.7 Samples

The recommendations given in Good manufacturing practices for biological products (22) should apply.

A.8 Labelling

The recommendations given in Good manufacturing practices for biological products (22) should apply, with the addition of the following:

- the words acellular pertussis vaccine;
- the word “adsorbed”;
- the name and address of the manufacturer;
- the recommended storage temperature and the expiry date if kept at that temperature;
- the recommended single human dose and route of administration.

In addition, the label printed on or affixed to the container, or the label on the carton, or the leaflet accompanying the container should contain the following:

- a statement that the vaccine satisfies the recommendations of this document;
- the nature and amount of any preservative present in the vaccine (if there is no preservative in single-dose containers, this should be stated);
- the nature and amount of the adsorbing agent, if applicable;
- the nature and amount of any substances added to the vaccine;
- the recommended conditions for storage and transport;
- a warning that the vaccine should not be frozen;
- a warning that the vaccine should be shaken before use;
- instructions for the use of the vaccine, and information on contraindications and the reactions that may follow vaccination.

A.9 Distribution and shipping

The recommendations given in Good manufacturing practices for biological products (22) should apply.

A.10 Stability, storage and expiry date

A.10.1 Stability

Stability evaluation is an important part of the quality assessment. The purpose of stability studies is to ensure that the vaccine at the end of its shelf-life, storage

period or period of use, still has the required characteristics supporting quality, safety and efficacy. The recommendations given in the WHO Guidelines for stability evaluation of vaccines should apply (36).

Adequate stability studies form an essential part of vaccine development. The stability of the vaccine in its final containers, maintained at the recommended storage temperature, should be demonstrated to the satisfaction of the NRA.

For each of the antigens claimed to contribute to protective efficacy, real-time stability studies should support immunological activity and lack of specific toxicity of the product up to the expiry date.

The product must be manufactured in such a way that reversion to toxicity of the inactivated PT in the vaccine does not occur during the period of validity provided that the product is stored under the conditions stated on the label.

The desorption of antigens from the adjuvant, which may occur over time, should be investigated and, where possible, limits should be agreed with the NRA.

Accelerated stability studies may provide additional evidence of product stability, but cannot replace real-time studies.

When any changes that may affect the stability of the product are made in the production procedure, the stability of the vaccine produced by the new method should be demonstrated.

A.10.1.1 **Stability for licensure**

Studies that support stability of a vaccine for the purpose of licensure have to be performed as real-time studies under the intended storage conditions. Stability-indicating parameters should be carefully selected. They should always include, but should not be limited to, the tests for immunological activity. Tests should be conducted at appropriate time intervals during storage to determine the loss of immunological activity. Final containers from at least three lots of vaccine derived from different bulks should be tested on the expiry date to demonstrate stability during storage.

Accelerated stability data for products stored for limited periods at temperatures that may affect stability may support preliminary data from ongoing real-time stability studies but should not replace them. Any modification of the shelf-life approved as part of licensure requires additional stability data to support proposed modification and should be approved by the NRA. Following licensure, stability should be monitored throughout the proposed shelf-life.

A.10.1.2 **Stability at different stages of the manufacturing process**

Stability testing should be performed at different stages of production, namely single harvests, bulk materials, final bulk and final lot on at least three lots each. Suitable parameters indicating stability should be selected according to the stage of production. Manufacturers are encouraged to assign a shelf-life to all materials

during vaccine production, particularly intermediates such as single harvests, purified antigen bulk and final bulk.

A.10.1.3 **Stability for clinical trial approval**

For vaccines under development, stability data, such as those described above, are expected for the purpose of clinical trial approval. However, for such vaccines under development, the stability data are generally available for a limited period. Appropriate documentation to support the stability profile of a vaccine should be submitted to the competent NRA at all stages mentioned above.

A.10.2 **Storage conditions**

Recommended storage conditions and defined maximum duration of storage should be based on stability studies, as described in section A.10.1 above, and approved by the NRA. For acellular pertussis vaccines, a temperature of 2–8 °C is generally considered to be satisfactory. This should ensure that the minimum potency specified on the label of the container or package will be maintained after release and until the end of the shelf-life if the conditions under which the vaccine is stored are in accordance with what is stated on the label.

The manufacturer should recommend conditions of storage and transport that ensure that the vaccine satisfies the potency requirements until the expiry date stated on the label.

The vaccine must not be frozen.

A.10.3 **Expiry date**

The expiry date should be defined on the basis of shelf-life supported by the stability studies, as described in section A.10.1 above, and approved by the NRA.

Part B. Nonclinical evaluation of acellular pertussis vaccines

Nonclinical testing of vaccines is a prerequisite for initiation of clinical studies in humans. There is no laboratory test or series of tests that will unequivocally assure that a newly developed acellular pertussis vaccine will be adequately safe and effective. With this limitation, these Recommendations describe a sequential approach to the collection of supporting evidence, beginning with a comprehensive programme of nonclinical testing, followed by a progression of clinical evaluations. This part describes the recommended nonclinical testing. The extent to which nonclinical studies will be required depends on the clinical experience that has already been gained with the different vaccine components. Animal studies, which aim to provide evidence that the vaccines induce functional

immune responses (e.g. induction of PT neutralizing antibodies, protection against bacterial challenge), form an essential part of the development of the vaccines. For vaccines containing acellular pertussis components that have not previously been evaluated for efficacy in clinical trials, the results of nonclinical testing represent only a part of the aggregate of data that needs to be considered when assessing the likelihood that the vaccine will prove to be effective when used in the clinical setting. Other considerations include manufacturing methods, control of the manufacturing process and clinical immunogenicity of the vaccine.

New vaccine formulations that have not been evaluated in safety and efficacy trials require extensive characterization, including assessment in vaccination/challenge studies in animal models (proof of concept) and safety testing in animals. However, extensive nonclinical testing may not be required for vaccines that use pertussis antigens that are the same (i.e. from the same manufacturer and produced by the same methods) as those in vaccines that have already been approved on the basis of their safety and efficacy.

For vaccines based on novel pertussis antigens or on formulations for which the pertussis components are produced using a new manufacturing process that is different from the established one, the characterization should include detailed evaluation and testing of: 1) the purified antigens prior to chemical treatment (e.g. detoxification), 2) the individual antigens prior to formulation, and 3) the formulated product. Although characterization is more difficult for pertussis antigens that are co-purified than for those that are individually purified, co-purified antigens should undergo similar evaluation and testing before and after chemical treatment (e.g. detoxification) and in the final formulated product.

Lots of individual antigens and formulated vaccine used in nonclinical studies should be adequately representative of those intended for clinical investigation and, ideally, should be the same lots as the ones to be used in clinical studies. If this is not feasible, then the lots used clinically should be comparable to those used in the nonclinical studies with respect to manufacturing, immunological activity, stability and other characteristics of quality. Details of the design, conduct, analysis and evaluation of nonclinical studies are available in the WHO Guidelines on nonclinical evaluation of vaccines (37).

B.1 Nonclinical characterization and testing of pertussis antigens and in-process materials

In the case of vaccines for which the acellular pertussis antigen is new (in terms of range of antigens and/or manufacturing processes for one or more antigens) an extensive preclinical evaluation should be undertaken. This should include thorough characterization of purified antigens before and after any chemical treatment, as well as any relevant in-process intermediates and final product materials. Characterization should evaluate purity, integrity and functional activity using a variety of approaches including physicochemical evaluation,

bioassays, measurement of residual toxicity/lack of reversion to toxicity, and active protection models. Given the complexity of acellular pertussis vaccines produced by co-purification methods, additional testing to fully define and specify the composition should also be conducted. For example, an assessment of the proportion of each antigen (e.g. the PT:FHA ratio) should be established to characterize clinical lots and to monitor product consistency.

The inclusion of a novel antigen – i.e. an antigen other than those that have already been tested in previous clinical efficacy trials (i.e. PT, FHA, PRN, FIM) – would require additional considerations. So would, for instance, the inclusion of the PRN antigen purified from *B. bronchiseptica* since this is not the same antigen as PRN from *B. pertussis*. When possible, the individual antigens should be evaluated in active protection animal models (16, 18, 19, 38, 39). Pertussis toxoid (PTxd) is effective in most active protection models, and therefore the demonstration of an additional benefit for antigens mixed with PTxd may be challenging. In such cases, the antigen should be examined in protection models with either no PTxd or suboptimal amounts of PTxd. The other antigens (FHA, PRN and FIM) are not necessarily active in all protection models, so it is important to consult the literature (see Table A4.1 below) to identify relevant models for each antigen.

The following describes the testing strategy that could be considered for the antigens that are contained in currently approved acellular pertussis vaccines, either produced by co-purification processes or by individual purifications of each component (PTxd, FHA, PRN, FIM2 and FIM3). When included in an acellular pertussis vaccine, the FIM2 and FIM3 are typically co-purified and processed as a single antigen (FIM2/3).

