WHO Technical Report Series, No. 878, 1998

Annex 2
Guidelines for the production and control of the acellular pertussis component of monovalent or combined vaccines

1. General considerations 57

2. Scope 62
2.1 Control of starting materials 62
2.2 Control of the manufacturing process 63
2.3 Control of final bulk 65
2.4 Control of final lot 67
2.5 Stability, storage and expiry date 68
2.6 Reference materials 68

Authors 69

Acknowledgements 70

References 70

Appendix
Methods currently in use in some countries for quality control of acellular pertussis vaccines 72

1. General considerations

Coordinated efforts on the part of manufacturers, research institutions and national control authorities have led to the development of a variety of acellular pertussis vaccines that appear to be effective in clinical studies. These positive developments have led to the need for international guidelines to assure the quality of this new generation of pertussis vaccines.

These guidelines are concerned with the acellular pertussis component of monovalent and combined vaccines. They are intended to cover the production and control of pertussis vaccines composed of one or more individually purified or co-purified antigens. The vaccines tested in studies of clinical efficacy have been mainly products where the acellular pertussis component has been formulated with diphtheria and tetanus antigens.

There is as yet no consensus about the antigenic composition of an ideal acellular pertussis vaccine. Acellular pertussis vaccines currently available from different manufacturers should be considered as different and unique products because of the presence of one or more different components (chemically or genetically detoxified pertussis toxin, filamentous haemagglutinin (FHA), 69 kDa outer-membrane protein (also known as pertactin), fimbrial-2 and fimbrial-3 antigens) in different concentrations, and with different degrees of adsorption to different adjuvants. In addition, these individual antigens may be derived from different strains of *Bordetella pertussis* and have been purified by different methods. For these reasons the protective efficacy in humans of various manufacturers' products may be based on different mechanisms, which complicates the direct comparison of the protective activity of various products and new formulations by means of simple laboratory tests. Indeed, no unequivocal immunological correlates of protection against pertussis have yet been demonstrated, nor has a generally accepted animal model to predict clinical efficacy been validated.

In the light of this difficulty, any change in the manufacture or formulation of an acellular pertussis vaccine that has been shown to be safe and effective in clinical studies should be treated with the utmost caution. While, ideally, the safety and efficacy of a new formulation should be demonstrated in the target population, it is becoming increasingly difficult to undertake efficacy trials. Consequently, other criteria may be accepted by a national control authority as predictors of clinical performance. One possibility is to demonstrate the induction of immune responses equivalent to those induced by an approved homologous acellular pertussis vaccine of proven safety and efficacy. However, additional information about the physicochemical and immunological characteristics of a new vaccine formulation will also be necessary to demonstrate its equivalence with a homologous approved pertussis vaccine. Such criteria should be discussed with the national control authority.

In view of these considerations, it is essential that research to identify immunological markers of protection against pertussis be actively supported and pursued, and that there be rigorous post-licensing monitoring of vaccines for safety and effectiveness.

Consistency of manufacture

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 to prove efficacy and safety in clinical trials. In addition, laboratory tests should show equivalence in safety, potency, physicochemical and immunological characteristics of new vaccine lots compared with a homologous reference vaccine. Such a reference vaccine should in turn show equivalence to lots of known clinical efficacy. Only by these means can maximum assurance be given of the safety and efficacy of vaccine production lots. Manufacturers should ensure that sufficient quantities of homologous reference vaccine of adequate stability are available for routine in-house testing and for confirmatory tests undertaken by the national control authority. This approach is only meaningful when the immunological and physicochemical characteristics of the reference vaccine are the same as they were when clinical trials demonstrating efficacy were performed.

Characterization of antigens

The immunological, biological and biochemical characterization of the individual antigens claimed to contribute to vaccine efficacy is critical for the demonstration of the structural and/or functional integrity of these components in vaccine production lots. The relevant tests must be done before any procedure, such as detoxification or chemical treatment, known to modify the immunological or biological characteristics of the components in carried out. Other tests, for example the test for residual activity of pertussis toxin, are more appropriately undertaken after detoxification or chemical treatment of antigen lots. If antigen lots are not detoxified or chemically modified, then all tests indicated in section 2.2.3 should be performed on the purified antigen lots.

The quantification of individual antigens in the final bulk and assessment of their adsorption to the adjuvant are important for assuring consistency of formulation. However, the effect of the detoxification process and the potential interference of adjuvant with certain quantitative tests, such as enzyme-linked immunosorbent assays (ELISAs) for the individual antigens, may complicate standardization of the final bulk. For this reason manufacturers are encouraged to develop reliable methods for monitoring individual antigens in the final bulk.

