

Annex 6

Recommendations for whole-cell pertussis vaccine

These Recommendations provide information and guidance to national regulatory authorities and vaccine manufacturers concerning the characterization, production and control of whole-cell pertussis vaccines to facilitate their international licensure and use. Each of the following sections constitutes a recommendation. The parts of each section printed in large type have been written in the form of requirements so that if a national regulatory authority so desires these parts may be adopted as definitive national requirements. The parts of each section printed in small type are comments and recommendations for guidance. It is recommended that modifications to these Recommendations be made only on condition that the modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the Recommendations set out below. It is desirable that the World Health Organization should be kept informed of any such differences.

To facilitate the international distribution of vaccine made in accordance with these recommendations, a summary protocol is given in the Appendix.

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Introduction

The WHO Requirements for whole-cell pertussis vaccine were first formulated in 1963 and the requirements for diphtheria and tetanus vaccines were prepared in 1964. Since diphtheria, tetanus and whole cell pertussis (DTwP) vaccines have been commonly used in a combined form, the requirements revised in 1978 contained separate sections on all three components as a followed by a final section that applied to a combination. The last revision of the requirements for diphtheria, tetanus, pertussis and combined vaccines was made in 1989 and published in 1990 (1).

Since that time a number of developments have taken place in the production, standardization and quality control of DTwP vaccines, as well as in the understanding of *Bordetella pertussis*, and it was considered that the existing requirements should be reviewed and where appropriate revised and updated. An amendment to the diphtheria and tetanus sections concerning single dilution and in vitro potency assays was adopted by the Expert Committee on Biological Standardization in 2004 (2). The present revision of the requirements for whole-cell pertussis vaccine should therefore be considered as part of the revision of the overall requirements for DTP. In 1998 the title WHO Requirements was changed to WHO Recommendations to better reflect the nature of these documents. These recommendations for whole-cell pertussis vaccine supersede those published in 1990 (1) and should be read in conjunction with the recommendations for diphtheria and tetanus vaccines when whole-cell pertussis vaccine is part of DTwP combined vaccine (1, 2). Once both the diphtheria and tetanus sections have also been fully revised, it is the intention to combine all three sections into one document.

Since 1989 a variety of combination vaccines based on DTP and involving a number of additional antigens have been developed and licensed. Many countries have already included tetravalent and pentavalent vaccines containing hepatitis B, *Haemophilus influenzae* type b conjugate (Hib) and inactivated polio vaccines (IPV), in addition to DTwP, in their immunization programmes. A need for further guidance on the evaluation of combination vaccines based on DTwP has been recognized and will be considered as a separate document.

In addition, a number of acellular pertussis vaccines have been licensed and used in combination with other vaccines for more than 20 years. Separate Guidelines for the production and control of the acellular pertussis component of monovalent or combined vaccines were developed in 1996 (3) (and their revision is also being undertaken separately).

General considerations

Pertussis is an important cause of infant death worldwide and continues to be a public health concern even in countries with high vaccination coverage.

Recent estimates from WHO suggest that in 2002 about 18 351 000 cases of pertussis occurred worldwide, the vast majority in developing countries, and that about 294 000 of those infected died. It is further estimated that in 2002, global vaccination against pertussis averted more than 37 million cases and 587 000 deaths (WHO/IVB database at http://www.who.int/immunization_monitoring/burden/estimates_burden/en/index.html).

It is clear that immunization programmes with high coverage have significantly reduced mortality and morbidity from the disease in many countries. However, despite its efficient prevention of clinical disease, the vaccine appears to have had limited impact on the circulation of *B. pertussis* even in countries with high vaccination coverage. The impact of vaccines on the circulating strains is not fully understood. In addition, during the 1990s, a significant epidemiological shift towards higher incidences of pertussis among schoolchildren previously vaccinated, adolescents and adults has been observed in many industrialized countries (4). This led to the consideration of a potential need for immunization of adolescents and adults to improve current control of whooping cough.

The optimal immunization schedule and the appropriate time for booster dose of DTwP vaccine should be assessed in individual national programmes taking into account the current epidemiological situation (4). Careful epidemiological surveillance of pertussis is encouraged worldwide to monitor disease burden and the impact of vaccination and particularly to compare different products and vaccination schedules.

Also, a shift in the antigenic properties of *B. pertussis* strains in circulation has been reported (5–7) and the continued monitoring of its potential impact on the overall immunity of a population is crucial in controlling the disease. Therefore, monitoring of genetic and antigenic characteristics of the pathogen in the context of the appropriateness of the strains of *B. pertussis* used in the production of both whole cell and acellular pertussis vaccines is encouraged.

Whole-cell pertussis vaccines have been used worldwide as part of combined DTP vaccine in national childhood immunization programmes for decades. Although concerns about possible adverse events following their administration have led to the adoption of acellular pertussis vaccines in some countries, whole-cell pertussis vaccines are still widely produced and used globally in both developed and developing countries. Whole-cell pertussis vaccines that comply with WHO requirements, administered according to an optimal schedule have a long and successful record in the control of whooping cough. Furthermore, the excellent efficacy of some currently available whole-cell pertussis vaccine has also been shown, not only in recent clinical trials, but also on the basis of the resurgence of disease where vaccination has been interrupted or when coverage has markedly decreased

(8). Therefore, WHO continues to recommend whole-cell pertussis vaccines for use in national immunization programmes. Further details are available in a WHO position paper on pertussis vaccines (4).