PT: purity and bioactivity of PT before chemical treatment, residual bioactivity after chemical treatment, lack of reversion to toxicity, activity in animal protection models, binding and functional activity of antibodies induced in animals, detection of known epitopes using monoclonal antibodies.

FHA: purity and functional integrity (e.g. haemagglutinating activity) of FHA before chemical treatment, residual activity after chemical treatment, activity in animal protection models, binding activity of antibodies induced in animals, detection of known epitopes using monoclonal antibodies.

PRN: purity of PRN, activity in animal protection models, binding activity of antibodies from immunized animals, detection of known epitopes using monoclonal antibodies.

FIM2, FIM3, or FIM2/3: purity of FIM, relative content of FIM2:FIM3, activity in animal protection models, binding and whole-cell agglutinating activity of antibodies from immunized animals and detection of known epitopes using monoclonal antibodies.

Special consideration should be given to vaccines based on genetically inactivated PT. Characterization studies of these vaccines should include evaluation of genetic stability of the production strain, consistency of the genetic sequence and attenuation of the toxic bioactivity (40).

Table A4.1 provides an overview of results from the published literature. The specific references should be consulted for details and additional information.

Table A4.1

Summary of published studies evaluating the ability of purified pertussis antigens to protect in mouse challenge models

Animal model protection studies					
Antigen	Respiratory challenge models		Intracerebral challenge models		
	Purified antigen		Purified antigen		Purified antigen plus small, nonprotective, amount of active PT
	Active immunization	Passive immunization	Active immunization	Passive immunization	Active immunization
PTxd	Yes References: 18, 19, 41–47	Yes References: 17, 43–51	Yes References: 17, 19, 31, 42, 44, 45	Yes References: 48, 49	Yes Reference: 31
FHA	Yes References: 18, 19, 44–46, 52–57	Yes References: 17, 44, 45, 51, 55	No References: 17, 19, 31, 44–46	No Reference: 39	Yes Reference: 31
PRN	Yes References: 53, 58–61	Yes Reference: 61	?	?	?
FIM	Yes References: 45, 62–65	Yes Reference: 45	No References: 31, 45, 65	?	Yes References: 31, 65

PT = pertussis toxin; PTxd = pertussis toxoid; FHA = filamentous haemagglutinin; PRN = pertactin; FIM = fimbriae; "Yes" = protection was demonstrated; "No" = protection was not observed; "?" = no information was found.

Despite advances in knowledge regarding the mechanisms of toxicity of PT and other potentially reactogenic components produced by *B. pertussis*, uncertainty remains concerning the exact role played by these substances in the pathogenesis of pertussis and in vaccine reactions. This lack of information has hampered the establishment of scientifically sound limits for the residual activity of these components in vaccines containing pertussis antigens. However, vaccines containing chemically or genetically inactivated PT require thorough characterization to assess residual PT activity and, where appropriate, the possible reversion of this toxoid during storage. Manufacturers should demonstrate to the satisfaction of the NRA that chemically inactivated PT present in the final bulk does not revert to its toxic form before the vaccine expiry date. In addition, as part of the validation of the manufacturing process, manufacturers are required to submit evidence that the purification steps reduce the levels of LOS endotoxin, as well as the residual activities of heat-labile (dermonecrotic) toxin, tracheal cytotoxin and adenylate cyclase toxin, to acceptable levels.

In some countries, during development of the vaccine, the production process should be validated to demonstrate that it yields consistently an antigenic fraction that complies with the purity requirements listed below. After demonstration of consistency, the tests need not be applied routinely to each lot (66).

Adenylate cyclase: not more than 500 ng in the equivalent of 1 dose of the final vaccine, determined by immunoblot analysis or another suitable method.

Tracheal cytotoxin: not more than 2 pmol in the equivalent of 1 dose of the final vaccine, determined by a suitable method such as a biological assay or liquid chromatography.

Absence of residual dermonecrotic toxin: inject intradermally into each of three unweaned mice, in a volume of 0.1 ml, the amount of antigenic fraction equivalent to 1 dose of the final vaccine. Observe for 48 hours. No dermonecrotic reaction is demonstrable.

The mouse weight-gain test and the leukocytosis promotion test, which are currently used to monitor the toxicity of whole-cell pertussis vaccines, are considered to be of insufficient sensitivity to demonstrate residual PT activity in acellular pertussis vaccines. Specific tests for residual PT activity (see sections A.3.3 and A.3.4.2.5) are preferred.

B.2 Nonclinical characterization and testing of final vaccine formulation

Given that there are currently no stand-alone acellular pertussis vaccines, the following studies should be carried out using the final formulation (i.e.

formulation that includes diphtheria and tetanus toxoid and other components). The capacity of an acellular pertussis vaccine to protect mice against a *B. pertussis* challenge may be used to establish a nonclinical proof of concept for new vaccine formulations. Two models have been developed to assess acellular pertussis vaccines: the MICA (Part A and Appendix 1) and the INCA (by instillation or by aerosol) (Part A and Appendix 5). However, as noted above (section B.1), PTxd is an effective antigen in both models; therefore, for vaccines that contain PTxd, the contribution of antigens other than PTxd may be difficult to discern in these models. Moreover, because residual active PT can influence the outcome of the MICA (32), care should be taken in interpreting the results of that assay. The assessment of functional antibodies, such as PT-neutralizing antibodies as evaluated in the CHO cell assay (for anti-PT) or whole-cell agglutinating antibodies (for anti-FIM), would provide further nonclinical evidence of the potential protective efficacy against *B. pertussis* in humans.

Additional toxicity and other testing should follow the recommendations outlined in the WHO guidelines on nonclinical evaluation of vaccines (37).

Part C. Clinical evaluation of acellular pertussis vaccines

This part of the Recommendations provides guidance on issues related to the design and evaluation of clinical studies. Most studies are expected to be comparative in nature, so the choice of a comparator vaccine is discussed in some detail. Importantly, only those vaccines with adequate nonclinical testing should be considered for clinical evaluation, with the local NRA being responsible for evaluating adequacy of nonclinical information. Because efficacy trials appear very difficult, the trials for assessing safety and immunogenicity are emphasized.

Clinical evaluations conducted over the past 30 years provide models for the clinical evaluations of new vaccines. Most importantly, in the period between 1986 and 1996, several acellular pertussis-containing vaccines, including both vaccine types (vaccines composed of purified antigens and vaccines composed of co-purified antigens) were evaluated in a series of efficacy trials. In the first acellular pertussis vaccine efficacy trial in Sweden (1986–1987), a PTxd and a PTxd/FHA vaccine from the same Japanese manufacturer were evaluated (67). The efficacy estimates for the primary case definition (culture-confirmed pertussis with at least one day of cough) were 69% (95% CI 47–82) for the two-component vaccine and 54% (95% CI 26–72) for the PTxd alone. Secondary analyses of this trial revealed the critical importance of case definition, in particular the marked influence on vaccine efficacy estimates of the laboratory and clinical criteria used to define a case. For example, markedly different efficacy estimates for the one- and two-component vaccines could be obtained depending on whether or not mild

clinical cases were included (68). To address the problems with case definition, WHO convened an expert group in 1991 to recommend case definitions that could be used for subsequent efficacy studies (69). The recommended primary case definition required 21 days of paroxysmal cough and laboratory confirmation by culture, serology or household contact with a confirmed case. However, because this primary case definition provided incomplete information, evaluation of secondary end-points was strongly encouraged. The evaluation of efficacy against milder illness (e.g. fewer than 21 days of paroxysmal cough) was considered of particular importance.

Additional trials were conducted between 1991 and 1996 (11, 12, 70–79). In these trials, DTaP vaccines containing 1–5 pertussis components were investigated. Different study designs were employed in these trials, namely: 1) randomized placebo-controlled cohort trials, 2) household contact studies, and 3) case–control studies. The different calculated vaccine efficacies were affected by study design as well as case definition. The most reliable estimates of absolute vaccine efficacies were obtained for those trials that used a double-blind format with an unvaccinated control group (80). Blinding was not possible in the case–control studies and in most of the household contact studies, and thus the efficacy estimates for such trials have more potential for bias. The exceptions were household contact studies which were nested within some randomized controlled cohort trials.

This series of trials revealed that all the tested acellular pertussis vaccines protected children against pertussis to at least some degree (11, 12). However, unless the vaccines were tested in parallel within the same trial, comparing the efficacy of the different acellular vaccines must be done with caution, as all the trials varied with respect to design, case ascertainment methodology, and case definition. For instance, in placebo-controlled cohort trials, culture-confirmation was more likely to occur in unvaccinated than in vaccinated subjects, leading to inflated vaccine efficacy estimates. This bias was overcome to a great extent by employing appropriate serological tests to confirm the cases. Similarly, mild cases were proportionally more frequent in vaccinees than in controls; thus efficacy estimates were inflated when milder cases were excluded or were deflated when they were included. Some of the randomized placebo-controlled cohort trials investigated two different acellular pertussis vaccines and, from these, some comparisons could be made. In two studies, an acellular pertussis vaccine containing five components provided better protection than the specific (and never licensed) two-component vaccine included in that trial (72, 75). However, the vaccine composition that optimally protects against both mild and severe disease remains uncertain. Epidemiological investigations have shown that disease has been controlled by vaccines of varying composition (1, 10, 81, 82).