There is as yet no internationally accepted direct method of measuring the potency of acellular pertussis vaccine that can guarantee protective immunity will be elicited in the target population. However, the characteristics of the various antigens claimed to contribute to vaccine efficacy, together with data on vaccine composition and

dosage, consistency of production, and conformity with the specifications of the vaccine used in clinical trials give some indication, though not definitive proof, of the ability to elicit protective immunity.

At the present time, immunogenicity assays in mice that compare test vaccine with homologous reference vaccine are widely used for the lot release of the vaccine final bulk. The adoption of this approach should not be interpreted to imply that such assays possess all the desirable characteristics of a potency assay. However, in the absence of laboratory correlates of human protection, the immunogenicity test in mice is a simple means of complementing other tests for consistency of manufacture. To standardize the antibody tests used to evaluate the immunogenicity of individual antigen components in mice, manufacturers are encouraged to include appropriate mouse reference sera.

Research to identify assays that better predict protective efficacy in humans is strongly encouraged.

Recent information suggests that evidence of induction of cell-mediated immunity, cytokine induction, and protection against aerosol or intranasal challenge with *B. pertussis* may give useful information for further characterization of the vaccine. In addition, a modified intracerebral protection test in mice, used in some countries for lot release, may merit further investigation.

Toxicity testing

Despite advances in the immunochemical knowledge of the toxins and other potentially reactogenic components produced by *B. pertussis*, uncertainty remains regarding the exact role played by these substances in the pathogenesis of pertussis and in vaccine reactions. This has hampered the establishment of scientifically sound limits for the residual activity of these components in vaccines containing pertussis antigens.

Chemically or genetically inactivated pertussis toxin is a component of all acellular pertussis vaccines currently produced. For that reason, toxicity tests to monitor residual pertussis toxin activity and, where appropriate, the possible reversion of pertussis toxoid to pertussis toxin during storage are critical for ensuring the safety of the product.

Acceptable limits should be based on consistency of manufacture, i.e. the amount of active pertussis toxin in a new production lot should not exceed that present in lots shown to be safe in clinical studies. The histamine-sensitizing test appears to be suitable for lot release.

The histamine-sensitizing test is based on the fact that exposure of certain strains of mice to active pertussis toxin increases their sensitivity to the lethal effect of histamine by a factor of approximately 100. Although the physiological basis of this phenomenon is not well understood, an assay based on histamine sensitization has been shown to be specific and adequately sensitive for the detection of active pertussis toxin in acellular-pertussis-vaccine preparations. Many factors influence the histamine-sensitizing activity of pertussis toxin, including the strain, age and sex of mice used for the assay, the route of administration of the preparations, the amount of histamine used for the challenge and a number of less-well-characterized environmental factors.

The manufacturing process should be designed to reduce the level of lipooligo-saccharide (LOS) endotoxin from *B. pertussis* associated with the antigens constituting the vaccine. The LOS content of the final bulk pertussis vaccine is usually measured for lot release by means of the *Limulus* amoebocyte lysate (LAL) test. LOS content should not exceed the amount present in lots shown to be safe in clinical trials.

In addition to these lot-release tests, manufacturers are required to submit evidence of the absence of residual activity of heat-labile toxin, tracheal cytotoxin, adenylate cyclase toxin, as well as the lack of reversion to toxicity in the final product, as part of the validation of the manufacturing process. Where appropriate, detailed information about the kinetics of the detoxification of the pertussis toxin must also be submitted. The Chinese-hamster ovary-cell (CHO) toxicity test is a convenient and sensitive test for this purpose, and can be used instead of the histamine-sensitizing test.

It should be noted that the mouse weight-gain test and leukocytosis-promotion test, which are currently in use to monitor the toxicity of whole-cell pertussis vaccine, are considered to be of insufficient sensitivity to demonstrate residual pertussis-toxin activity in acellular pertussis vaccines.

Purity

Tests for the presence of residual levels of reagents used in bacterial culture or antigen purification (e.g. cyclodextrin or fetuin) should be included, and limits should be set. In addition, the purity of preparations of individually purified antigens should be assessed either for every lot or as an important part of process development and validation.

2. Scope

The guidelines apply to the production of acellular pertussis vaccines. Where the acellular pertussis component is to be combined with other antigens (e.g. diphtheria, tetanus toxoids), tests recommended for the final bulk of acellular pertussis vaccine must be performed on the final bulk of the combination vaccine.

The guidelines cover control of the following three areas:

- the starting materials
- the manufacturing process
- the final product.

The general manufacturing requirements contained in Good Manufacturing Practices for Pharmaceutical (*1*) and Biological (*2*) Products apply to the production of the acellular pertussis component of monovalent or combined vaccines.