In terms of severe adverse events, acellular pertussis and whole-cell pertussis vaccines appear to have acceptable safety, whereas mild to moderate adverse reactions are more commonly associated with the whole-cell pertussis vaccine. The latter is not recommended for use in adolescents and adults. So far, no clinically significant immunological interference has been documented between whole-cell pertussis vaccines and other antigens when they are offered in a combination formulation, or with other vaccines simultaneously administered at different injection sites. This is in contrast to the reduced antibody levels to Hib vaccine that have been observed when given in combination with some acellular pertussis vaccines (9).

Recent developments in the production, standardization and quality control of pertussis vaccines were reviewed by a Center for Biologics Evaluation and Research (CBER)/WHO working group on pertussis vaccines in November 2000 and at a WHO consultation in July 2003. However, the scientific basis for the present revision of the requirements for whole-cell pertussis vaccines was developed at a WHO consultation of national regulatory authorities, vaccine manufacturers and other experts, in March 2005. Key areas covered included vaccine composition, potency evaluation and toxicity testing.

Considerable progress has been made in understanding the nature of some of the agglutinogens of *B. pertussis* (10). These are surface proteins which, on infection, elicit the production of antibodies that cause the agglutination of the organism in vitro. Some have been identified as fimbriae. The presence of fimbriae 2 and 3, formerly identified as agglutinogens 2 and 3, in whole-cell pertussis vaccines is believed to contribute to their protective efficacy, and a test has been included in these revised Recommendations for the purpose of determining whether fimbriae 2 and 3 are present, before adjuvant is added.

The evidence that vaccines shown to protect mice against intracerebral challenge also protected immunized children against whooping cough when such children were exposed to the disease in the home by infection from a sibling was published in the 1950s. This correlation was the basis for the establishment of the current potency test (11). Although the potency test has a long record of use, it has often been criticized, especially on its reproducibility. However, a recent WHO proficiency study involving 13 laboratories in 12 countries confirmed that the intracerebral challenge assay was effective in distinguishing potent and sub-potent batches of vaccine and gave consistent results both between repeat tests and between different laboratories (12).

Nevertheless, the mouse protection test is technically demanding and efforts have been made to develop alternative *in vitro* potency assays, such as serological assays. However, the lack of understanding of the mechanisms of protection in humans afforded by whole-cell pertussis vaccines, in particular, of the value of neutralizing antibodies, the nature of the critical antigens and the role of cell-mediated immunity, it is difficult to design an acceptable alternative. The serological approach was extensively discussed at an European Directorate for the Quality of Medicines/European Centre for the Validation of Alternative Methods (EDQM/ECVAM) consultation in 2005 (13) where the issue of the relevance of simple antibody measurements to human clinical protection was considered. It was concluded that such tests cannot yet be considered as validated alternatives to the mouse protection potency test for whole-cell pertussis vaccines. However, correlation between production of agglutinins in mice and protection in children demonstrated as early as the Medical Research Council (MRC) trials in the 1950s should be further explored as a potential alternative or a complementary test to the currently recommended potency test. There was also a strong recommendation from the EDQM /ECVAM consultation to use validated humane end-points in the mouse protection test.

The use of the WHO Opacity Standard has also been much discussed. Comments from many manufacturers and discussion at the WHO Consultation in 2005 indicated that the estimation of the number of bacteria using the opacity of the bacterial suspension prior to inactivation is still a valuable parameter in the in-process control of whole-cell pertussis vaccines. Manufacturers are encouraged to continue to express opacity in International Units and to specify the range of values for their own vaccine product.

The role of different toxins, such as pertussis toxin, heat labile (dermonecrotic) toxin, tracheal cytotoxin, adenylate cyclase toxin and endotoxin in immunity to the natural infection or in immunization is not fully understood. A potential link between the presence of some of these toxins and reactogenicity in humans has been reported, but the mechanisms of their action and the contribution of individual toxins to overall toxicity remains unclear. Nevertheless, the determination of residual toxic activity remains an important aspect of the safety assessment. Residual levels of active pertussis toxin and endotoxin are likely to be a major contributor to the reactogenicity of whole-cell pertussis vaccines in humans and limits have been established for active pertussis toxin in acellular pertussis vaccines. The First International Standard for pertussis toxin has been established and various methods for the determination of residual levels of this toxin in vaccine preparations have been developed. At present, there is no scientific basis for setting specifications for pertussis toxin and endotoxin in whole-cell pertussis vaccine preparations, but monitoring their levels for consistency during production is encouraged.

In recent years, safety concerns have been raised over the use of thiomersal in vaccines, especially those given to infants. These concerns have been based primarily on data regarding the toxicity of a related substance, methyl mercury, and from data on chronic exposure to mercury via the food chain. Such safety concerns have led to initiatives in some countries to eliminate, reduce or replace thiomersal in vaccines, both in single dose and multidose presentations. It is important to note that the concerns about the toxicity of thiomersal are theoretical and there is no compelling scientific evidence of a safety problem with its use in vaccines, although a public perception of risk remains in some countries. WHO policy is clear on this issue, and the Organization continues to recommend the use of vaccines containing thiomersal for global immunization programmes because the benefits of using such products far outweigh any theoretical risk of toxicity (14). In the case of whole-cell pertussis vaccines, thiomersal has been used in the production process as an inactivating agent as well as a preservative. Potential changes in its content, following licensing, may affect quality, safety and efficacy of the vaccine. In the event of any change, WHO Guidelines on regulatory expectations related to the elimination, reduction or replacement of thiomersal in vaccines (15) should be followed.