Several of these trials included both whole-cell and acellular pertussis vaccines. Some tested whole-cell vaccines provided less protection than the acellular vaccines (71, 72). However, in other trials other whole-cell vaccines appeared to be more efficacious than most acellular vaccines, particularly against mild pertussis (75, 77). Heterogeneity among whole-cell vaccines has been reported since the 1950s, and emphasizes the importance of monitoring the efficacy of any whole-cell or acellular pertussis immunization programme.

Two of the efficacy trials were designed to determine the antibody values at the time of exposure, and thus were able to evaluate whether the presence of specific antibodies was correlated with protection from disease (13, 14). Both studies showed that the presence of antibodies to PRN, PT and fimbriae correlated with protection. Neither study, however, found a correlation with antibody to FHA. However, a role for an immune mechanism other than serum antibody cannot be ruled out for this antigen.

Following the completion of these trials, many countries began exclusive use of acellular pertussis vaccines. Several studies have attempted to evaluate the duration of protection (82–84). To date, studies support a conclusion that efficacy is retained for at least five years after a three- or four-dose series of acellular pertussis vaccine. Further evaluations should be able to define the duration of protection more clearly and thus provide guidance to public health officials on the optimal time for administration of booster doses.

With respect to safety, several head-to-head studies have demonstrated that primary immunization with DTaP vaccines caused fewer local reactions and less fever than DTwP vaccines (11, 12). However, no clinically significant differences in safety have been demonstrated among acellular pertussis vaccines with differing numbers of components. Several studies evaluating booster doses have indicated that the frequency of significant redness and swelling (e.g. redness and/or swelling greater than five centimetres or swelling of the entire limb) increases in those subjects who have received multiple doses of DTaP vaccines. With respect to more serious events, the literature provides no reliable basis for a causal relationship between vaccination and the handful of other serious adverse effects described in case reports or national adverse event reports (12).

C.1 General considerations for clinical studies

This section addresses some issues that are specific to, or particularly relevant to, the clinical development of new acellular pertussis vaccines. The recommendations made should be considered in conjunction with the general principles described in the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (85).

These Recommendations should be viewed in the light of further data on the safety, immunogenicity and effectiveness or any relevant data on other types of acellular pertussis vaccines that may become available in the future.

Manufacturers should provide justification for the choice of the vaccine formulation (e.g. the pertussis components included in the formulation) and the design of the clinical development programme used to evaluate the vaccine, including the size of studies and the end-points for evaluation.

For vaccines that contain new acellular pertussis vaccine components rather than established components – with established components defined as the same pertussis purified antigen(s) manufactured by the same company, using the same process, and formulated in the same way as components tested in clinical efficacy studies – the immunogenicity data obtained in clinical studies should be considered along with manufacturing information and nonclinical data when assessing the likelihood that the vaccine will prove to be effective in the clinical setting. Clinical investigations should be initiated only with vaccines that have undergone thorough nonclinical evaluations, as described in Part B. Consistency of manufacturing for the vaccine lots used in clinical trials should be demonstrated and well documented. It is expected that several lots with the same formulation intended for marketing will be used in the late stages of the clinical development programme.

C.1.1 **Scope of the studies**

Placebo-controlled protective efficacy studies are no longer feasible for ethical reasons, and trials designed to measure efficacy relative to that of a licensed acellular pertussis vaccine with proven efficacy would need to be very large in order to provide adequate precision in the relative efficacy estimates. Therefore, the approval of any new acellular pertussis vaccines would be likely to rely on data from comparative immunogenicity and safety trials of adequate size and design to provide reasonable assurance of their clinical benefit. When applicable and when relevant, studies documenting the performance of the investigational vaccine when co-administered with other routinely recommended infant and toddler vaccines should be performed.

Infants less than three months of age are at highest risk of hospitalization and death from pertussis. There is a growing interest in approaches that could provide improved protection for these very young infants. Such approaches, include, for example, the development of stand-alone acellular pertussis vaccines to be used as a birth dose and the immunization of mothers before or during pregnancy. However, the unique considerations that apply to the clinical evaluation of vaccines in these special populations are not included in this document.

C.1.2 Comparator vaccine

The predictive relationship between the concentration of antibody induced and protection against pertussis has not been established for each antigen. Therefore, one of the critical aspects when designing clinical trials for licensure is the choice of a comparator vaccine. When doing this, the points made below should be considered.

The choice of the comparator vaccine will vary according to the characteristics of the new acellular pertussis vaccine under development. However, the comparator should in general be the vaccine most similar to the new vaccine with respect to content and composition of the acellular pertussis component. Three potential scenarios are proposed when assessing a new vaccine formulation:

- **Scenario 1** – the new acellular pertussis combination vaccine contains an established acellular pertussis component (i.e. same purified pertussis antigen(s) manufactured by the same company using the same processes, and formulated the same way) that has been found suitably efficacious in a clinical efficacy trial. In this scenario, the most appropriate comparator vaccine would be the most similar licensed product from same manufacturer. This scenario applies to the evaluation of different combination vaccines based on the same DTaP components (e.g. DTaP-IPV, DTaP-HepB, DTaP-HepB-Hib etc.) or DTaP with different amounts of pertussis components (e.g. booster formulations Tdap). The evaluation of a combination vaccine in this scenario is based on a non-inferiority clinical trial of immune responses relative to the separately administered licensed DTaP or DTaP-based combination vaccine.
- **Scenario 2** – the new acellular pertussis-containing vaccine has a composition which is the same as, or very similar to, that of an established acellular pertussis component that has been found suitable in a clinical efficacy trial. However, some or all antigens are made by a different manufacturer and/or by a different process than the vaccine tested in a previous protective efficacy study. In this scenario, the most appropriate comparator would be the licensed product (with proven efficacy) with matching composition (same acellular pertussis antigens, similar amounts).

NOTE: Vaccines evaluated in efficacy studies include one-component (PTxd) (67, 70), two-component (PTxd/FHA) (67, 72, 74, 75, 77), three-component (PTxd/FHA/PRN) (71, 73, 75), four-component (PTxd/FHA/PRN/FIM2) (78), and five-component (PTxd/FHA/PRN/FIM2/FIM3) (72, 75) acellular pertussis vaccines.

- **Scenario 3** – the new acellular pertussis-containing vaccine has an acellular pertussis antigen composition that is not the same as that of an already licensed acellular pertussis vaccine that has been found suitably efficacious in a clinical efficacy trial. There are at least two ways this could occur, namely: 1) the vaccine could be based on the currently used antigens but present in different combinations such as PTxd/PRN or PTxd/PRN/FIM2/FIM3, or 2) the vaccine could include novel antigens in combination with one or more of the currently used antigens. In this scenario, the most appropriate comparator would be the licensed product (with proven efficacy) with the most similar composition.

It is important to highlight that, for scenarios 2 and 3, manufacturers should justify the choice of comparator vaccine and the non-inferiority margin used, particularly when there are differences in the content and composition of the acellular pertussis components.

C.2 **Assessment of immune responses**

C.2.1 **Assays to assess antibody responses**

Serological assays used in clinical immunogenicity studies in support of vaccine licensure require validation (85). An international reference pertussis antiserum has been established to assist in the standardization of serological methods (86). Thus, to ensure the comparability and acceptability of the serological data across trials, results of immunogenicity should be expressed in IU in reference to this International Standard for pertussis antiserum. A rigorous assessment of assay specificity is essential prior to initiating validation studies. Formal validation should assess all appropriate performance criteria – including accuracy, linearity, precision and range – and robustness studies are also recommended. The validation studies should be designed to demonstrate that the range (including the lower limit of quantitation) is suitable for the clinical study, and should consider the way in which the vaccines are to be compared to each other (e.g. whether the criteria for evaluation are based on percentages with post-primary series titres above a threshold, seroconversion rates or geometric mean antibody titres).

The immune response in clinical trials should be assessed by using a small range of validated assays. Selection of the assays for evaluation of the immune response to the vaccine should be justified by the vaccine developer. When feasible, assays that measure functional immune responses should be employed. Suitable assays are unlikely to be commercially available.

Specific antibody responses to different components of the vaccine (e.g. PT, PRN, FIM etc.) can be assessed by methods that measure the concentration

of antibody binding to a specific antigen (e.g. ELISA) or, when applicable, the functional biological activity by measuring the PT neutralizing titre (e.g. CHO cell assay) (87) or the *B. pertussis* agglutination titre (88–90).

