EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION
Geneva, 17 to 21 October 2011

Recommendations to Assure the Quality, Safety and Efficacy of Acellular Pertussis Vaccines

Proposed replacement of: TRS 878, Annex 2

NOTE:

This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). The text in its present form does not necessarily represent an agreed formulation of the Expert Committee. Comments proposing modifications to this text MUST be received by 23 September 2011 and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Quality Safety and Standards (QSS). Comments may also be submitted electronically to the Responsible Officer: Dr Dianliang Lei at email: leid@who.int.

The outcome of the deliberations of the Expert Committee will be published in the WHO Technical Report Series. The final agreed formulation of the document will be edited to be in conformity with the "WHO style guide" (WHO/IMD/PUB/04.1).

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Introduction

Pertussis immunization is an integral part of immunization programs 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 also have been shown to be safe and effective and which have been successfully introduced into many national immunization programs. 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. 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 standardization of quality control methods have prompted the WHO to update the current WHO Guidelines for the production and control of acellular pertussis component of monovalent or combined vaccines.

The WHO Guidelines for acellular pertussis vaccines were approved in 1996, with the recognition that further improvements on production and evaluation of these vaccines would follow. Since 1996, stakeholders have gained additional experience with these vaccines, and limitations of the original guidelines have been identified. Acellular pertussis vaccines are almost exclusively administered in combinations with diphtheria and tetanus toxoids 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 the clinical section (part C). The goal of this revision is to address these issues of 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 B. 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 in two protein components that were protective in animal models, namely pertussis toxin (PT) and filamentous hemagglutinin (FHA) and introduced for routine use in Japan in about 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 ultra centrifugation, and all contained FHA and PT that were treated with formaldehyde to inactivate PT. These vaccines also contained various amounts of other B. pertussis proteins as minor components, including pertactin (PRN or 69 KD) and fimbriae (FIM). Epidemiological evaluations indicated that these vaccines effectively controlled pertussis disease when introduced for routine immunization.

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, the PT
detoxification procedures and the antigen purification processes.

In the period between 1986 and 1996, several acellular pertussis containing vaccines,
including some composed of purified antigens as well as some composed of co-purified
antigens, were evaluated in a series of efficacy trials. These trials evaluated acellular pertussis
vaccines containing one to five pertussis components, and utilized different study designs
including randomized placebo-controlled cohort trials, household contact studies, and case-
control studies. This series of trials revealed that all of the tested acellular pertussis
vaccines provided some protection against pertussis, although the studies suggested
differences among the vaccines. Additional detail is provided in Part C of these
Recommendations. Unless vaccines of different type 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 programs.

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 (IC) 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 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 *E. coli*) they are
not being 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, Hib, IPV, Hep B). Therefore, the tests
recommended for the final bulk or final product of acellular pertussis vaccines shall be performed on the final bulk or final product of the combined vaccines.

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

1. Improvement of quality control of existing vaccines based on new information and experience.
2. Evaluation of new products and new combinations through control of manufacturing, and evaluation of the vaccines in both nonclinical and clinical studies

The major changes made to the 1996 version are the following:

(1) The title of the document is upgraded from Guidelines to Recommendations.
(2) Advice on clinical and nonclinical evaluation of acellular pertussis vaccines sections is added to guide the National Regulatory Authorities and vaccine manufacturers in approaches that can be used to assess the safety, efficacy and quality of vaccines.
(3) Details are provided for the modified intracerebral mouse protection assay used to evaluate the potency of the acellular pertussis vaccine and for the histamine sensitization test based on temperature measurement to determine the residual activity of PT in the vaccine.
(4) Information is provided for the performance of the mouse respiratory challenge method. Although the method currently is not recommended for routine potency testing, the test may have an important role in nonclinical testing discussed in part B of these Recommendations.

General considerations

Written description 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 National Regulatory Authority as part of the license application. Proposals for any variations to the manufacturing and/or control methods should be submitted for approval to the National Regulatory Authority according to the national regulatory requirements before they are implemented.

There is as yet no consensus about an 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. 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, for example, diphtheria and tetanus toxoids.
In the absence of internationally accepted indicators of protective efficacy, 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 safety, potency, physicochemical and immunological characteristics of new vaccine lots compared with lots evaluated in clinical studies. Special care should be taken in validating the test procedures 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 National Regulatory Authority.

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. An example of conditions which have been used are 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).

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. With these limitations, these Recommendations describe a sequential approach to collection of supporting evidence beginning with a comprehensive program of nonclinical testing, 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, FIM2/3) that is produced by new process, new strain, and/or from 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. This document assumes that only those vaccines with extensive nonclinical testing would be considered for clinical evaluation, with agreement with the local National Regulatory Authority 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 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. 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 international standards and reference materials (http://www.who.int/biologicals/IBRP/Catalogue.htm).

Key standards and reference materials include:

(1) The First International Standard for Acellular Pertussis Vaccine for use in modified mouse intracerebral challenge assay (MICA) and other protection assays (code No. JNIH 3 with assigned unitage per ampoule of 34 IU).

(2) The First International Standard for Pertussis Toxin for standardization of assays used to monitor the residual pertussis toxin activity in pertussis vaccines, e.g. histamine sensitization tests and Chinese Hamster Ovary (CHO) cell assay (code No. JNIH 5 with assigned unitage per ampoule of 10,000 IU).

(3) The First 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).

(4) The First WHO 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).

(5) The First International Standard monoclonal antibody to *B. pertussis* fimbriae type 2 for the determination of serotype of *B. pertussis* strains (Code No. 06/124).

(6) The First International Standard monoclonal antibody to *B. pertussis* fimbriae type 3 for the determination of serotype of *B. pertussis* strains. (Code No. 06/128).

(7) The First WHO 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 International Standards/reference materials and other reagents from the WHO International Standards Laboratory are in the custody of the National Institute for Biological Standards and Control, Health Protection Agency, Potters Bar, Hertfordshire, EN6 3QG, United Kingdom (web site: http://www.nibsc.ac.uk).

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

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 shall 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.

Antigens currently used in the vaccines are inactivated PT, 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 for pharmaceutical and biological products 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

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 National Regulatory Authority. The method should 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 need extensive validation and approval of the National Regulatory Authority.