Changes made and issues addressed

The main changes made, and issues addressed, in the present revision are as follows:

- Final vaccine bulk should be examined to ensure it contains predominantly phase I bacteria that display fimbriae 2 and 3. Strains of *B. pertussis* used in production should be well characterized emphasizing phase I organisms. Markers for phase I strains (e.g. haemolytic activity) are suggested in small print.
- The reference reagents currently available are listed and include reagents for the determination of fimbriae 2 and 3.
- Determination of bacterial concentration is considered to be an important in-process control test and the International Reference Preparation of Opacity is still considered to be a valuable tool.
- The recommendation to use the mouse weight gain test to assess specific toxicity of vaccine preparations has been upgraded to large print whereas the details of the methodology and refined methodology are displayed in small print.
- During monitoring of detoxification processes, as well as when validating methods used for detoxification and establishing consistency of production, manufacturers are encouraged to monitor levels of pertussis toxin and endotoxin. International Standards for pertussis toxin and endotoxin are available and results should be expressed in IU.

- The estimation of potency has been upgraded from small to large print clarifying that it should not be less than 4.0 IU per single human dose with a lower fiducial limit of the estimated potency being not less than 2.0 IU.
- Manufacturers and control laboratories are encouraged to use validated humane end-points in recording results of potency testing.
- A new section on the stability evaluation of vaccines has been included which emphasizes the importance of real-time studies under intended storage conditions and discusses the extent of stability studies needed for different purposes and at different stages of manufacturing.
- Specific issues for nonclinical and clinical evaluation of new pertussis vaccines as well as a need for the improvements in postmarketing surveillance are also discussed in separate sections of this document.

Part A. Manufacturing recommendations

A.1 Definitions

A.1.1 *International name and proper name*

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

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

A.1.2 *Descriptive definition*

Whole-cell pertussis vaccine is a suspension of the whole cells of one or more strains of killed *Bordetella pertussis* which have been appropriately treated to minimize toxicity and retain potency. The preparations for human use should satisfy all the recommendations formulated below.

A.1.3 *International reference materials*

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>).

The third International Standard for Pertussis Vaccine was established in 1998 with a potency of 46 IU of pertussis vaccine per ampoule.

The fifth International Reference Preparation of Opacity was established in 1975 with an opacity of 10 International Units. It consists of plastic rods simulating the optical properties of a bacterial suspension.

The WHO reference reagents of monoclonal antibodies for *B. pertussis* anti- fimbriae serotype 2 and 3 were established in 2004. They are intended for the determination of serotype of *B. pertussis* strains.

The First International Standard for Pertussis Toxin was established in 2003 with an activity of 10 000 IU per ampoule. It is intended for the determination of residual pertussis toxin in pertussis vaccine.

The above-mentioned International Standards/reference materials and other reagents from the WHO Pertussis reagent Bank are in the custody of the National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, EN6 3QG, England (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.

A.1.4 **Terminology**

The following definitions are given for the purpose of these recommendations only.

Seed lot. A quantity of bacterial suspension that is derived from one strain, has been processed as a single lot and has a uniform composition. It is used for preparing the inoculum for the production medium.

Single harvest. A suspension of bacteria prepared from cultures of one strain of *B. pertussis* inoculated, harvested and processed together.

Final bulk. The homogeneous finished vaccine from which the final containers are filled either directly or through one or more intermediate containers.

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

A.2 **General manufacturing recommendations**

The general manufacturing recommendations contained in good manufacturing practices for pharmaceuticals (16) and biological products (17) should apply to establishments manufacturing whole-cell pertussis vaccine.

A.3 **Production control**

A.3.1 **Control of source materials**

A.3.1.1 *Strains of Bordetella pertussis*

Strains of *B. pertussis* used in preparing vaccines should be identified by a full record of their history, including their origin, characteristics on isolation, and particulars of all tests made periodically to verify strain characteristics.

The strains should be well characterized and chosen in such a way that the final vaccine contains predominantly phase I cells that display fimbriae 2 and 3. They should have been shown to the satisfaction of the national regulatory authority, to yield safe and immunogenic vaccines when inactivated.

The reference preparations of antibodies for detection of fimbriae 2 and 3 should be used.

Since haemolytic activity has been suggested as a marker for phase I cells, colonies of *B. pertussis* can be examined for this characteristic on a suitable solid medium containing blood. Alternatively, some culture media (e.g. meat extract agar plates) support the growth of phase III/IV isolates of *B. pertussis*, but not that of phase I bacteria, and these media can be used to confirm phase I status of cultures. When culture methods for phase I assessment are used, the media composition, blood type and concentration, and incubation time need to be properly defined.

The strains should be maintained by a method that will preserve their ability to yield potent vaccine.

Freeze-drying or storage in liquid nitrogen is a satisfactory method of maintaining strains.

A.3.1.2 *Seed lot system*

The production of pertussis vaccine should be based on a seed lot system. Cultures of the working seed should have the same characteristics as those of the strain from which the parent seed lot was derived.

A.3.1.3 *Culture media for production of bacteria*

The media chosen for growing *B. pertussis* should be carefully selected and enable the organism to grow well and to retain phase I characteristics. Given that different media have an impact on the quality of the vaccine, every effort should be made to use media proved as a substrate for manufacturing a vaccine that consistently meets the potency requirements. Once the media have been demonstrated as appropriate they should be consistently used. Every change of media should be validated and the national regulatory authority notified.