Cell-mediated immune (CMI) responses play a role in protecting against *B. pertussis* infection. However, immunological assays to evaluate CMI responses following immunization have not been standardized and have not been used to support licensure. Nevertheless, the exploratory assessment of CMI should be encouraged in order to enlarge the body of knowledge regarding all aspects of the immune response to pertussis antigens.

C.2.1.1 ELISA to assess antibody responses to acellular pertussis components

The assessment of antibody responses to specific pertussis components included in the vaccine should be regarded as the primary means of assessing the immune response to new acellular pertussis vaccines. The standardization of serological methods for *B. pertussis* has been pursued not only for the purpose of licensure of new pertussis vaccines but also for clinical diagnosis of pertussis infection and for seroepidemiological studies (91). However, it is important to note that assays developed and optimized for diagnostic and epidemiological purposes, including most commercially available ELISA kits, are unlikely to have the performance characteristics needed for vaccine immunogenicity studies. For instance, diagnostic kits are unlikely to provide the specificity required to assess each of the pertussis components individually (e.g. PT, FHA, etc.) and the accuracy to determine geometric mean concentration (GMC) (92).

Collection, recording, analysis and interpretation of data should be conducted according to good clinical practice guidelines (93). Methodological and statistical considerations described in WHO guidelines should be taken into account (85).

C.2.1.2 Assessment of functional antibody titres

Functional activities of antibodies against pertussis components have been identified as important additional parameters to consider, particularly when evaluating new formulations containing PTxd and FIM which are known to induce antibodies with functional activity such as toxin neutralization and bacterial agglutination respectively (89). Assays to measure PT neutralizing and whole-cell *B. pertussis* agglutinating antibodies have been established (87, 88, 90). Although no functional thresholds have been found to correlate directly with the protective efficacy of pertussis vaccines, there are nevertheless important immune parameters to determine as part of the overall comparison of new vaccine formulations to those proven to be safe and effective.

When feasible, functional antibodies should be measured, at a minimum, in a subpopulation of the comparator and test vaccine groups.

C.2.2 Criteria for evaluation of immune responses

The preferred method for evaluating new vaccine formulations is the direct clinical comparison of licensed vaccines with proven pre-licensure clinical efficacy, with the new product through randomized controlled trials.

C.2.2.1 Primary immunization of infants and young children

In comparative studies of post-primary immune responses, the main analysis will be based on demonstrating that the response in subjects immunized with the test vaccine is not inferior to that in the comparative vaccine group(s). The selection of the primary parameter for the assessment of non-inferiority, the predefined margin of non-inferiority and hence the total sample size for a comparative study, will need careful justification (85). Although studies that compare immune responses between candidate and licensed acellular pertussis vaccines are essential, comparisons with historical data that were generated during previous protective efficacy studies using similar assays may be used to provide supportive evidence. While a demonstration that the new candidate vaccine is immunogenic in humans is important, the data should be interpreted with some caution. In particular, when evaluating immunogenicity data comparing vaccines produced by different manufacturers or produced using different methods, equivalent efficacy cannot be directly inferred from equivalent immunogenicity.

The study objectives must be taken into account when defining appropriate time intervals for assessing the immune response. In most cases, clinical studies for new vaccines for infants are designed to determine the antibody response to acellular pertussis components at approximately four weeks following the final dose. Predefined non-inferiority criteria using an appropriate acceptability limit are used to compare the responses in subjects immunized with the test vaccine versus the licensed comparator vaccine (see section C.1.2) using the end-points described below.

The following co-primary analyses are recommended:

- **Percentage of responders** – in one primary analysis, the percentage of responders with a significant increase (e.g. fourfold increase) above pre-immunization for each of the acellular pertussis components is compared between subjects immunized with test vaccine and the licensed comparator vaccine. Alternative definitions for responders could be considered if well justified. The groups should be compared using an appropriate predefined non-inferiority limit; generally the lower bound of the two-sided 95% confidence interval of the observed difference should not be less than the criterion approved by the NRA, most commonly 10 percentage points.

- **Magnitude of the response** – in a second primary analysis, the magnitude of the response is compared – on the basis of the GMC induced by the new vaccine and the licensed comparator using a predefined margin of non-inferiority – to evaluate the antibody response (GMC ratios) to each acellular pertussis component. The lower bound of the two-sided 95% confidence interval of the observed ratio of the GMC of the new vaccine relative to the control should not be less than the criterion approved by the NRA (most commonly 0.50 or 0.67).

In case of failure to meet the predefined non-inferiority criteria, detailed investigation of the immune response and the reasons for not meeting the criteria may be considered. In particular, the NRA may take into consideration the results from the antibody responses to each of the antigens, as well as any differences in composition between the test and the comparator vaccines, and the available information about the contribution of that antigen (i.e., the antigen to which the antibodies are directed) in protection.

C.2.2.1.1 *Secondary analysis*

Functional antibody response. When evaluating new vaccine formulations, it is important to assess as many immune parameters as possible. Therefore, functional antibody responses (i.e. PT neutralizing titres or *B. pertussis* agglutination titre if PTxd or FIM are part of the formulation, respectively) should be determined in a randomized subset of vaccinated subjects within some or all of the clinical studies. At present, the interpretation of functional antibody data is made difficult by the fact that a titre that might correlate with protection against pertussis infection is unknown. For this reason it is recommended that comparisons of functional antibody titres between the new vaccine and the licensed comparator should focus on GMT ratios.

C.2.2.1.2 *Additional information*

Reverse cumulative distribution (RCD) curves (94). Use of RCD curves which display the accumulated proportion of individuals with an antibody concentration greater than or equal to a given level have been shown to be useful when comparing the response to the test and licensed comparator vaccines. RCD plots should be generated for ELISA data and for functional antibody response. The review of these data should be viewed as exploratory.

It is recommended that subsets of subjects are identified for longer-term follow-up of persistence of immunity. These data may be provided after first approval. Waning of antibody concentrations over time is inevitable and should not be interpreted per se to indicate the need for a booster dose. It is important

that longer-term antibody concentrations are viewed in conjunction with observed effectiveness data in order to assess the potential need for additional doses later in life to maintain protection.

C.2.2.2 **Booster immunization of older children, adolescents and adults**

In most instances, the emphasis for initial use and evaluation will be for the primary immunization of infants. However, acellular pertussis vaccines may also be used for booster doses in the second year of life, and in preschool children, adolescents and adults. Currently, different immunization schedules are employed in different countries for primary and booster immunization. In all cases, the chosen schedule should be supported by appropriate immunogenicity studies. Previous experience has demonstrated that vaccines intended solely for booster immunization may require lower amounts of one or more of the pertussis antigens.

An active comparator vaccine may not be needed when evaluating the immunogenicity of the acellular pertussis components used as a booster vaccine, such as in older children and adults. In such cases, it may be possible to compare the immune response of adolescents and adults following a single dose to that of infants who received primary immunization with the corresponding DTaP vaccine (historical comparator). In addition, the ability of these vaccines to induce immunological memory assessed by an anamnestic response following immunization should be evaluated for each acellular component.

C.2.3 **Combined vaccines and concomitant administration with other vaccines**

C.2.3.1 **Combined vaccines**

In the case of combination of acellular pertussis components with other antigens, potential interference between the pertussis components and the other antigens and/or excipients should be investigated, as described in the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (85).

In particular, it has been demonstrated that some acellular pertussis components can have a negative impact on immune responses to some polysaccharide conjugated antigens when administered in pre-formulated products or when vaccines are mixed only immediately before injection (e.g. *Haemophilus influenzae* type b conjugate vaccine responses in some combination products containing PRP-T and some acellular pertussis components). Therefore, the immune responses to all the antigens in the final combined formulation should be shown to be satisfactory through well-designed randomized comparative trials. If there is any immune interference observed with respect to any of the combined antigens, the possible clinical implications should be carefully considered before proceeding with clinical development.

C.2.3.2 Concomitant administration with other vaccines

In recent years, it has become apparent that concomitant administration of acellular pertussis components with other vaccines in routine use, including conjugated vaccines, may give rise in some situations to detectable immune interference, although the clinical significance of the observed phenomena is not always clear. Examples include decreased antibody responses to *Haemophilus influenzae* type b conjugate vaccine and to meningococcal C monovalent vaccine. Thus it is important that immune responses to candidate acellular pertussis vaccines should be evaluated on co-administration with other vaccines that are representative of types that, for convenience and compliance reasons, are very likely to be given at the same clinic visits. Responses to other co-administered antigens should also be evaluated. The approach to these studies is based primarily on demonstrating non-inferiority of responses to antigens when vaccines are co-administered, compared to each vaccine given alone, with careful justification of predefined non-inferiority margins.

These studies might compare concomitant administration with administrations made in a staggered fashion (e.g. together at two, four and six months compared to the usual antigens at this schedule and the new vaccine at three, five and seven months).