### 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 good 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, neither for seed lots nor 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 National Regulatory Authority. If any materials of animal origin are used in seed preparation or preservation, or in production, they should comply with the guidelines on medicinal and other products in relation to human and animal transmissible spongiform encephalopathies and national regulatory requirements. When animal blood or blood products are used, they should be removed in the process of production by appropriate methods.

If any materials of animal origin are used in seed preparation or preservation, or in production, they should comply with the guidelines on medicinal and other products in relation to human and animal transmissible spongiform encephalopathies. Any change of media should be submitted for approval to the National Regulatory Authority.

### 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, pH and the rate of production of the desired antigen(s). Acceptance specifications should be established and agreed with the National Regulatory Authority.

#### 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.

#### 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 lypooligosacharide (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.

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 sulphate 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 physico-chemical separation methods.

The tests used for determination of consistency of yield and purity and their performance characteristics shall be approved by the National Regulatory Authority.

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 sulphate polyacrylamide gel electrophoresis (SDS-PAGE), single radial immunodiffusion, immunoblotting, the CHO cell toxicity test for detection of active PT, haemagglutination and high-performance liquid chromatography (HPLC). The specific activity of PT (IU/ng) should be determined.

**Antigenic 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 individual product as found for vaccine lots shown to be safe and effective in clinical trial or other lots used in support of licensing. Specifications should be set during the product development and validation process and established by agreement with the National Regulatory Authority.

**Residual levels of endotoxin.** The antigens should be tested for residual endotoxin content by means of the 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 National Regulatory Authority.

**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. Antigenic activity 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 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 National Regulatory Authority.

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 National Regulatory Authority. 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 shall be subjected to appropriate detoxification methods. Other antigens may also be treated with agents to detoxify any residual PT remaining 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 shall be prepared using antigen preparations processed either in a single run or a pool of those prepared in multiple runs. With the approval of the National Regulatory Authority, 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 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 suitably validated procedure may be used to estimate the amount of individual antigens based on the amounts measured before detoxification. The amount of each antigen present in the vaccine should be consistent with levels found in vaccine lots shown to be safe and effective in the clinical trials.
or other lots used in support of licensing. Specifications for antigen content should be set
during the product development and validation process and approved by the National
Regulatory Authority.

*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, for example the histamine sensitization test (HIST) or the CHO-cell
assay. Adjuvants and other vaccine components may interfere with the adequate performance
of the CHO-cell assay. Special care must be taken to ensure that it does not interfere with
the tests (e.g. by adequate dilution of test solutions). At the concentration of vaccine strength,
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 National Regulatory
Authority.

There is no reliable information about the relation between PT residual
activity and the clinical safety of the vaccine; therefore, residual activity
should be permanently reduced as far as feasible, without damaging antigen
immunogenicity.

*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. The limits applied to
the vaccine concentration of individual components should be agreed with the National
Regulatory Authority.

There is no definitive information on the maximum safe limits for endotoxin
in pertussis vaccines. It is therefore recommended that the endotoxin contents
of the final bulk materials be kept as low as feasible and that acceptability
limits be based on production performance and approved by the National
Regulatory Authority.

*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 National Regulatory Authority.

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

A.3.4 **Control of final bulk**

At the present time, there is no standalone acellular pertussis vaccine, so the tests described
below are undertaken at the final bulk stage of combined vaccines.

If a standalone 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 shall 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 other appropriate adjuvant prior to or after blending with other components e.g.
Diphtheria toxoid, Tetanus toxoid, IPV etc. 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 upon agreement with the National Regulatory Authority.

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 National Regulatory Authority.

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 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 National Regulatory Authority. The amount of preservative in the vaccine dose should be shown not to have any deleterious effect on the antigen(s) nor impair the safety of the product for humans. The preservative, its use at different stages of the manufacturing process, as well as its concentration or residual amount should be approved by the National Regulatory Authority. 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.

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 National Regulatory Authority. 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 National Regulatory Authority. 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 production lots should be demonstrated to be within the range of values found for vaccine lots shown to be clinically safe and effective or for lots used in support of licensing.

A.3.4.2.4 Sterility

Each final bulk shall 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 National Regulatory Authority.

If a preservative has been added to the vaccine, appropriate measures shall 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 histamine sensitization test (HIST) or other test that is sufficiently sensitive to detect the level of toxin activity agreed with the National Regulatory Authority. For products containing genetically detoxified PT, this test may not be necessary as agreed with the National Regulatory Authority. Currently the HIST method is in use for the determination of residual bioactive PT in licensed acellular pertussis vaccines is based on response of mice injected with the vaccine on test, to the histamine challenge. Two possible test outcomes (end-points) are actually in use. One is based on the lethal effect of the histamine challenge dose and the other on the decrease in body temperature post-histamine challenge. If an alternative assay(s) is used, it should be validated against the histamine sensitization test and agreed with the National Regulatory Authority.

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 international standard (currently JNIH-5) and expressed in IU, because the nominal protein masses of the toxin preparations do not necessary predict their biological activity in HIST. The detection limit for PT of the test should be defined and accepted by the National Regulatory Authority.

The acceptance criteria for content of residual bioactive PT should be based on the results for lots shown to be safe and effective in clinical trials or other lots used in support of licensing. Specification should be established in agreement with the National Regulatory Authority.

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 feasible without undue compromise to immunogenicity. There is no internationally agreed upper limit for active PT in acellular pertussis vaccines. In some countries upper limits of 1.09 or 2.18 IU of PT per single vaccine dose are a requirement for DTaP vaccines \(^{27, 28}\). A recent study of 8 DTaP based combination vaccines using the HIST lethal end-point method suggests that these vaccines contained approximately 2 IU/SHD \(^{29}\).

The HIST lethal end-point method 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 has been successfully used in some countries. And it has been optimized to provide a quantitative estimate of the activity of a test vaccine relative to the activity of a PT standard.
The tests are described in Appendices 1 and 2.

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 replacement for the HIST. Any alternative assay to current HIST to determine the residual pertussis toxin should be validated and approved by the National Regulatory Authority.

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 4 weeks at 37°C, may be used to demonstrate that it is unlikely that the chemically inactivated PT will regain at least some of its toxicity before the vaccine expiry date. Some National Regulatory Authorities 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 National Regulatory Authority.

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 modified intracerebral challenge assay (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 National Regulatory Authority. 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, and assay conditions.