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. *B. pertussis* should be grown in media free from substances likely to cause toxic or allergic reactions in humans. 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 (18). When animal blood or blood products are used, they should be removed by washing the harvested bacteria.

In some countries, the use in the medium of blood from any source is not permitted. Manufacturers are encouraged to explore the use of media derived from non-animal sources.

Human blood or blood products should not be used in culture media for propagating bacteria, either for seed or for vaccine.

A.3.2 Control of single harvests

A.3.2.1 Monitoring consistency of production

Consistency of production should be demonstrated. Parameters to be measured include, but are not limited to, bacterial growth rate and some characteristics of phase I organisms in the culture, such as haemolytic activity and presence of fimbriae 2 and 3.

Criteria for acceptance or rejection of harvests should be defined.

A.3.2.2 Control of bacterial purity

Samples of single harvests taken before killing should be tested for purity by microscopic examination of stained smears or by inoculation into appropriate culture media. Single harvests should not be used for the final bulk if contamination has occurred at any stage in their production.

A.3.2.3 Control of opacity

The opacity of each single harvest should be measured not later than 2 weeks after harvesting and before the bacterial suspension has been subjected to any process capable of altering its opacity. It should be measured by comparison with the International Reference Preparation of Opacity or an equivalent reference preparation approved by the national regulatory authority. The opacity of bacterial suspensions should be expressed in International Units and specifications set for each vaccine.

A bacterial suspension having the same opacity as the International Reference Preparation of Opacity has a bacterial concentration providing 10 IU of opacity. The relationship between such units and actual numbers of bacterial cells may vary from vaccine to vaccine.

A spectrophotometric method validated against the opacity reference may also be used for this purpose.

A.3.2.4 Killing and detoxification

After samples of single harvests have been taken for purposes of purity control and opacity measurement, the bacteria shall be killed and detoxified by a method approved by the national regulatory authority. To ensure that the organisms have been killed, a sample should be tested in an appropriate culture medium.

B. pertussis can be killed by a number of methods whose effectiveness depends on the concentration of the chemicals used and the temperature, time and pH at which killing is carried out. The aim is twofold: to kill all bacterial cells and to achieve an appropriate level of detoxification without adversely affecting the potency or the physical characteristics of the vaccine. The methods used and kinetics of inactivation should be validated to the satisfaction of, and approved by, the national regulatory authority.

After killing and detoxification, the opacity of the suspension will be different from what it was originally. Each single harvest should, however, still be regarded as containing the same number of bacteria.

No biologically active heat-labile toxin (dermonecrotic toxin) should be detectable in a vaccine. The method of manufacture should be validated to ensure that no active dermonecrotic toxin is present in the final product. The method of detoxification should ensure vaccine safety. At present, it is not possible to recommend limits for levels of pertussis toxin, endotoxin, tracheal cytotoxin and adenylate cyclase in whole-cell pertussis vaccines. Manufacturers are encouraged to appropriately validate tests for these factors, and to ensure consistency of production.

A.3.3 Control of final bulk

A.3.3.1 Preparation

The final bulk may consist of a single harvest or a pool of single harvests from one or more strains. If a vaccine is prepared from two or more strains, the proportion of each strain in the pool, as calculated in opacity units, should remain consistent for each batch of the final bulk. The single harvest or pool should be diluted such that the number of bacteria in a single human dose of the final bulk is equivalent to the number of bacteria in the same volume of a suspension showing an opacity of no more than 20 IU. The opacity measured on the single harvests (before killing, see part A, section A.3.2.3) should be used to calculate the bacterial concentration in the final bulk.

A.3.3.2 Fimbriae

Each bulk should be examined, before adjuvant is added, for the presence of fimbriae 2 and 3 to ensure that appropriate expression has occurred during bacterial growth.

A.3.3.3 Preservative

If the vaccine is to be dispensed into multidose containers, a suitable antimicrobial preservative should be added. 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. If a preservative has been added to the vaccine, 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 nor impair the safety of the product in humans. The preservative, its use at different stages of the manufacturing process as well as its residual amount should be approved by the national regulatory authority.

If any modification of the preservative 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 (15).

Phenol should not be used as a preservative.

A.3.3.4 *Adjuvants*

If an adjuvant has been added to the vaccine, its nature, purity and concentration should be determined by a method approved by the national regulatory authority.

Either aluminium or calcium compounds may be used as mineral carriers.

Where aluminium compounds are used as adjuvants the concentration of aluminium should not exceed 1.25 mg. When calcium adjuvants are used, the concentration of calcium should not exceed 1.3 mg per single human dose.

In some countries, an upper limit of 1.25 mg of aluminium is considered to be excessive for products containing a pertussis component and such vaccines therefore contain only 0.1–0.3 mg of aluminium per single human dose.

If other substances have been used as adjuvants or those with adjuvanted effect, specifications should be set and agreed by the national regulatory authority.

The formulation should be such that the homogeneous suspension appear after shaking and remains as such for a specified time (e.g. time needed for vaccine administration).

A.3.3.5 *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) (19) or by a method approved by the national regulatory authority. If a preservative has been added to the

vaccine, appropriate measures should be taken to prevent it from interfering with the test.