C.3 Safety evaluation

As stated in section C.1.1, placebo-controlled efficacy studies which would also deliver a large safety database are not feasible. Nevertheless, the pre-licensure assessment of vaccine safety is a critically important part of the clinical programme, and should be developed to meet the general principles described in the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (85). A comparison of rates of adverse events between test and approved comparator vaccines is commonly a predefined secondary end-point in study protocols. The minimum acceptable size of the safety database at the time of approval should take into account the vaccine composition (including all antigens and adjuvants), the presence of novel antigens, and any past experience with vaccines with the same or similar composition of the acellular pertussis component.

For new vaccines, a total safety database (combined from all trials in the same targeted age group) of approximately 3000–5000 subjects is commonly expected because this allows for the evaluation of uncommon adverse events – i.e. those that occur at a rate between 1 in 100 and 1 in 1000 subjects (85). However, depending on the data available for the vaccine, the NRA may accept a smaller number or may request a larger database prior to first approval.

Information on adverse events such as extensive limb swelling syndrome should be carefully monitored in studies evaluating the safety of booster doses.

C.4 Post-marketing studies and surveillance

Every effort should be made to improve current scientific understanding of the protection in humans afforded by acellular pertussis vaccines by providing data from active post-marketing surveillance. Vaccine effectiveness in the population should be reported wherever possible. In addition, given that limited safety data are obtained in pre-licensure studies, all relevant safety-indicating parameters should be monitored as part of post-marketing surveillance programmes. In particular, the impact of routine vaccination on pertussis infection needs to be assessed in comprehensive studies of vaccine performance. Ongoing surveillance programmes should be in place to monitor for longer-term protection and for evidence of any changes in vaccine effectiveness.

In reality, sound and comprehensive safety and effectiveness data cannot be collected by the manufacturers alone. Therefore, there should be discussions between vaccine manufacturers responsible for placing the product on the market and national and international public health bodies regarding the feasibility of estimating effectiveness and safety in the post-marketing period. Reliable estimates of effectiveness can be obtained only in geographical locations where appropriate vaccine campaigns are initiated and where there is already a suitable infrastructure in place to identify cases of pertussis disease.

General WHO guidelines for continued oversight of vaccines after licensure should be followed (85). All data collected should be submitted to the responsible NRAs at regular intervals so that any implications for the marketing authorization can be assessed.

Part D. Guidelines for NRAs

D.1 General

The general recommendations for control laboratories contained in the Guidelines for national authorities on quality assurance for biological products (95) and Guidelines for independent lot release of vaccines by regulatory authorities (96) should apply.

The detailed production and control procedures and any significant changes in them should be discussed with and approved by the NRA.

Consistency of production has been recognized as an essential component in the quality assurance of acellular pertussis vaccines. In particular, the NRA should carefully monitor production records and quality control test results for clinical lots as well as a series of consecutive lots of the final bulk.

D.2 Official release and certification by the NRA

A vaccine should be released only if it fulfills national requirements and/or satisfies Part A of these Recommendations.

A statement signed by the appropriate official of the NRA should be provided at the request of the manufacturing establishment and should certify that the lot of vaccine in question satisfies all national requirements as well as Part A of the present Recommendations. The certificate should state the number under which the lot was released by the NRA, and the number appearing on the labels of the containers. The official national release document should be provided to importers of pertussis vaccines.

The purpose of the certificate is to facilitate exchange of pertussis vaccines between countries. A model of a suitable certificate is given in Appendix 7.

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Appendix 1

Modified intracerebral challenge assay

1. Materials

Strain 18323 of *Bordetella pertussis* (hereafter referred to as the challenge strain in this appendix) should be used for challenge. The diluent for the test sample and standard should be sterile physiological saline (0.85% NaCl).

The challenge strain should be cultured on Bordet–Gengou medium for approximately 24 hours and suspended in 1% w/v casamino acid solution containing 0.6% w/v of sodium chloride (pH 7.0–7.2) to a concentration of approximately 200 LD₅₀ per 0.025 ml (or approximately 1×10^5 organism/challenge dose) to serve as the suspension for challenge. Alternatively, a stable frozen stock can be used for direct challenge after appropriate dilution.

2. Test procedures

The test sample and standard should be diluted serially to make at least three levels of fourfold or other suitable logarithmic dilutions. Each dilution should be given by intraperitoneal injection at a dose of 0.5 ml to at least 16 mice aged approximately four weeks. The animals should be of the same sex or both sexes in equal numbers for each dose. The challenge suspension should be given by intracerebral injection into the animals at a dose of 0.025 ml 21 days after immunization. The animals should be observed for 14 days. Any animals dying within three days after challenge should be excluded from the test. Any animals showing paralysis or swelling of the head at the end of the observation period should be counted as deaths.

At least three appropriate serial dilutions of the challenge suspension should be injected into a group of at least 10 mice to titrate the virulence. The bacterial count for the LD₅₀ per 0.025 ml of the challenge suspension should be no more than 300 CFU.

3. Criterion for judgement

Assay data are analysed using parallel line analysis following probit transformation of the proportion of mice responding. The dose–response curves of the test and reference vaccines are checked for significant deviations from linearity and parallelism. If there is a significant ($p < 0.05$) regression of probit response on log dose and there are no significant deviations ($p > 0.05$) from linearity or parallelism, the potency and its 95% limits are calculated. The First WHO International Standard for acellular pertussis vaccine (JN1H-3) has been

established and an in-house reference used in this test should be calibrated in terms of the International Standard. The specifications should be established with the agreement of the NRA. Where used currently, the potency of the test sample is considered to be passed if it is no less than 8 IU/ml (4 IU/human dose) upon statistical analysis.

4. Retest

If a test vaccine did not pass in the first test, the test should be repeated using the same number or an increased number of mice. Results for all statistically valid assays should be combined. Weighted mean log potency should be calculated for homogeneous results using log potencies obtained in repeated tests using inverse of variance estimate for each log potency value as weight.

Appendix 2

Histamine sensitization test by temperature measurement

Groups of mice (with no fewer than 10 mice each) defined with respect to strain, sex and age, should be randomly allocated to the different treatments. Samples should include the test sample(s) and a Reference Preparation. If reversion to toxicity testing is required by the NRA the test also should include a sample of the test vaccine incubated at 37 °C for four weeks. All mice should be challenged post-sensitization with histamine dihydrochloride. The reference and histamine dihydrochloride should be diluted with physiological saline. If PT is used as reference, physiological saline or phosphate buffered saline, both containing 0.2% (w/v) gelatin, should be used as diluent to prevent possible loss of PT activity by adsorption to the container. Appropriate thermometers with recommended precision of 0.1 °C and capable of measuring temperatures between 25 °C and 40 °C should be used for the test.

The Reference Preparation should be diluted to give a suitable dose-response. The test sample (usually a single human dose) and each dilution of the Reference Preparation in a volume of 0.5 ml should be given by intraperitoneal injection to each group of mice. Four days after injection, 4 mg of histamine dihydrochloride should be intraperitoneally injected into each mouse. The rectal or dermal temperature should be measured 30 minutes after histamine injection for all mice. Temperature should be recorded for all mice, including those that die within the 30-minute observation period.

Temperature responses are analysed using a suitable statistical method to give an estimate of the residual activity of PT in the test vaccine, in relation to the Reference Preparation.

The test lot passes the test if the estimated residual activity of PT in the test group is not higher than the value specified by agreement with the NRA. There is currently no internationally agreed upper limit for active PT in acellular pertussis vaccines. In some countries an upper limit of 1.09 or 2.18 IU (0.2 or 0.4 HSU) of PT per single vaccine dose is a requirement for DTaP vaccines.

Appendix 3

Histamine sensitization test by lethal end-point assay

Groups of mice, each of an appropriate number defined with respect to strain, sex and age, should be randomly allocated to the different treatments. For assays performed for validation purposes and initially after vaccine licensure, the positive control set consists of groups that should be injected intraperitoneally with three or more serial dilutions of a Reference Preparation of PT of suitable specific activity (IU/ng). The dilution factor should be chosen so as to obtain a graded response; however, it should be no greater than five. An additional group of mice (the negative control group) should be injected intraperitoneally with diluent. One group should be injected intraperitoneally with the test vaccine and, if reversion to toxicity testing is required, another group should receive the test sample incubated at 37 °C for four weeks. A single human dose (some methods allow up to two single human doses) of the final bulk is used as the test dose for both groups. The position of the cages on the shelves during the testing period should be allocated at random in order to reduce the influence of positional effects on the assay outcome. All mice should be challenged by injection with a defined dose of histamine (1 or 2 mg of histamine base is most commonly used) at four or five days after sensitization or injection with diluent. The histamine challenge dose may be administered intraperitoneally or intravenously; however, the injection route should be defined and the same route should be used for all testing within the laboratory. Histamine challenge should follow the place order of the cages on the shelves. Deaths within 24 hours of histamine injection should be recorded.