Mouse immunogenicity test (MIT): The mouse immunogenicity test 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 5 antigens is available to monitor consistency of the ELISA stage.

MIT is designed to assess consistency of manufacture by evaluating whether the mouse immunogenicity of lots manufactured post-licensure is consistent with that of lots with acceptable performance in clinical trials. Due to heterogeneity of vaccine products in composition, 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 product on test.

In the absence of international reference vaccine for MIT, each manufacturer is responsible for development of a reference vaccine that can allow for the meaningful assessment of the immunogenic activity of the test vaccine with respect to the established specifications. Although a clinical lot with established efficacy should be considered as the reference vaccine, this is usually impractical due to considerations including availability and long-term stability. However the vaccine lot selected as reference should be sufficient similar to the
clinical lots in composition, manufacturing process, immunogenicity and/or protective effect. Stabilization of the reference vaccine is recommended but careful attention shall 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. Testing approaches that allow for higher precision (e.g. more tests, more animals per test, or more dilutions per test) are encouraged for stability monitoring and for testing of candidate replacement references.

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 each antibody response to the antigens claimed to contribute to efficacy should be established and approved by the National Regulatory Authority. Additional details on the method are provided in Appendix 3.

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 maximum allowable deviation from the reference vaccine and the minimum precision of the test.

**Modified intracerebral mouse protection assay (MICA):** The modified intracerebral protection assay (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 International Standard for acellular pertussis vaccines (currently JINH-3) \(^{33}\) and the protective activity should be expressed in International Units.

The assay method and the specifications should be approved by the National Regulatory Authority.

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 assay**

An alternative assay, the guinea pig immunogenicity test, is under evaluation \(^{34, 35}\). Adoption of this or other alternative methods would require extensive evaluation, validation, and approval by the National Regulatory Authority.

Alternative respiratory challenge method(s) such as the mouse intranasal challenge assay (INCA) (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 manufacturing process or/and formulation; new antigens and formulations; or potential interactions in new combinations. They also can be used for stability studies and assessing lot consistency. This model is an important tool in new product development as described in Part B in this document.

The requirements concerning filling and containers given in Good Manufacturing Practices for Biological Products 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 National Regulatory Authority to ensure that the final containers contain the appropriate amounts of each of the vaccine antigens as designed for the acellular vaccine formulation.

The following tests should be performed on each final lot of vaccine (i.e. in the final containers).

A.5.1 Identity

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

A.5.2 Sterility

Final containers shall be tested for sterility by a method approved by the national control authority. 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. If a preservative has been added to the vaccine, appropriate measures shall 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 National Regulatory Authority. 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 specification 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 National Regulatory Authority. 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 National Regulatory Authority. 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.
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 specification as approved by the National Regulatory Authority.

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 National Regulatory Authority.

This test may be omitted for routine lot release once consistency of production has been well established to the satisfaction of the National Regulatory Authority and when good manufacturing practices are 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 provided in Part A, section A.3.4.2.7, on each final lot, if such a test has not been done 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 should apply.

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

A.7. Samples

The recommendations given in Good Manufacturing Practices for biological products should apply.

A.8. Labeling

The recommendations given in Good Manufacturing Practices for biological products 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, and
• 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 shall 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, and
• 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 should apply.

A.10. Stability, storage and expiration 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.

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 National Regulatory Authority. Containers from at least three consecutive final lots, and derived from different antigen production lots, should be tested.

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 expiration 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 validity period, 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 to with the National Regulatory Authority.

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 intended storage condition. 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 to determine the loss of immunological activity at appropriate time intervals during storage. Final containers from at least three lots of vaccine derived from different bulks shall be tested on the expiration date to demonstrate stability during storage.

Accelerated stability data for product stored for limited periods at temperatures that may affect stability could 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 National Regulatory Authority. Following licensure, stability should be monitored throughout proposed shelf-life.

A.10.1.2. Stability at different stages of manufacturing process

Stability testing should be performed at different stages of production, namely single harvests, bulk materials, final bulk and final lot at least on three lots each. Suitable stability indicating parameters should be selected according to the stage of production. Manufacturers are encouraged to assign shelf life to all materials during vaccine production, in particular 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 vaccines under development, the stability data are generally available for a more limited period.

Appropriate documentation to support stability profile of a vaccine should be submitted to the competent National Regulatory Authority 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 10.1 above and approved by the National Regulatory Authority. 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 shall recommend conditions of storage and transport that shall ensure that
the vaccine satisfies the potency requirements until the expiration date stated on the label.

The vaccine must not be frozen.

A.10.3 Expiration date

The expiration date should be defined on the basis of shelf life supported by the stability
studies as described above (section 10.1) and approved by the National Regulatory Authority.
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 collection of supporting evidence beginning with a comprehensive program of nonclinical testing, followed by a progression of clinical evaluations; this part describes the nonclinical testing recommended. The extent to which nonclinical studies will be required depends on the clinical experience already existing with the different vaccine components. For vaccines containing acellular pertussis components that have not previously been evaluated for efficacy in clinical trials, non-clinical testing results represent only a part of the aggregate of data that need 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 than 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 rather than 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 those 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 on the design, conduct, analysis and evaluation of nonclinical studies are available in the WHO guidelines for nonclinical evaluation of vaccines. Animal studies form an essential part of the development of these vaccines to provide evidence that they induce functional immune responses (e.g. induction of pertussis toxin neutralizing antibodies, protection against bacterial challenge).

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 pre-clinical 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 physical-chemical 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. PT/FHA ratio) should be established to characterize clinical lots and to monitor product consistency.

The inclusion of a novel antigen, that is, an antigen other than those that have already been tested in previous clinical efficacy trials (i.e. PT, FHA, PRN, fimbriae), would require additional considerations, as would, for example, the inclusion of the PRN antigen purified from *B. bronchiseptica*, as this is not the same antigen as PRN from *B. pertussis*. When possible, the individual antigens should be evaluated in active protection animal models. Pertussis toxoid (PTxd) is effective in most active protection models, thus, the demonstration of an additive 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 sub-optimal 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 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 the 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 typically are co-purified and processed as a single antigen (FIM2/3).

PT: purity and bioactivity of PT before chemical treatment, residual bioactivity after toxoiding, 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., hemagglutinating 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 considerations 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.