A.3.3.6 *Specific toxicity*

Each final bulk should be tested for toxicity using the mouse weight gain test. The final bulk is considered satisfactory if the following conditions are met:

- (a) at the end of 72 hours the average weight of the group of vaccinated mice is not less than that preceding the injection,
- (b) at the end of 7 days the average weight gain per mouse is not less than 60% of that per control mouse, and
- (c) no deaths occur when 10 mice are used and no more than one death occurs when 20 mice are used.

A satisfactory method of carrying out the assay is as follows: at least 10 healthy mice each weighing 14–16 g are used for each vaccine group and for the saline control group. Mice should be of the same sex or segregated males and females should be distributed equally between all groups. Mice should have access to food and water for at least 2 h before injection, and continuously after injection for the duration of the test. The total weight of each group of mice should be measured immediately before injection. Each mouse is given an intraperitoneal injection of 0.5 ml of 0.85% NaCl aqueous solutions containing half of the recommended single human dose. The mice in the control group are inoculated with 0.5 ml of physiological saline, preferably containing the same amount of preservative as the inoculum injected into the test mice. The total weight of each group of mice is measured or calculated at 72 h and again at 7 days after injection.

If vaccine fails to meet the requirements in a first test, it can be retested once, and the results of the two valid tests should be combined.

If the average weight gain per mouse in the vaccine group is greater than 150% of that per control mouse, ascites production should be suspected and the test should be considered invalid.

Manufacturers are encouraged to develop refinements and alternatives to the mouse weight gain test.

In some countries a refinement of the mouse weight gain test is used. Mice are weighed individually immediately before injection, and 16–24 h, 72 h and 7 days after injection. On day 7 blood samples are taken from the tail vein and leukocytes are counted. Weight change at 16–24 h is considered to reflect the presence of lipo-oligosaccharide and an increase in the leukocyte count is considered to reflect the presence of pertussis toxin in the vaccine.

Other tests:

Cell harvests of *B. pertussis* to be used in the manufacture of pertussis vaccine contain a number of biologically active molecules which may contribute to the toxicity of the final product. Assays for some of these substances can be used to monitor and validate the methods used for detoxification and may also be useful in assessing final products. In the process of validating the manufacturing procedures, manufacturers are encouraged to monitor the following:

Pertussis toxin. A Chinese hamster ovary cell (CHO-cell) assay, based on the clustering of cells after treatment with pertussis toxin is used in some countries to measure pertussis toxin in vaccine. A pertussis toxin standard is included in the assay, and a vaccine reference is used as a positive control. All samples are serially diluted to obtain an end-point and the concentration of the pertussis toxin in the test sample is calculated in relation to the toxin reference. Tests for histamine sensitizing activity in mice may also be used.

Endotoxin. *B. pertussis* is a Gram negative organism, thus whole-cell pertussis vaccines contain lipo-oligosaccharide endotoxin. The endotoxin content of vaccines can be estimated by the limulus amoebocyte lysate assay or the rabbit pyrogen test. The limulus amoebocyte lysate assay is preferred. Although there is no agreement as to what constitutes an acceptable level of endotoxin in whole-cell pertussis vaccines, monitoring of endotoxin level on a lot-to-lot basis is encouraged as a monitor of consistency of production.

A.3.3.7 *Potency*

The potency of each final bulk (or of each final lot) should be determined by comparison with that of a reference vaccine calibrated against the International Standard for Pertussis Vaccine or an equivalent standard vaccine approved by the national regulatory authority. The assay should be performed by the intracerebral mouse protection test. The assay method and the method of calculating the results should be approved by the national regulatory authority. The potency is estimated in terms of IU in the volume recommended for a single human dose. The vaccine passes the recommendations for potency if the result of a statistically valid test 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. Additional tests may be done, but in this case the results of all valid tests must be combined in the weighted geometric mean estimate and its lower fiducial limit.

In some countries, an upper limit of potency is also specified.

A satisfactory method of carrying out the assay is as follows:

Mice. Healthy mice from a strain and colony capable of giving an adequate immune response are used. They should preferably be of the same sex but, if this is not possible, both sexes should be distributed equally throughout the test and the sexes segregated. Mice should be consistent for age and weight. An example of a criterion for consistency which has been used is that mice should weigh at least 10 g and not more than 18 g and in a single test the weight of the mice should not differ by more than 4 g.

The mice are randomly allocated to the different groups, and the shelf position of the cages, the order of immunization, and the order of challenge are also randomized. Groups of at least 16 mice should be used for each dilution of the standard vaccine and of the vaccines under test, and at least 10 mice should be used for each dilution of the culture in the estimation of the number of median lethal doses (LD_{50}) in the challenge dose.

Immunization of mice. At least three dilutions of the reference vaccine and of each lot of vaccine should be tested. Serial dilutions, not greater than fivefold, of the vaccine to be tested and of the standard vaccine should be made in a suitable diluent. The median effective dose (ED_{50}) for each preparation should be tested by the dilutions used. Each mouse in each immunization group should be injected intraperitoneally with 0.5 ml of the appropriate dilution.

The interval between immunization and challenge should be 14–17 days. At least 94% of the mice immunized by each dilution of both the reference vaccine and the test vaccines should survive until challenged, and each mouse challenged should appear healthy prior to challenge.

The challenge. The strain used for challenge (generally *B. pertussis* 18323) should be approved by the national regulatory authority. To ensure consistency of virulence from test to test, a large working challenge lot prepared from the master culture is dispensed into ampoules and freeze-dried or stored in liquid nitrogen.