The assay sensitivity and other validity criteria should be defined in agreement with the NRA. For the assay to be considered valid, mice injected with diluent (negative control) must not show, in general, sensitization to the lethal effect of histamine. However, experience has shown that, with low frequency, a small percentage of mice (i.e. less than 5%) in the negative control group may die following histamine challenge. Thus some laboratories consider a test valid if there is no more than one death in a negative control group of 20 or more. Each test should also meet the criteria set to demonstrate its sensitivity. Several strains of mouse (all with Swiss-Webster ancestry) are highly responsive to histamine sensitization but a number of strains, both inbred and outbred, are weakly responsive. The susceptibility to sensitization of the strain chosen for the test should be defined during assay validation studies and approved by the NRA. Adequate susceptibility of mice used in a HIST should be verified by demonstrating that the sensitizing dose of the PT control meets criteria established during assay

validation. Once linearity has been established by repeated experiments using multiple doses of a control PT, a suitable dose of the reference toxin, chosen in the linear region of the dose–response curve and giving a positive response considered appropriate by the NRA, should subsequently be included in each assay as the positive control group to demonstrate assay sensitivity.

In some laboratories, the test also includes a reference group of mice injected with PT at a dose previously set as the allowable upper limit of PT in the product or with a reference vaccine with established clinical safety.

A lot passes the test if the proportion of animals that die following sensitization with the test dose of vaccine and the subsequent histamine challenge do not surpass the maximum proportion approved by the NRA. This proportion should be related to the performance in the test of lots shown to be safe and effective in clinical trials or those used in support of licensure. When a reference group is included, the lot passes the test if the percentage of deaths in the test group is not greater than that in the reference group.

If a vaccine lot fails in a single test, it should pass two additional, consecutive and independent assays to be considered suitable for release.

Appendix 4

Mouse immunogenicity test

The mouse immunogenicity test (MIT) for an acellular pertussis vaccine is an assay designed to demonstrate consistency between vaccine lots on the basis of the induction of antibody in mice by each antigen in the vaccine. This test is product-specific and a suitable product-specific reference (or control) vaccine is required for a meaningful assay. Immunogenicity can be measured either in terms of the amount of antibody produced in mice injected with a defined test dose, or as the dose of antigen that induces a defined measurable antibody response in a certain proportion of mice (e.g. the median effective immunizing dose, ED₅₀).

For each antibody, the linear-response region of the dose-response curve (vaccine dose versus antibody production) should be determined. In the first method, a group of mice is injected once with a preselected dose of vaccine that is within the linear-response region. For preparations containing multiple pertussis antigens, more than one test dose of vaccine, and therefore more than one group of mice per lot, may be required because of the differential immunogenicity of these antigens in mice. In the second method, groups of mice are injected with a suitable range of dilutions of vaccine, and the proportion of responding animals is determined at each dose. After consistency in manufacturing and testing has been demonstrated to the satisfaction of the NRA, the serial-dose method may be simplified to an appropriate single-dose (e.g. ED₅₀ for the antigen) assay.

Regardless of test design, the antibody content of test sera is calculated relative to a stabilized reference serum by means of a validated ELISA.

For all antigens, reproducibility of the antibody response in the chosen strain of mice should be verified in every test by the inclusion of group(s) of mice injected with homologous reference (or control) vaccine. The reference (or control) vaccine ensures that the test mice respond in a way that is consistent with previous testing. The stability of the reference (or control) vaccine should be monitored. Appropriate stabilization of the reference (or control) vaccine, preferably by lyophilization, is recommended. An example of conditions for lyophilization that have been successfully used are as follows: 3.5% polygelin (1:1) under freeze-drying cycle at -50 °C load, -50 °C freeze over 2.5 hours, then primary drying at 35 °C (100 µbar vacuum) and secondary drying at 30 °C (30 µbar vacuum). The reference vaccine does not need to be a clinical lot because acceptance criteria are values reflecting the behaviour in the test of clinical lots or those lots used in support of licensure, either in absolute terms or in terms relative to the reference vaccine. However, the reference vaccine should be sufficiently similar to the clinical lots in composition and manufacture to

serve as an adequate control in the test. The response of the test vaccine may be reported either in absolute terms or in terms relative to the reference vaccine. Calibration of replacement reference vaccines for the MIT should make use of sound statistical principles to prevent drift in the efficacy of acellular pertussis vaccines in the market.

The specifications for evaluating vaccines containing acellular pertussis are product-specific and are based on an appropriate statistical analysis of the responses observed in the MIT test for clinical vaccine or other lots used in support of licensure. Specifications must be carefully justified and defined with the agreement of the NRA. Specifications should be defined for each antigen claimed to contribute to vaccine efficacy. Specifications based on a simple failure to reject the null hypothesis of equivalency of immunogenicity between a reference lot and a manufacturing lot, or between two consecutively manufactured lots, are not recommended.

Two components of the test require careful attention:

Mouse: strains of mouse (if necessary more than one) should be selected so that a sufficient antibody response is obtained for each antigen. The optimal age for mouse immunization (e.g. more than five weeks of age), the optimal time for bleeding (e.g. 4–6 weeks after immunization), and the isotype of the antibody response should be thoroughly studied. The test design should be agreed with the NRA.

Antibody detection system: the ELISA used for the detection of antibodies should be subjected to thorough validation and standardization studies. These studies should include determination of the biochemical integrity and immunological purity of antigens used for coating assay plates and determination of the optimal antigen-coating concentration. For this purpose, the production and standardization of a working-reference mouse serum is of utmost importance. Calibration of the working-reference mouse serum in terms of the international reference serum (97/642) may provide a suitable control and facilitate inter-laboratory comparisons. Studies for reference serum standardization should include an evaluation of the parallelism of the titration curves of reference and test sera. Another component of the antibody detection system requiring careful study is the anti-mouse-immunoglobulin-enzyme conjugate. This reagent should be characterized in terms of isotype specificity and subclass reactivity, and a suitable working dilution should be determined.

The reproducibility (intra-assay and inter-assay) of the assay for sera containing different amounts of antibody and the limits of detection and quantitation (LOD and LOQ, respectively) should be studied.

The development of criteria for acceptance of a vaccine lot subjected to the immunogenicity test should take into account the following ELISA validity criteria:

- The average absorbance value for normal mouse serum should be below a historically defined upper limit. Normal mouse serum should be obtained from mice injected with diluent and housed with vaccinated mice for the duration of the immunization period. The absorbance of normal mouse serum should be measured in the same ELISA as the sera of immunized mice.
- The parameters of the curve relating absorbance to dilution for the reference serum should be within historically defined upper and lower limits.
- A control serum with characteristics similar to the test sera and stored in a separate location from the reference serum should be included in every ELISA plate. The ratio of the ELISA units calculated for the control serum to those for the reference serum should be within historically defined upper and lower limits.

If the ELISA meets these validity criteria, the antibody values should be calculated for mice immunized with the reference (or control) vaccine and the test vaccine. Sera with ELISA unit values below the LOQ should be qualified as belonging to non-responder mice. For the purpose of calculating geometric mean antibody level, an arbitrary value (e.g. 1/2 LOQ) may be assigned to such sera. Alternatively, the geometric mean antibody level could be calculated using only those values above the LOQ, provided a limit is in place for the minimal acceptable number of values to use for the calculation. If immunogenicity is being expressed in terms of dose of vaccine, then the number of mice responding to each antigen is used to calculate the ED₅₀. If the ELISA validity criteria are not met, the ELISA should be repeated.

After either a geometric mean or ED₅₀ has been calculated for the reference (or control) vaccine, the value should be compared with the criteria for sufficient antibody response that were established when the assay was validated. If these validity criteria are met, the results for the test vaccine should be evaluated as described below. If the validity criteria for the reference vaccine are not met, the ELISA should be repeated on all sera (from mice inoculated with both reference and test vaccine). If the criteria are not met after a second ELISA, immunization should be repeated.

To pass the immunogenicity test, the geometric mean antibody levels or ED₅₀ for mice immunized with test vaccine should meet the criteria that were established when the assay was validated. Alternatively, immunogenicity of the test vaccine can be expressed relative to the immunogenicity of the reference vaccine. Acceptance criteria should be determined by performing multiple tests on several lots (preferably three or more) that have shown acceptable performance (i.e. efficacy, immunogenicity, or both) in clinical studies. If geometric mean antibody levels, in absolute terms or relative to the geometric mean antibody

levels induced by the reference vaccine, are below the established limit, or if the ED_{50} or ED_{50} ratio fails to meet the established limit, immunizations and ELISAs should be repeated for those antigens that fail the test. After a second test (if valid), the geometric mean antibody levels, geometric mean ratio, ED_{50} or ED_{50} ratio should be calculated, and results of the two tests may be combined by appropriate statistical methods. The acceptance criteria to consider when two tests are performed should be statistically adjusted. If the results of single or double tests for all antigens in the vaccine satisfy their corresponding limits, the vaccine passes the immunogenicity test. If any antigen does not satisfy its adjusted limit after two assays, the vaccine fails the test.

The test – including the specifications, the method used to calculate antibody response, and the treatment of non-responder mice in the calculation of vaccine potency – should be approved by the NRA.