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

Table 1: Summary of published studies evaluating the ability of purified pertussis antigens to protect in mouse challenge models
<table>
<thead>
<tr>
<th>Antigen</th>
<th>Respiratory Challenge Models</th>
<th>Intra-cerebral Challenge Models</th>
<th>Animal Model Protection Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Purified antigen</td>
<td>Purified antigen</td>
<td>Purified antigen plus small, non-protective, amount of active PT</td>
</tr>
<tr>
<td></td>
<td>Active Immunization</td>
<td>Active Immunization</td>
<td>Passive Immunization</td>
</tr>
<tr>
<td>PTxd</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>18, 19, 40-46</td>
<td>17, 42-50</td>
<td>17, 19, 31, 41, 43, 44</td>
</tr>
<tr>
<td>FHA</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>18, 19, 43-45, 51-56</td>
<td>17, 43-44, 50, 54</td>
<td>17, 19, 31, 43-45</td>
</tr>
<tr>
<td>PRN</td>
<td>yes</td>
<td>yes</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>52, 57-60</td>
<td>60</td>
<td>?</td>
</tr>
<tr>
<td>FIM</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>44, 61-64</td>
<td>44</td>
<td>31, 44, 64</td>
</tr>
</tbody>
</table>

Note: “yes” means protection was demonstrated; “no” means protection was not observed, and “?” no information found.

Despite advances in the 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 National Regulatory Authority that chemically inactivated PT present in the final bulk does not revert to its toxic form before the vaccine expiration 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 lipooligosaccharide (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 shall 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.

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 3 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 h. No dermonecrotic reaction is demonstrable.
The mouse weight-gain test and the leukocytosis promotion test, which are currently in use 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 above) are preferred.

B.2 Nonclinical characterization and testing of final vaccine formulation

Given that there are currently no standalone 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 4) 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, care shall 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.

Part C. Clinical evaluation of acellular pertussis vaccines

As noted in the Introduction, this part of the Recommendations provides guidance on issues related to the design and evaluation of the clinical studies. Most studies are expected to be comparative in nature, thus the choice of a comparator vaccine is discussed in some detail below. 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, this section emphasizes the trials to assess safety and immunogenicity.

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 (1986-87), a PTxd and a PTxd/FHA vaccine from the same Japanese manufacturer were evaluated. 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. 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. 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 endpoints was strongly encouraged. The evaluation of
efficacy against milder illness (e.g. less than 21 days of paroxysmal cough) was considered of
particular importance.

Additional trials were conducted between 1991 and 1996\textsuperscript{11, 12, 69-78}; in these trials DTaP
vaccines containing one to five pertussis components were investigated. Different study
designs were employed in these trials: 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\textsuperscript{79}. Blinding was not possible in the case-control studies
and in most of household contact studies, and thus the efficacy estimates for such trials have
more potential for bias. The exceptions of this were household contact studies which were
nested within some randomized controlled cohort trials.

This series of trials revealed that all of the tested acellular pertussis vaccines protected
children against pertussis to at least some degree\textsuperscript{11, 12}. However, unless having been tested in
parallel within the same trial, comparing the efficacy among 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 example, 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 case. 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\textsuperscript{71, 74}. However, the vaccine composition that
optimally protects against both mild and severe disease remains uncertain. Epidemiologic
investigations have shown that disease has been controlled by vaccines of varying
composition.\textsuperscript{1, 10, 80, 81}

In addition, several of these trials included both whole-cell and acellular pertussis vaccines.
Some tested whole-cell vaccines provided less protection than the acellular vaccines\textsuperscript{70, 71},
however, in other trials other whole-cell vaccines appeared to be more efficacious than most
acellular vaccines, particularly against mild pertussis\textsuperscript{74, 76}. Heterogeneity among whole-cell
vaccines has been reported since the 1950’s, and emphasizes the importance of monitoring the
efficacy of any whole-cell or acellular pertussis immunization program.

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\textsuperscript{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.\textsuperscript{81-83} 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 immunisation with DTaP vaccines caused fewer local reactions and less fever than DTwP vaccines\textsuperscript{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., greater than 5 millimetres) 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\textsuperscript{12}.

### C.1 General considerations for clinical studies

This section addresses some issues that are specific to, or particularly relevant for, 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.\textsuperscript{84}

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 program used to evaluate the vaccine.

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 only be initiated 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 are used in the late stages of the clinical development program.

#### 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 or post-market effectiveness, 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 likely 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.

The size of such studies and the end-points for evaluation require justification. The immunogenicity end-points should be set according to the sensitivity and specificity of the assays and in the light of experience regarding natural variation between individuals.

Newborn infants less than 3 months of age are at highest risk of hospitalization and death from pertussis. There is a growing interest in the development of standalone acellular pertussis vaccines to be used as a birth dose and the possibility to assess the clinical efficacy. However, the unique considerations that apply to the clinical evaluation of vaccines in the newborn 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 specific antigen. Therefore, one of the critical aspects when designing clinical trials for licensure is the choice of a comparator vaccine. To do so, the following points should be considered:

The choice of the comparator vaccine will vary depending on the characteristics of the new acellular pertussis vaccine under development; however, the comparator should be, in general, the vaccine most similar to the new vaccine. Three potential scenarios are proposed when assessing a new vaccine formulation:

**Scenario 1.**

The new acellular pertussis combination vaccine containing 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 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-HBsAg, DTaP-HBsAg-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 vaccine.

**Scenario 2.**

The new acellular pertussis-containing vaccine has a composition which is the same, or very similar, as 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 as 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 antigens; similar amounts). NOTE: Vaccines evaluated in efficacy studies include one-component (PTxd) 66, 69, two-component
(PTxd/FHA) \textsuperscript{66, 71, 73, 74, 76}, three-component (PTxd/FHA/PRN) \textsuperscript{70, 72, 74}, four-component (PTxd/FHA/PRN/FIM2) \textsuperscript{77}, and five-component (PTxd/FHA/PRN/FIM2/FIM3) \textsuperscript{71, 74} acellular pertussis vaccines.

\textit{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: 1) the vaccine could be based on the currently used antigens, but present in different combinations, for example, 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, in particular when there are differences in the amount of antigen and proportion of the pertussis components.