The bacterial suspension used for challenge is prepared from a 20–24 h culture grown on Bordet–Gengou medium, or other suitable medium that has been seeded from a rapidly growing culture not more than 30 h old. Alternatively, aliquots of the challenge suspension may be frozen and kept in liquid nitrogen; after thawing and dilution, they can be used directly as the challenge culture. The suspension is diluted with a diluent in which the organisms will remain viable, e.g. an aqueous solution containing 10 g/l casein peptone and 6 g/l sodium chloride adjusted to a pH of 7.1 ± 0.1 . The suspension, free from particles of agar or clumps of bacteria, is adjusted in such a way that each challenge dose of not more than 0.03 ml contains 100–1000 times the LD_{50} .

Mice immunized with the reference vaccine and the test vaccines are challenged at random under mild narcosis by intracerebral injection of the challenge dose. To obtain an estimate of the LD₅₀, dilutions of the challenge dose are then injected into control mice by the intracerebral route and an appropriate dilution of the challenge dose is cultured on Bordet–Gengou medium to determine the number of colony-forming units contained therein.

Recording of results. The mice are observed for 14 days. Mice that die within 72 hours should be excluded from the test. To determine the ED₅₀ of the vaccines, records should be kept of the number of mice that die after 72 hours. Animal welfare regulations should be followed.

The use of validated humane end-points is encouraged.

Calculation of results. The ED₅₀ values for each preparation are determined by a statistical method that includes the transformation of the mouse survival data into a form capable of consistently producing a linear regression. Probits, logits and angle transformation have been shown to be suitable. Similar methods should be used to determine the LD₅₀ of the challenge suspension.

Validity of the test. The test is valid if the ED₅₀ of each vaccine is intermediate between the largest and the smallest immunizing doses, and the regressions do not show significant deviation from linearity and parallelism ($P < 0.05$). The challenge dose should contain 100–1000 LD₅₀ and the LD₅₀ should contain no more than 300 colony-forming units.

Estimate of potency. The ED₅₀ of the vaccine under test and the standard vaccine are calculated by a method that provides an estimate of the limits of the 95% confidence intervals. The potency is estimated in terms of IU in the volume recommended for a single human dose.

A.3.3.8 pH

The pH of each final bulk should be measured and specifications set.

In some countries this test is applied to the final filled vaccine (A 5.7).

A.4 Filling and containers

The requirements concerning filling and containers given in *Good manufacturing practices for biological products (17)* should apply to vaccine filled in the final form.

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

A.5 **Control of pertussis component in final lot**

A.5.1 **Identity**

An identity test should be performed on at least one container from each final lot.

The identity test may be based on an immunological reaction (for example, agglutination of the organisms) with a specific antipertussis serum. Alternatively, vaccines may also be inoculated into animals to show that pertussis-specific antibodies (e.g. agglutinins) are present in their serum.

A.5.2 **Sterility**

Final containers should be tested for sterility by a method approved by the national regulatory 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 (19). If a preservative has been added to the vaccine, appropriate measures should be taken to prevent it from interfering with the sterility test.

A.5.3 **Potency**

A potency test should be carried out as provided in Part A, section A.3.3.7, on each final lot, if such a test has not been done on the final bulk.

A.5.4 **General safety (innocuity) test**

Each final lot should be tested for unexpected toxicity (sometimes called abnormal toxicity) using a general safety (innocuity) test 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 general safety.

A.5.5 **Adjuvant content**

If an adjuvant has been added to the vaccine, its content 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 were used as adjuvants, appropriate specifications should be set for the substance with adjuvant effect.

A.5.6 **Preservative content**

If a preservative has been added to the vaccine, 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 or to impair the safety of the product in humans. The preservative, its use at different stages of the manufacturing process as well as its 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 (15).

A.5.7 **pH**

The pH of each final lot should be measured and specifications set.

In some countries this test is applied to the final bulk only (A 3.3.8).

A.5.8 **Inspection of final containers**

Each container in each final lot should be inspected visually, and those showing abnormalities — such as improper sealing, lack of integrity, clumping or the presence of particles — should be discarded.

A.6 **Records**

The recommendations given in Good Manufacturing Practices for biological products (17) should apply.

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

A.7 **Retained samples**

The recommendations given in Good Manufacturing Practices for biological products (17) (Annex 1) should apply.

A.8 **Labelling**

The recommendations given in Good Manufacturing Practices for biological products (17) should apply, with the addition of the following.

- the words whole-cell pertussis vaccine;
- the word “adsorbed”, if applicable;
- 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 requirements 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 transport**

The recommendations given in Good Manufacturing Practices for biological products (17) should apply.

A.10 **Stability, storage and expiry date**

A.10.1 **Stability**

Stability evaluation is an important part of the quality assessment. The purpose of stability studies is to ensure that the vaccine at the end of its shelf life, storage period or period of use, still has the required characteristics supporting quality, safety and efficacy.

For licensing

Studies that support stability of a vaccine for the purpose of licensing have to be performed as real-time studies under the intended storage conditions. Stability-indicating parameters should be carefully selected. They should always include, but should not be limited to, the potency test. Tests should be conducted to determine the loss of potency at appropriate time intervals during storage. Final containers from at least three batches of vaccine derived from different bulks should be tested on the expiry 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 licensing requires additional stability data to support the proposed modification and should be approved by the national regulatory authority. Following licensure, stability should be monitored throughout the proposed shelf-life.