Appendix 5

Method for respiratory challenge

The respiratory challenge model is designed to demonstrate the protective effect of immunizing mice with acellular pertussis vaccines or candidate antigens. However, it is important to note that the activity of a vaccine or antigen in this model is not an index of clinical efficacy. In general, it involves immunizing mice, which have the capacity to give an adequate immune response to pertussis vaccine, with pertussis vaccine at appropriate doses. Mice are then challenged with live *B. pertussis* suspension. Two challenge routes/methods have been reported, namely intranasal or aerosol administration of challenge. The response to challenge is measured by dissecting out mouse lungs after a suitable time and determining the number of bacterial colony-forming units (CFU). The mouse protective effect of a test vaccine can be determined by comparison of its responses with the responses of a vaccine of known clinical efficacy or an appropriate Reference Preparation. The aerosol challenge method requires specialized aerosol equipment and this may not be readily available in most laboratories. The intranasal challenge method using a harmonized protocol has been proved to be transferable between laboratories in international collaborative studies. However, the current assay is not designed as a routine test for determining vaccine potency. Nevertheless, by comparing with a reference vaccine included in the assay, the respiratory challenge method may be useful to assess the potential impact of changing the manufacturing process and/or formulation; to evaluate new formulations; to investigate potential interactions in combinations; to monitor stability of product and to assess lot consistency. A number of designs are possible with variation in age of the mice, sampling times, and so on. A brief outline of the procedure for a harmonized intranasal challenge method is given below.

Mice

Balb/c mice, three weeks old; 15 mice are to be ordered per vaccine group (i.e. five mice at each time of sampling. Sampling time can be at two hours, five days and eight days post-challenge; or alternatively on other days after the validation study).

Immunization

First immunization:

Prepare vaccine doses: one vaccine dose = $\frac{1}{4}$ of a human dose (e.g. 125 μ l) per mouse and per immunization. The mice are immunized using a 1 ml syringe.

The syringe is divided into 125 µl sections with a marker. The vaccine is injected subcutaneously. When the vaccination is correct, a liquid-filled blister should be visible under the skin.

The second immunization is carried out two weeks after the first immunization, and as described above.

The challenge

All animals are challenged two weeks after the second immunization.

Preparation of challenge suspension:

The bacterial suspension of *B. pertussis* 18323 used for challenge is prepared from an 18–24 hrs culture grown on Bordet–Gengou medium (alternatively, charcoal agar plates containing blood may also be used). Colonies are picked and resuspended in fresh Stainer–Scholte medium or in 1% casamino acids solution. The opacity of the bacterial suspension is adjusted to $OD_{650nm} = 1$, which corresponds to 3×10^9 CFU/ml (this may vary according to the individual laboratory) and further dilution is carried out to obtain a suspension containing 10^8 CFU/ml. Fifty µl of this suspension is used for infection of each mouse.

One aliquot of this suspension is serially diluted to 10^{-4} , 10^{-5} and 10^{-6} , and 100 µl of the latter two dilutions are plated on Bordet–Gengou plates, in duplicate, in order to enumerate the CFU/ml content of the bacterial suspension used to infect the mice.

Intranasal challenge:

The mice immunized by each vaccine under test should survive until challenge, and each mouse should appear healthy prior to challenge.

All mice are anaesthetized before the challenge. A total of 50 µl of the bacterial suspension is delivered in the nostril, or 25 µl into each nostril, with an automatic 50 µl pipette (in some laboratories, the nose of the mouse is dried with a paper towel before the challenge).

Sampling and CFU count

Five lungs from each group of mice are removed two hours, five and eight days post-challenge following terminal anaesthesia and deposited into tubes containing 1 or 2 ml of saline or 1% casamino acids. The lungs are homogenized individually and plated out under appropriate dilution on a Bordet–Gengou plate or charcoal agar plate. Plates are incubated at 36–37 °C for 4–5 days.

Result

For one animal the lungs homogenate (in 1 ml) is normally diluted to 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} , and up to 10^{-6} may be needed for the control group.

For each point, the number of colonies on each plate is counted.

Mean of CFU/lungs:

$$m = \frac{\Sigma \text{ CFU on the 3 plates} \times \text{dilution factor}}{\Sigma \text{ volumes used}}$$

Note: dilution factor: 10 if the lungs were homogenized in 1 ml or 20 if the lungs were homogenized in 2 ml.

Example:

For one animal the lungs homogenate (in 1 ml) was diluted 10^{-2} , 10^{-3} and 10^{-4} :

For 10^{-2} : 500 colonies were counted

For 10^{-3} : 47 colonies were counted

For 10^{-4} : 6 colonies were counted

$$M = \frac{500 + 47 + 6}{0.01 + 0.001 + 0.0001} \times 10 = 5.53 \times 10^5 \text{ CFU/ml}$$

$\log_{10}$ is calculated for each mouse and the arithmetic mean is calculated for each vaccine group.

The curve is then traced: mean of $\log_{10}$ CFU/lungs versus day after infection.

Suggested validity criteria of the test

Several important criteria have to be met in order to validate the assay.

- Respect of the protocol: there is no technical problem in assay performance.
- The number of bacteria used to challenge the mouse is not below 10^5 .
- The calculated $\log_{10}$ mean number of CFU/lung for the negative control group at two hours after challenge should be above 5.4.
- The calculated $\log_{10}$ mean number of CFU/lung for the positive reference group at five days after challenge should be below 3.75.
- A significant difference of at least 3.1 log CFU between the reference group and the negative control group should be detected at five days after challenge.

Both reference and test vaccines should be included in the assay. The $\log_{10}$ CFU/lung for the test and reference vaccines can be used for quantitative analysis, either for comparison of results for groups of mice treated with single doses or, if suitable doses of each vaccine have been used, interpretation of potency using a parallel line assay could be achievable.

Appendix 6

Summary protocol for production and testing of acellular pertussis vaccine

1. Summary information on final lot

Name and address of manufacturer: _____
 Lot no.: _____
 Date of filling: _____
 Date of manufacture: _____
 Nature of final product (absorbed): _____
 Volume of each recommended single human dose: _____
 No. of doses per final container: _____
 No. of final containers: _____
 Expiry date: _____

2. Detailed information on manufacture and control

Strain

Identity of *B. pertussis* strains used in vaccine: _____
 Serological types of strains: _____
 Reference no. of seed lot: _____
 Date(s) of reconstitution of ampoule(s) for manufacture: _____

Culture media for production

Name of the culture medium: _____

Control of bacterial purity

Result: _____
 Date: _____

Control of antigen purification

Purification of PT: _____
 Purification of FHA: _____
 Purification of pertactin: _____
 Purification of FIM 2/3: _____

Identification: _____
 Volume: _____

Test on purified antigens

For purified antigens

Methods: _____

Purity: _____

Date: _____

For co-purified antigens

Purity of claimed antigens: _____

Proportion of each antigen claimed: _____

Methods: _____

Date: _____

Residual level of endotoxin

Methods: _____

Content: _____

Date: _____

Antigen content

Methods: _____

Content: _____

Date: _____

Sterility test:

Tests for bacteria and fungi

Method: _____

Media: _____

Number of containers tested: _____

Volume of inoculum per container: _____

Volume of medium per container: _____

Temperatures of incubation: _____

Date of test (start, end): _____

Result: _____

Detoxification

Detoxifying reagent: _____

Detoxifying conditions: _____

Control of bulk

Identification: _____

Volume: _____

Test for antigen content

Methods: _____

Content: _____

Date: _____

Residual activity of pertussis toxin

1. HIST by temperature measurement

Date: _____

Strain of mice/Sex of mice: _____

No. of mice per dilution: _____

No. of mice dilutions injected: _____

Age range or weight range on day of immunization: _____

Immunization route/Immunization dose: _____

Challenge route/Challenge dose: _____

Interval between immunization and challenge: _____

Results (IU/SHD or HSU/SHD): _____

Calculation method: _____

Rectal temperature or dermal temperature: _____

	Temperature	
	average	variance
Reference dilution 1		
Reference dilution 2		
Reference dilution n		
Test vaccine		
Negative		

- Dilution must contain 1.09 or 2.18 IU (0.2 or 0.4 HSU)/SHD.