\section*{C.2 \textit{Assessment of immune responses}}

\subsection*{C.2.1 \textit{Assays to assess antibody responses}}

Serological assays used in the clinical immunogenicity studies in support of vaccine licensure require validation \textsuperscript{84}. An international reference pertussis antiserum has been established to assist in the standardization of serological methods \textsuperscript{85}. 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; 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, for example, whether the criteria for evaluation is based on percentages with post-primary series titers above a threshold, seroconversion rates or geometric mean antibody titers.

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 used 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) \textsuperscript{86} or the \textit{B. pertussis} agglutination titer \textsuperscript{87-89}. Cell-mediated immune (CMI) responses play a role in protecting against \textit{B. pertussis} infection, however immunological assays to evaluate CMI responses following immunization...
have not been standardized and they have not been used to support licensure. Nevertheless, the exploratory assessment of CMI should be encouraged to enlarge the body of knowledge regarding all aspects of the immune response to pertussis antigens.

C.2.1.1 ELISA to assess antibody concentration 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. However, it is important to note that assays developed and optimized for diagnostic and epidemiologic purposes, including most commercially available ELISA kits, are unlikely to have the performance characteristics needed for vaccine immunogenicity studies. For example, 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 GMCs.

Collection, recording, analysis and interpretation of data should be conducted according to GCP guidelines. Methodological and statistical considerations described in WHO guidelines should be taken into account.

C.2.1.2 Assessment of functional antibody titers

Functional activity of antibodies against pertussis components have been identified as important additional parameters to consider, in particular 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. Assays to measure pertussis toxin neutralizing and whole-cell *B. pertussis* agglutinating antibodies have been established. Although there is no functional threshold that has been found to correlate directly with the protective efficacy of pertussis vaccines, they are nevertheless an important immune parameter to determine as part of the overall comparison of new vaccine formulations to those proven to be safe and effective.

Functional antibodies should be measured, at a minimum, in a subpopulation of the comparator and the 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 or post-licensure effectiveness, with the new product through randomized controlled trials.

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 non-inferior to that in the comparative vaccine group(s). The selection of the primary parameter for the assessment of non-inferiority, the pre-defined margin of non-inferiority and hence the total sample size for a comparative study will need careful justification. Although studies that
compare immune responses between candidate and licensed acellular pertussis vaccines are essential, comparisons to 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. Specifically, 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 appropriate time intervals for assessing the immune response should be defined taking into account the study objectives. In most cases, clinical studies for new vaccines for infants are designed to determine the antibody response to acellular pertussis components at approximately 4 weeks following the final dose. Pre-defined non-inferiority criteria using an appropriate acceptability limits are used to compare the responses in subjects immunized with the test vaccine versus the licensed comparator vaccine. (see Comparator vaccine section 1.2) using the following endpoint:

The following co-primary analyses are recommended:

- Percentage of responders: In one primary analysis the percentage of responders with significant increase (e.g. 4 fold 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 pre-defined non-inferiority limit; generally the lower bound of the two-sided 95% confidence interval of the observed difference should be less than 10 percentage points.

- Magnitude of the response: In a second primary analysis, the magnitude of the response is compared based on the geometric mean concentration (GMC) induced by the new vaccine and the licensed comparator using a pre-defined margin of non-inferiority to evaluate the antibody response (GMC ratios) to each acellular pertussis component (e.g. the lower bound of the two sided 95% confidence interval of observed ratio of the GMC of the new vaccine relative to the control should not be less than 0.50 or 0.67).

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 titers or B. pertussis agglutination titer 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. The functional assays used should be validated. At present, the interpretation of functional antibody data is made difficult by the fact that a titer that might correlate with protection against pertussis infection is unknown. For this reason it is recommended that comparisons of functional antibody titers between the new vaccine and the licensed comparator should focus on GMT ratios.

Additional information:

- Reverse cumulative distribution (RCD) curves: 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 effectiveness data to assess the potential need for additional doses later in life to maintain protection.

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, pre-school 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.

Once an acellular pertussis vaccine is licensed for use in infants, the evaluation of same formulations (e.g. co-purified vaccines) or formulations with lower amounts of pertussis components may be used as booster vaccines in adolescents and adults.

An active comparator vaccine may not be needed when evaluating the immunogenicity of the acellular pertussis components when used as a booster vaccine, for example, 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 also 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 exipients should be investigated as described in the WHO guidelines for clinical evaluation of vaccines.

In particular, it has been demonstrated that some acellular pertussis components can negatively impact the immune responses to some polysaccharide conjugated antigens when administered in pre-formulated products or when vaccines are mixed only immediately before injection (e.g. Hib 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 also become apparent that concomitant administration of acellular
pertussis components with other vaccines in routine use, including conjugated vaccines, may
give rise to detectable immune interference, although the clinical significance of the observed
phenomena is not always clear. Examples include decreased antibody responses to Hib and to
Men C monovalent vaccine. Therefore it is important that immune responses to candidate
acellular pertussis vaccines 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 pre-defined non-inferiority margins.

These studies might compare concomitant administration with administrations made in a
staggered fashion (e.g. together at 2, 4 and 6 months compared to the usual antigens at this
schedule and the new vaccine at 3, 5 and 7 months).

C.3 Safety evaluation

Although a demonstration of clinical efficacy is very difficult for new acellular pertussis
vaccines, preapproval clinical assessment should include a thorough assessment of vaccine
safety. The assessment of safety, with appropriately defined objectives, should be part of the
comparability study mentioned above, and should have sufficient power to provide reliable
rates of frequent or very common adverse events. The rates of specific adverse events
should be formally compared and should be based on anticipated rates from the trials
conducted in the past. It is important to note that the number of subjects enrolled in one
immunogenicity trial will not be sufficiently large to identified rare events of new vaccine
formulations. Therefore, it is expected that the safety database of pre-licensure clinical studies
be of a minimum of 3000 subjects, information on adverse events such as extensive limb
swelling syndrome should be carefully monitored.

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
programs. In particular, the impact of routine vaccination on pertussis infection needs to be
assessed in comprehensive studies of vaccine performance. Ongoing surveillance programs
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 only be obtained in geographical locations in which 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. All data collected should be submitted to the responsible National Regulatory Authorities at regular intervals so that any implications for the marketing authorization can be assessed.