At different stages of manufacturing process

Stability testing should be performed at different stages of production, namely single harvests, final bulk and final lot. Stability indicating parameters should be selected according to the stage of production. Manufacturers are encouraged to assign a shelf-life to all materials during vaccine production, in particular to intermediates such as single harvests, purified bulk and final bulk.

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, the stability data for such vaccines are generally available for a limited period.

Appropriate documentation to support the 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 pertussis vaccines, a temperature of 2–8 °C has been found satisfactory. This should ensure that the minimum potency specified on the label of the container or package will still be maintained after release until the end of the shelf-life, if the conditions under which the vaccine is stored are in accordance with what is stated on the label.

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

The vaccine must not be frozen.

A.10.3 *Expiry date*

The expiry date should be defined on the basis of the 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 whole-cell pertussis vaccines

Nonclinical evaluation of new pertussis vaccines

For a new whole-cell pertussis vaccine, a new formulation, or for a vaccine produced by a manufacturer with no previous experience of such vaccines and which has not been previously tested in humans, proof of concept in a relevant animal model in terms of both potency and safety should be demonstrated. In addition, a safety assessment should be undertaken before initiation of the clinical evaluation of a new vaccine. General principles for the design, conduct, analysis and evaluation of nonclinical data are available in the WHO guidelines for nonclinical evaluation of vaccines (20). In particular, studies on safety pharmacology intended to investigate the effects of a vaccine on vital functions should be undertaken.

Part C. Clinical evaluation of whole-cell pertussis vaccines

New whole-cell pertussis vaccines, vaccines with a new formulation, or those intended to use a new route of administration and /or produced by a manufacturer with no previous experience with such vaccines should undergo clinical evaluation. This section is intended to indicate some of the specific issues which need to be considered in the clinical testing of such vaccines, as monocomponent vaccines or as a part of a combination. Issues to be considered in designing clinical studies for licensing as well as those for monitoring clinical effectiveness and safety in postmarketing surveillance studies are discussed.

In general, clinical trials should adhere to the principles described in good clinical practice (21) as well as to those formulated for the design, conduct and analysis of vaccine clinical trials described in the WHO guidelines for clinical evaluation of vaccines (22). Data generated in clinical trials should be submitted to the national regulatory authority as described in the Summary protocol for vaccine evaluation (22). All clinical trials should be approved by the relevant national regulatory authority.

However, there are issues which apply specifically to pertussis clinical trials and these should be considered in addition to the general principles mentioned above. First, prospective randomized controlled studies of protective efficacy (i.e. testing against a placebo) cannot be performed for ethical reasons. Second, trials designed to measure efficacy relative to that of a licensed whole-cell pertussis vaccine with proven efficacy, would need to be very large in order to provide adequate precision in the efficacy estimates.

An additional complexity is that many different antigens are expressed by *B. pertussis* and there are many different assays that might be used for the assessment of the immunogenicity. However, without any established immunological correlate(s) of protection the data cannot be used to predict efficacy.

C.1 Clinical evaluation of new whole-cell pertussis vaccines for licensing

C.1.1 Compliance with the recommendations for production and control

Candidate vaccine should comply with the recommendations for production and control described in part A of this document.

C.1.2 Immunogenicity and safety assessment in humans

C.1.2.1 A comparability study using a “new” wP preparation which meets these requirements for potency and safety, and a wP-containing vaccine that has been licensed for some years and used extensively in countries with reliable postmarketing safety surveillance schemes may be an appropriate approach.

C.1.2.2 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. The assays used are unlikely to be commercially available, and thus validation issues must be addressed.

C.1.2.3 For each assay used, the immunogenicity data obtained should be compared both in terms of percentage of vaccinees who demonstrate a response (e.g. the percentage who reach a specified threshold or achieve a significant increase in antibody concentration) and in geometric mean concentrations (GMCs).

C.1.2.4 Every effort should be made to determine antibody response to individual, specific antigens rather than relying solely on the measurement of antibodies against whole cells or whole-cell extracts. Because of the historical link to clinical efficacy, the measurement of whole-cell agglutinins is recommended. Additionally, at least one assay used should determine antibodies against pertussis toxin.

C.1.2.5 The size of such a study and the end-points for evaluation require justification. The immunogenicity end-points need to be set according to the sensitivity and specificity of the assays and in the light of experience regarding natural variation between individuals.

C.1.2.6. In the case of combination with other antigens, potential interactions between the whole cell pertussis component and the others

should be investigated as described in the WHO guidelines for clinical evaluation of vaccines (22).

C.1.2.7 Safety assessment should be part of the comparability study mentioned above with defined objectives of the study. The study should have sufficient power to provide reliable rates of frequent or very common adverse events (22).

C.1.2.8 The rates of specific adverse events should be formally compared: the non-inferiority margin should be based on anticipated rates from the trials conducted in the past.

C.2 **Monitoring vaccine effectiveness and safety in the population**

Every effort should be made to improve current scientific understanding of the protection in humans by providing data from active postmarketing surveillance.

Vaccine effectiveness in the population should be reported wherever possible.

Given that limited safety data are obtained in pre-licensure studies, all relevant safety indicating parameters should be monitored as part of postmarketing surveillance.

Data generated in postmarketing surveillance should be submitted to the national regulatory authority.

Part D. Recommendations for National Regulatory Authorities

D.1 **General**

The general recommendations for National Regulatory Authorities contained in the Guidelines for National Authorities on Quality Assurance for Biological Products (23) should apply.