2. HIST by lethal end-point assay

Date: _____

Strain of mice/Sex of mice: _____

No. of mice per dilution: _____

No. of mice dilutions injected: _____

Age range or weight range on day of immunization: _____

Immunization route/Immunization dose: _____

Challenge route/Challenge dose: _____

Interval between immunization and challenge: _____

Result: _____

	No. of deaths/No. of animals inoculated
Reference dilution 1	/
Reference dilution 2	/
Reference dilution n	/
Test vaccine	/
Negative	/

Residual level of endotoxin

Methods: _____

Content: _____

Date: _____

Sterility test

Tests for bacteria and fungi

Method: _____

Media: _____

Number of containers tested: _____

Volume of inoculum per container: _____

Volume of medium per container: _____

Temperatures of incubation: _____

Date of test (start, end): _____

Result: _____

Control of final bulk

Identification: _____

Volume: _____

Detoxifying agent

Methods: _____

Content: _____

Date: _____

Preservative content

Methods: _____

Content: _____

Date: _____

Adjuvant

Methods: _____

Content: _____
Date: _____

Sterility

Tests for bacteria and fungi

Method: _____
Media: _____
Number of containers tested: _____
Volume of inoculum per container: _____
Volume of medium per container: _____
Temperatures of incubation: _____
Date of test (start, end): _____
Result: _____

Residual activity of pertussis toxin

1. HIST by temperature measurement

Date: _____
Strain of mice/Sex of mice: _____
No. of mice per dilution: _____
No. of mice dilutions injected: _____
Age range or weight range on day of immunization: _____
Immunization route/Immunization dose: _____
Challenge route/Challenge dose: _____
Interval between immunization and challenge: _____
Results (IU/SHD or HSU/SHD): _____
Calculation method: _____
Rectal temperature or dermal temperature: _____

	Temperature	
	average	variance
Reference dilution 1		
Reference dilution 2		
Reference dilution n		
Test vaccine		
Negative		

- Dilution must contain 1.09 or 2.18 IU (0.2 or 0.4 HSU)/SHD.

2. HIST by lethal end-point assay

Date: _____

Strain of mice/Sex of mice: _____

No. of mice per dilution: _____

No. of mice dilutions injected: _____

Age range or weight range on day of immunization: _____

Immunization route/Immunization dose: _____

Challenge route/Challenge dose: _____

Interval between immunization and challenge: _____

Result: _____

	No. of deaths/No. of animals inoculated
Reference dilution 1	/
Reference dilution 2	/
Reference dilution n	/
Test vaccine	/
Negative	/

Reversion to toxicity

Incubation period: start date _____ end date _____ temperature _____

Methods: _____

1. HIST by temperature measurement

Date: _____

Strain of mice/Sex of mice: _____

No. of mice per dilution: _____

No. of mice dilutions injected: _____

Age range or weight range on day of immunization: _____

Immunization route/Immunization dose: _____

Challenge route/Challenge dose: _____

Interval between immunization and challenge: _____

Results (IU/SHD or HSU/SHD): _____

Calculation method: _____

Rectal temperature or dermal temperature: _____

	Temperature	
	average	variance
Reference dilution 1		
Reference dilution 2		
Reference dilution n		
Test vaccine		
Negative		

- Dilution must contain 1.09 or 2.18 IU (0.2 or 0.4 HSU)/SHD.

2. HIST by lethal end-point assay

Date: _____
Strain of mice/Sex of mice: _____
No. of mice per dilution: _____
No. of mice dilutions injected: _____
Age range or weight range on day of immunization: _____
Immunization route/Immunization dose: _____
Challenge route/Challenge dose: _____
Interval between immunization and challenge: _____
Result: _____

	No. of deaths/No. of animals inoculated
Reference dilution 1	/
Reference dilution 2	/
Reference dilution n	/
Test vaccine	/
Negative	/

Immunological activity

1. MIT

Strain of mice: _____
No. of mice per dilution: _____
No. of dilutions injected: _____
Volume and route of injection: _____
Identification of reference: _____
Date of bleeding: _____
Antibody titration: _____

Result for test vaccine: _____
 GMT value of anti-PT: _____
 GMT value of anti-FHA: _____
 GMT value of anti-PRN: _____
 GMT value of anti-Fims: _____
 Result for reference vaccine: _____
 GMT value of anti-PT: _____
 GMT value of anti-FHA: _____
 GMT value of anti-PRN: _____
 GMT value of anti-Fims: _____
 Or ratio of test vaccine to reference vaccine: _____
 Ratio for anti-PT: _____
 Ratio for anti-FHA: _____
 Ratio for anti-PRN: _____
 Ratio for anti-Fims: _____
 Date: _____

2. MICA

Strain of mice: _____
 No. of mice per dilution: _____
 No of dilutions injected: _____
 Volume and route of injection: _____
 Date of injection: _____
 Identification of reference: _____
 LD₅₀ in challenge dose: _____
 No. of colony-forming units in challenge dose: _____
 Date of challenge: _____
 Date of end of observation: _____
 Results (IU/SHD): _____
 Calculation method: _____

	Dilution	No. of survivors/ No. of animals inoculated	Median effective dose (ED ₅₀)
Reference:	_____	_____	
Vaccine:	_____	_____	_____ ml
(IU/ml)	_____	_____	
	_____	_____	
Test vaccine	_____	_____	_____ ml
	_____	_____	

Potency of test vaccine is _____ IU per single human dose. Limits of 95% confidence interval (in %) are: _____.

Final product

Identification: _____

Volume: _____

Identity test

Method of testing: _____

Result: _____

Date of test: _____

Sterility test

Tests for bacteria and fungi

Method: _____

Media: _____

Number of containers tested: _____

Volume of inoculum per container: _____

Volume of medium per container: _____

Temperatures of incubation: _____

Date of test (start, end): _____

Result: _____

Test for adjuvant

Nature and concentration of adjuvant/SHD: _____

Method of testing: _____

Specification: _____

Result: _____

Date of test: _____

Test for preservative

Nature and concentration of preservative: _____

Method of testing: _____

Specification: _____

Result: _____

Date of test: _____

pH

Method of testing: _____

Specification: _____

Result: _____

Date of test: _____

Endotoxin test

Method of testing: _____

Specification: _____

Result: _____

Date of test: _____

Immunological activity

If the test was not performed on the final bulk, indicate this and report the data obtained on the final product in the space provided for biological activity tests in the “final bulk” section.

Innecuity test

Tests in mice

Date of start of test: _____

Date of end of test: _____

No. of animals tested: _____

Route of injection: _____

Volume and route of injection: _____

Observation period: _____

Results (give details of deaths): _____

Tests in guinea-pigs

Date of start of test: _____

Date of end of test: _____

No. of animals tested: _____

Route of injection: _____

Volume and route of injection: _____

Observation period: _____

Results (give details of deaths): _____

Inspection of final containers

Date of inspection: _____

Organoleptic characteristics: _____

Number of containers inspected: _____

Percentage of rejected containers: _____

3. Certification by the manufacturer

Name of head of production (typed) _____

Certification by person from the control laboratory of the manufacturing company taking overall responsibility for the production and control of the vaccine

I certify that lot No. _____ of acellular pertussis vaccine, whose number appears on the label of the final containers, meets all national

requirements and satisfies Part A¹ of the WHO Recommendations to assure the quality, safety and efficacy of acellular pertussis vaccines (2013)² (if applicable).

Name (typed) _____

Signature _____

Date _____

4. Certification by the NRA

If the vaccine is to be exported, attach a certificate from the NRA as shown in Appendix 7, a label from a final container and an instruction leaflet for users.

¹ With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.

² WHO Technical Report Series, No. 979, Annex 4.

Appendix 7

Model certificate for the release of acellular pertussis vaccine by NRAs

This certificate is to be provided by the NRA of the country where the vaccine has been manufactured, on request of the manufacturer.

Lot release certificate

Certificate no. _____

The following lot(s) of acellular pertussis vaccine produced by _____¹ in _____² whose numbers appear on the labels of the final containers, complies with the relevant specification in the marketing authorization and provisions for the release of biological products³ and Part A⁴ of the WHO Recommendations to assure the quality, safety and efficacy of acellular pertussis vaccines (2013)⁵ and comply with WHO good manufacturing practices for pharmaceutical products: main principles;⁶ Good manufacturing practices for biological products;⁷ and Guidelines for independent lot release of vaccines by regulatory authorities.⁸

The release decision is based on _____⁹

The certificate may include the following information:

- Name and address of manufacturer
- Site(s) of manufacturing

¹ Name of manufacturer.

² Country of origin.

³ If any national requirements are not met, specify which one(s) and indicate why release of the lot(s) has nevertheless been authorized by the NRA.

⁴ With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.

⁵ WHO Technical Report Series, No. 979, Annex 4.

⁶ WHO Technical Report Series, No. 961, Annex 3.

⁷ WHO Technical Report Series, No. 822, Annex 1.

⁸ WHO Technical Report Series, No. 978, Annex 2.

⁹ Evaluation of summary protocol, independent laboratory testing, and/or specific procedures laid down in defined document etc. as appropriate.

- Trade name and common name of product
- Marketing authorization number
- Lot number(s) (including sub-lot numbers and packaging lot numbers if necessary)
- Type of container
- Number of doses per container
- Number of containers/lot size
- Date of start of period of validity (e.g. manufacturing date) and/or expiry date
- Storage condition
- Signature and function of the authorized person and authorized agent to issue the certificate
- Date of issue of certificate
- Certificate number.

The Director of the NRA (or other authority as appropriate):

Name (typed) _____

Signature _____

Date _____