**Part D. Recommendations for National Regulatory Authorities**

**D.1. General**

The general recommendations for control laboratories contained in the Guidelines for National Authorities on Quality Assurance for Biological Products and Guideline for Independent Lot Release of Vaccines by Regulatory Authorities should apply. The detailed production and control procedures and any significant changes in them should be discussed with and approved by the National Regulatory Authority.

Consistency of the production has been recognized as an essential component in the quality assurance of acellular pertussis vaccines. In particular, National Regulatory Authority should carefully monitor production records as well as 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 National Regulatory Authority**

A vaccine shall 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 National Regulatory Authority shall be provided at the request of the manufacturing establishment and shall certify that the lot of vaccine in question satisfies all national requirements as well as Part A of the present Requirements. The certificate shall state the number under which the lot was released by the National Regulatory Authority, and the number appearing on the labels of the containers. The official national release document shall 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

HIST by temperature measurement

Groups (not less than 10 of mice each) mice defined with respect to strain, sex and age, should be randomly allocated to the different treatments. Samples shall include the test sample(s) and a reference preparation. If reversion to toxicity testing is required by the National Regulatory Authority the test also should include a sample of the test vaccine incubated at 37°C for 4 weeks. All mice shall be challenged post-sensitization with histamine-dihydrochloride. The reference and histamine-dihydrochloride shall 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 and 40°C shall be used for the test.

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

Temperature responses are analyzed 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 National Regulatory Authority. There is at present 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 HSD) of pertussis toxin per single vaccine dose is a requirement for DTaP vaccines.
Appendix 2

HIST by lethal end-point assay

Groups of mice each of 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). 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 4 weeks. A single human dose (some methods allow up to two single human doses) of the final bulk is used as test dose for both groups. The position of the cages on the shelves during the testing period should be allocated at random, 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 histamine base is most commonly used) at 4 or 5 days after sensitization or injection with diluent. The histamine challenge dose can be administered intraperitoneally or intravenously, however, the injection route should be defined and the same route should be used for all testing within a 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 National Regulatory Authority. 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 size 20 or larger. 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 National Regulatory Authority. 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 National Regulatory Authority, shall be subsequently 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 pertussis toxin at a dose previously set as the allowable upper limit of pertussis toxin 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 National Regulatory Authority. 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 3

Mouse Immunogenicity Test (MIT)

The mouse immunogenicity test 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 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, ED$_{50}$.

For each antibody, the linear-response region of the dose response curve (vaccine dose versus antibody production) shall be determined. In the first method, a group of mice is injected once with a pre-selected 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 National Regulatory Authority, the serial-dose method may be simplified to an appropriate single-dose (e.g. ED$_{50}$ 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 vaccine. The reference vaccine ensures that the test mice respond in a way consistent with previous testing. The stability of the reference vaccine should be monitored. Appropriate stabilization of the reference 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 behavior in the test of clinical lots and those lots used in support of licensure, either in absolute terms or relative terms to the reference vaccine. However, the reference vaccine should be sufficiently similar to the clinical lots in composition and manufacture to act as an adequate control in the test. The response of the test vaccine may be reported either in absolute terms or in relative terms 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 in the MIT against which new lots of vaccines of acellular pertussis vaccine are evaluated are product-specific, and should be based on an appropriate statistical analysis of the responses observed in the test for vaccine clinical lots and other lots used in support of licensure. Specifications must be carefully justified and defined with the agreement of National Regulatory Authority. Specifications based on a simple failure to reject the null hypothesis of equivalency of immunogenicity between a reference lot and a
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 5 weeks of age), the optimal time for bleeding (e.g. 4 to 6 weeks after immunization), and the isotype of the antibody response should be thoroughly studied. The test design should be agreed with the National Regulatory Authority.

**Antibody detection system.** The ELISA used for detection of antibodies should be subjected to thorough validation and standardization studies. The 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- 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 for mice immunized with the reference vaccine and the test vaccine should be calculated. 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 ED$_{50}$. If the ELISA validity criteria are not met, the ELISA should be repeated.

After either a geometric mean or ED$_{50}$ has been calculated for the reference vaccine, the value should be compared with the criteria for sufficient antibody response 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 ED$_{50}$ for mice immunized with test vaccine should meet the criteria 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 (GM) antibody levels, in absolute terms or relative to the GM 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, GM 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 National Control Authority.
Appendix 4

Modified Intracerebral Challenge Assay

1. Materials

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

The challenge strain shall 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 LD$_{50}$ per 0.025 mL (or approximately $1\times10^5$ 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 shall be diluted serially to make at least three levels of four-fold or other suitable logarithmic dilutions. Each dilution shall be given by intraperitoneal injection at a dose of 0.5mL to at least 16 mice aged approximately 4 weeks. The animals shall be of the same sex or both sexes in equal numbers for each dose. The challenge suspension shall be given by intracerebral injection into the animals at a dose of 0.025 mL 21 days after immunization. The animals shall be observed for 14 days. Any animals dying within 3 days after challenge shall be excluded from the test. Any animals showing paralysis or swelling of the head at the end of the observation period shall be counted as deaths.

At least 3 appropriate serial dilutions of the challenge suspension shall be injected into at least 10 mice/group to titrate the virulence. The bacterial count for the LD$_{50}$ per 0.025 mL of the challenge suspension shall be no more than 300 CFU.

3. Criterion for judgment

Assay data are analyzed using parallel line analysis following probit transformation of the proportion of mice responding. The log dose – probit response lines are tested 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 IS for acellular pertussis vaccine (JNIH-3) has been established and an in-house reference used in this test should be calibrated in terms of the IS. The specifications should be established with the agreement of National Regulatory Authority. Where used currently the potency of the test sample is considered to be passed if the potency 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 shall be repeated using the same number or increased number of mice. Results for all statistically valid assays shall be combined. Weighted mean log potency shall 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 5

Method for respiratory challenge

The respiratory challenge model is designed to demonstrate the protective effect of immunising 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 immunising 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 formation units (CFU). The mouse protective effect of a test vaccine can be determined by comparison of its responses with 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 comparison with a reference vaccine included in the assay, it is useful for determination of protective activity of vaccines to assess potential impact of changing manufacturing process or formulation; during development of new formulations; potential interactions in combinations; stability studies and to assess lot consistency. A number of designs are possible with variation in age of mice, sampling times, and so on. A brief outline of the procedure for a harmonised intranasal/challenge method is given below:

Mice

Balb/c mice, three weeks old; 15 mice are to be ordered per vaccine group (i.e. 5 mice/time of sampling; 2 hours, 5 days and 8 days post-challenge; or alternatively on other days after validation study).