The detailed production and control procedures and any change in them that may affect the quality, safety or efficacy of whole-cell pertussis vaccine should be discussed with and approved by the National Regulatory Authority. The National Regulatory Authority should establish a national working reference preparation calibrated against the International Standard for Pertussis Vaccine.

Consistency of the production has been recognized as an essential component in the quality assurance of whole-cell pertussis vaccines. In particular,

National Regulatory Authority should carefully monitor results of tests performed on a series of consecutive batches of the final bulk.

D.2 **Official release and certification by the national regulatory authority**

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

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

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

Authors

The scientific basis for the revision of the Requirements published in 1990 was discussed at the meeting of the working group held at the World Health Organization, Geneva, in July 2003 attended by the following people: Dr J. Arciniega, Center for Biologics Evaluation and Research, Rockville, MD, USA; Dr M. Corbel, National Institute for Biological Standards and Control, Potters Bar, England; Dr R. Gaines-Das, National Institute for Biological Standards and Control, Potters Bar, England; Dr E. Griffiths, Centre for Biologics Research, Biologics and Genetic Therapies Directorate Health Canada, Ottawa, Canada; Dr J.G. Kreeftenberg, Netherlands Vaccine Institute (NVI), Bilthoven, The Netherlands; Dr S.S. Jadhav, Quality Assurance and Regulatory Affairs, Serum Institute of India Ltd, Pune, India; and Dr D. Xing, National Institute for Biological Standards and Control, Potters Bar, England.

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Taking into account comments on the first draft, a second draft was prepared by Dr Ivana Knezevic, Quality Assurance and Safety of Biologicals, World Health Organization, Geneva, Switzerland and Dr Elwyn Griffiths, Health Canada, Canada, in January 2005.

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Appendix

Summary protocol for whole-cell 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 _____

Single harvests used for preparing final bulk

List the single harvests and indicate the medium, dates of inoculation, temperature of incubation, dates of harvests, volumes, results of tests for bacterial purity, methods and dates of inactivation, opacity and fimbriae present.

Final bulk

Identification _____

Volume _____

No. of opacity units (calculated from opacities of
single harvests) _____

Test for fimbriae 2 and 3

Date and results (before addition of adjuvant) _____

Sterility test

Sample tested and volume _____

Media, volume and temperatures of incubation _____

Date(s) of inoculation _____

Date(s) of end of observation _____

Result of each test _____

Specific toxicity test (mouse weight-gain test)

Strain of mice _____

No. of mice _____

Volume and route of injection _____

Date of end of observation _____

Result of test: on a separate sheet of paper, give all relevant details on mice in the control and test groups (survival, mean weight on day of injection and three and seven days after injection) and indicate percentage weight of test group as compared with control group).

Other specific toxicity tests

Mention here date and results of any other specific toxicity test(s) which may have been performed (e.g. tests for heat-labile toxin, lymphocytosis promoting factor and endotoxin) _____

Potency test

Strain, weight and sex of mice _____

Date of immunization _____

LD₅₀ in challenge dose _____

No. of colony-forming units in challenge dose _____

Date of challenge _____

Date of end of observation _____

Results _____

Calculation method _____

	<i>Dilution</i>	<i>No. of survivors/ No. of animals inoculated</i>	<i>Median effective dose (ED₅₀)</i>
Reference vaccine (..IU/ml)	_____	_____	_____ ml
	_____	_____	
	_____	_____	

	<i>Dilution</i>	<i>No. of survivors/ No. of animals inoculated</i>	<i>Median effective dose (ED₅₀)</i>
Test vaccine	_____	_____	_____ ml
	_____	_____	
	_____	_____	

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

pH

Date of measurement _____

Result _____

Specification _____

Final product

Identity test _____

Date of test _____

Type of test and result _____

Sterility test

No. of times the test had to be performed _____

No. of containers tested in each test and volume _____

Media, volume and temperatures of incubation _____

Date(s) of inoculation _____

Date(s) of end of observation _____

Result of each test _____

Potency test

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 potency tests in the “final bulk” section.

Innoccuity test	Mice	Guinea-pigs
No. of animals	_____	_____
Route of injection	_____	_____
Volume of injection	_____	_____
Date of start of test	_____	_____
Date of end of test	_____	_____
Results	_____	_____

Test for adjuvant

Date of test _____

Nature and concentration of adjuvant per single human dose _____

Method of testing _____

Specification _____
Result _____

Test for preservative

Date of test _____
Nature and concentration of preservative _____
Method of testing _____
Specification _____
Result _____

pH

Date of measurement _____
Method of testing _____
Specification _____
Result _____

Inspection of final containers

Date of inspection _____
Organoleptic characteristics _____
Number of containers inspected _____
% of rejected containers _____

Stability test¹

Indicate separately all relevant details and (as a percentage) the calculated losses of potency per year at different temperatures, as determined by accelerated degradation tests, and actual titres² (with limits of 95% confidence intervals) after storage for the maximum period claimed for the product at the recommended temperature.

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 pertussis vaccine, whose number appears on the label of the final containers, meets all national requirements³ and satisfies Part A of the pertussis vaccine section of Requirements for Biological Substances Nos. 8 and 10, revised 1989 and (if applicable) addenda 19...

¹ Not required in summary protocols of every batch.

² Needed only for three batches to validate the production method.

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

Signature _____
Name (typed) _____
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 2, a label from a final container, and an instruction leaflet for users.