Immunisation

First immunization:

Prepare vaccine doses: one vaccine dose = ¼ of a human dose (e.g.125 µl) per mouse and per immunization. The mice are immunized using a 1ml syringe. The syringe is divided in 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.

Second immunization is carried out at 2-weeks post the first immunisation 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 plate containing
blood could be also 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 OD₆₅₀nm = 1, which corresponds to 3×10⁹ CFU/ml (this may vary according to each individual laboratory) and further dilution is carried out to obtain a suspension containing 10⁸ 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 with an automatic 50 µl pipette or 25µl into each nostril (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 2 hours, 5 and 8 days post-challenge following terminal anesthesia 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 1ml) was normally diluted 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:

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

**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

\[ M = \frac{500 + 47 + 6}{0.01 + 0.001 + 0.0001} \times 10 = 5.53 \times 10^4 \text{ CFU/ml} \]

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

Curve is then traced: mean of \( \log_{10} \text{CFU/lungs} \) versus day after infection.
Suggested validity criteria of the test

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

- 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 2 hours after challenge should be above 5.4
- The calculated $\log_{10}$ mean number of CFU/lung for the positive reference group at 5 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 5 days after challenge.

To demonstrate consistency, both reference and test vaccines must be used. 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, then interpretation of potency using a parallel line assay could be achievable.
Appendix 6

Summary protocol for acellular pertussis vaccine production and testing

Summary information on final lot

Name and address of manufacturer
Lot no.
Date of filling
Date of manufacturing
Nature of final product (absorbed)
Volume of each recommended single human dose
No. of doses per final container
No. of final containers
Container/closure system for the final lot
Expiry date

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
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<th>Purity of claimed antigens</th>
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<tr>
<td>1</td>
<td>Proportion of each antigen claimed:</td>
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</tr>
<tr>
<td>2</td>
<td>Methods:</td>
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</tr>
<tr>
<td>3</td>
<td>Date:</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Residual level of endotoxin</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Methods:</td>
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</tr>
<tr>
<td>6</td>
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</tr>
<tr>
<td>7</td>
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<tr>
<td>8</td>
<td>Antigen content</td>
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<td>Sterility test:</td>
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<td>13</td>
<td>Tests for bacteria and fungi</td>
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<tr>
<td>15</td>
<td>Media:</td>
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<tr>
<td>18</td>
<td>Volume of medium per container:</td>
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<td>40</td>
<td>Residual activity of pertussis toxin</td>
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</tr>
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</table>
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 HSD/SHD): ________________________
Calculation method: ________________________
Rectal temperature or Dermal temperature

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<tr>
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<td></td>
</tr>
<tr>
<td>Reference Dilution n</td>
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<td>Test vaccine</td>
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<tr>
<td>Negative</td>
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</table>

Dilution must contain 1.09 or 2.18 IU (0.2 or 0.4 HSD)/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

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Residual level of endotoxin

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Date: ________________________

Sterility test

Tests for bacteria and fungi

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Media: ________________________
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<td>Volume of inoculum per container:</td>
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<td>Volume of medium per container:</td>
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<td>Temperatures of incubation:</td>
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<td>Date of test (on, off):</td>
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<td>6</td>
<td>Result:</td>
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<td>Test for mycoplasmas</td>
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</tr>
<tr>
<td>16</td>
<td>Control of final bulk</td>
<td></td>
</tr>
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<tr>
<td>18</td>
<td>Identification</td>
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<tr>
<td>19</td>
<td>Volume</td>
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</tr>
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<td>21</td>
<td>Detoxifying agent</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>Methods:</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Content:</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Date:</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
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<td>26</td>
<td>Preservative content:</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>Methods:</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>Content:</td>
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</tr>
<tr>
<td>29</td>
<td>Date:</td>
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<tr>
<td>30</td>
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</tr>
<tr>
<td>31</td>
<td>Adjuvant</td>
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</tr>
<tr>
<td>32</td>
<td>Methods:</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>Content:</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>Date:</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>Sterility</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>Tests for bacteria and fungi</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>Method:</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>Media:</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>Number of containers tested:</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>Volume of inoculum per container:</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>Volume of medium per container:</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>Temperatures of incubation:</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>Date of test (on, off):</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>Result:</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>Test for mycoplasmas</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>Method:</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>Media:</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>Volume tested:</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>Temperature of incubation:</td>
<td></td>
</tr>
</tbody>
</table>
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 HSD/SHD): ________________________
Calculation method: ________________________
Rectal temperature or Dermal temperature

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Average</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference Dilution 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference Dilution 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference Dilution n</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test vaccine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Dilution must contain 1.09 or 2.18 IU (0.2 or 0.4 HSD)/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

<table>
<thead>
<tr>
<th>No of deaths/ No of animal inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference Dilution 1</td>
</tr>
<tr>
<td>Reference Dilution 2</td>
</tr>
<tr>
<td>Reference Dilution n</td>
</tr>
<tr>
<td>Test vaccine</td>
</tr>
<tr>
<td>Negative</td>
</tr>
</tbody>
</table>

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 HSD/SHD): ________________________
Calculation method: ________________________
Rectal temperature or Dermal temperature ________________________

<table>
<thead>
<tr>
<th>Temperature</th>
<th>average</th>
<th>variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference Dilution 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference Dilution 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference Dilution n</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test vaccine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilution must contain 1.09 or 2.18 IU (0.2 or 0.4 HSD)/SHD</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Test vaccine</th>
<th>No of deaths/ No of animal inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference Dilution 1</td>
<td>/</td>
</tr>
<tr>
<td>Reference Dilution 2</td>
<td>/</td>
</tr>
<tr>
<td>Reference Dilution n</td>
<td>/</td>
</tr>
<tr>
<td>Negative</td>
<td>/</td>
</tr>
</tbody>
</table>

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 ________________________
<table>
<thead>
<tr>
<th></th>
<th>Result for test vaccine</th>
<th>Result for reference vaccine</th>
<th>Or Test result (ratio to reference):</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>_______________________</td>
<td>_____________________________</td>
<td>____________________________</td>
</tr>
<tr>
<td>2</td>
<td>GMT value of anti-PT</td>
<td>GMT value of anti-PT</td>
<td>Ratio for anti-PT</td>
</tr>
<tr>
<td>3</td>
<td>GMT value of anti-FHA</td>
<td>GMT value of anti-FHA</td>
<td>Ratio for anti-FHA</td>
</tr>
<tr>
<td>4</td>
<td>GMT value of anti-PRN</td>
<td>GMT value of anti-PRN</td>
<td>Ratio for anti-PRN</td>
</tr>
<tr>
<td>5</td>
<td>GMT value of anti-Fims</td>
<td>GMT value of anti-Fims</td>
<td>Ratio for anti-Fims</td>
</tr>
<tr>
<td>6</td>
<td>_______________________</td>
<td>_____________________________</td>
<td>____________________________</td>
</tr>
<tr>
<td>7</td>
<td>Result for reference vaccine</td>
<td>Result for reference vaccine</td>
<td>Or Test result (ratio to reference):</td>
</tr>
<tr>
<td>8</td>
<td>_______________________</td>
<td>_____________________________</td>
<td>____________________________</td>
</tr>
<tr>
<td>9</td>
<td>GMT value of anti-PT</td>
<td>GMT value of anti-PT</td>
<td>Ratio for anti-PT</td>
</tr>
<tr>
<td>10</td>
<td>GMT value of anti-FHA</td>
<td>GMT value of anti-FHA</td>
<td>Ratio for anti-FHA</td>
</tr>
<tr>
<td>11</td>
<td>GMT value of anti-PRN</td>
<td>GMT value of anti-PRN</td>
<td>Ratio for anti-PRN</td>
</tr>
<tr>
<td>12</td>
<td>GMT value of anti-Fims</td>
<td>GMT value of anti-Fims</td>
<td>Ratio for anti-Fims</td>
</tr>
<tr>
<td>13</td>
<td>_______________________</td>
<td>_____________________________</td>
<td>____________________________</td>
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<td>14</td>
<td>Date:</td>
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<tr>
<td>15</td>
<td>_______________________</td>
<td>_____________________________</td>
<td></td>
</tr>
</tbody>
</table>

2. MICA

<table>
<thead>
<tr>
<th></th>
<th>Strain of mice</th>
<th>No. of mice per dilution</th>
<th>No of dilutions injected</th>
<th>Volume and route of injection</th>
<th>Date of injection:</th>
<th>Identification of reference</th>
<th>LD$_{50}$ in challenge dose</th>
<th>No. of colony-forming units in challenge dose</th>
<th>Date of challenge</th>
<th>Date of end of observation</th>
<th>Results (IU/SHD):</th>
<th>Calculation method:</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>___________________</td>
<td>________________________</td>
<td>________________________</td>
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<td>__________________</td>
<td>____________________________</td>
<td>____________________________</td>
<td>____________________________</td>
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<td>__________________</td>
<td>__________________</td>
<td>__________________</td>
<td>__________________</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Dilution</th>
<th>No of survivors/No of animal inoculated</th>
<th>Median effective dose (ED$_{50}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>Reference vaccine</td>
<td>1 __________________</td>
<td>1 ml</td>
</tr>
<tr>
<td>19</td>
<td>( IU/ml)</td>
<td>1 __________________</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Test vaccine</td>
<td>1 __________________</td>
<td>1 ml</td>
</tr>
<tr>
<td>21</td>
<td>__________________</td>
<td>1 __________________</td>
<td></td>
</tr>
</tbody>
</table>

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

**Final product**

<table>
<thead>
<tr>
<th></th>
<th>Identification</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
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<td>__________________</td>
</tr>
<tr>
<td>23</td>
<td>__________________</td>
<td>__________________</td>
</tr>
</tbody>
</table>

Identity test
1  Method of testing
2  Result
3  Date of test
4
5  **Sterility test**
6
7  Tests for bacteria and fungi
8    Method:
9    Media:
10   Number of containers tested:
11   Volume of inoculum per container:
12   Volume of medium per container:
13   Temperatures of incubation:
14   Date of test (on, off):
15   Result:
16
17  Test for mycoplasmas
18    Method:
19    Media:
20    Volume tested:
21    Temperature of incubation:
22    Positive controls:
23    Date of test (on, off):
24    Result:
25
26  **Test for adjuvant**
27    Nature and concentration of adjuvant/SHD
28    Method of testing
29    Specification
30    Result
31    Date of test
32
33  **Test for preservative**
34    Nature and concentration of preservative
35    Method of testing
36    Specification
37    Result
38    Date of test
39
40  **pH**
41    Method of testing
42    Specification
43    Result
44    Date of test
45
46  **Endotoxin test**
47    Method of testing
48    Specification
49    Result
50    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.

**Innocuity 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
- % of rejected containers

**Certification by the manufacturer**

Name of head and 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 A of the Recommendations to assure the quality, safety and efficacy of acellular pertussis vaccines (if applicable)

Name (typed)
Signature
Date

**Certification by the National Regulatory Authority**

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

Model certificate for the release of acellular pertussis vaccines by National Regulatory Authorities

This certificate is to be provided by the National Regulatory Authority of the country where the vaccines have been manufactured, on request by the manufacturer.

Lot release certificate

Certificate no. ________________

The following lot(s) of acellular pertussis vaccine produced by ________________ in ________________ whose numbers appear on the labels of the final containers, meet all national requirements\(^1\) and Parts A\(^4\), B and C of Recommendations to Assure the Quality, Safety and Efficacy of Acellular Pertussis Vaccines, (2012)\(^5\) and comply with Good Manufacturing Practices for Pharmaceutical Products\(^6\) and Good Manufacturing Practices for Biological Products\(^7\).

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

The certificate may include the following information:

- Name and address of manufacturer;
- Site(s) of manufacturing;
- Trade name and/common name of product;
- Marketing authorization number;
- Lot number(s) (including sub-lot numbers, 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; and
- Certificate number.

The director of the National Regulatory Authority (or authority as appropriate):

Name (typed) _______________________________________________

Signature __________________________________________________

Date ______________________________________________________

Footnote

\(^1\)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 National Regulatory Authority.
\(^4\)With the exception of provisions on distribution and shipping, which the National Regulatory Authority may not be in a position to assess.