Written descriptions of the standard operating procedures used for the preparation and testing of the acellular pertussis component of monovalent or combined vaccines, together with evidence of appropriate validation of each production step, should be submitted for approval to the national control authority as part of the licensing application. Proposals for any modifications of the manufacturing and/or control methods should be submitted for approval to the national control authority before they are implemented.

2.1 Control of starting materials

2.1.1 *Strains of B. pertussis*

Strains of *B. pertussis* used in preparing vaccine should be identified by a full record of their history, including origin and 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 control authority.

2.1.2 *Seed-bank system*

The production of the acellular pertussis component of monovalent or combined vaccines should be based on a well characterized seed-bank system. Cultures from the working seed bank should have the same characteristics as cultures from the master seed bank. If genetically modified *B. pertussis* is used, the relevant modified DNA sequences should be reconfirmed at the working-seed-bank level.

The strains should be maintained by a method approved by the national control authority. The method should preserve the ability of the seed to yield potent vaccine in terms of the quality of the antigens produced.

If validated, freeze-drying or storage in liquid nitrogen is a satisfactory method of maintaining strains.

2.1.3 *Culture media for production of bacteria*

The media used should enable *B. pertussis* to grow and produce the antigens of interest in good yields. Human blood or blood products should not be used in culture media, neither for seed banks nor for vaccine production. Where possible, animal blood or blood products should likewise not be used, but, if they are, they should be derived from animals in good general health, and the final product should be tested for the presence of contaminating antigens and allergenic substances.

Media constituents or other materials of bovine origin should comply with the guidelines given in the *Report of a WHO Consultation on Medical and other Products in relation to Human and Animal Transmissible Spongiform Encephalopathies* (*3*) and should be approved by the national control authority.

2.2 Control of the manufacturing process

2.2.1 *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 or antigens. Acceptance specifications should be established.

2.2.2 *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.

2.2.3 *Control of purified antigens*

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 these characteristics. Particular attention should be given to employing a range of analytical techniques based on different principles. Suitable assays include sodium-dodecyl-sulphate–polyacrylamide-gel electrophoresis (SDS-PAGE), single radial immunodiffusion, immunoblotting, the CHO-cell toxicity test, haemagglutination and high-performance liquid chromatography (HPLC).

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 efficacious in clinical trials.

Antigenic purity of the vaccine component(s). The purity of the individual or co-purified antigens should be determined by SDS-PAGE, HPLC or other appropriate analyses before detoxification. It is important for the techniques used to be 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 found for vaccine lots shown to be efficacious in clinical trials.

Residual levels of endotoxin. The antigens should be tested for residual endotoxin content by means of the LAL test or other appropriate assay. Endotoxin content should be consistent with levels found to be acceptable in vaccine lots used in clinical trials and approved by the national control authority.

Tests undertaken after detoxification/chemical treatment

Residual activity of pertussis toxin. The amount of residual biologically active pertussis toxin in the individually or co-purified antigens should be estimated after detoxification by means of a sufficiently sensitive test, for example the CHO-cell test. When diluted to vaccine strength, the total amount of residual pertussis toxin from all pertussis antigens should not exceed that found in vaccine lots shown to be safe in clinical trials and approved by the national control authority.

Antigen content. The amount of individually or co-purified antigens that have been characterized, purified and detoxified, as appropriate, and are ready for formulation of the final bulk, 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.

Sterility test. Each purified antigen lot 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) (*4*), or by a method approved by the national control authority.

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

2.3 Control of final bulk

2.3.1 *Preparation*

The final bulk is prepared by mixing the adjuvant (as appropriate) with suitable quantities of individually or co-purified antigens so as to meet the specifications of vaccine lots shown to be safe and efficacious in clinical trials. A preservative may be added.

2.3.2 *Detoxifying/stabilizing agents*

The content of free residual detoxifying or stabilizing agents in the final bulk should be determined by methods approved by the national control authority. Limits should be specified. If formaldehyde has been used, the residual content should not exceed 0.2 g/l.

2.3.3 *Preservative*

If preservative is added, the content should be determined by a method approved by the national control authority.

The amount of preservative in the final bulk should be shown not to have any deleterious effect on the antigens for which a claim of protective efficacy is made, and should be shown not to cause any unexpected adverse reaction in humans. The preservative and its concentration should be approved by the national control authority.

2.3.4 *Adjuvant*

When adjuvant is added, its content in the final bulk (or final lot) should be determined by a method approved by the national control authority.

When aluminium or calcium compounds are used as adjuvants, the concentration of aluminium should not exceed 1.25 mg per single human dose, and that of calcium 1.3 mg. Adsorption of antigens to the adjuvant should be investigated, where 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 effective.

2.3.5 *Sterility*

Each final bulk should be tested for bacterial and mycotic sterility as indicated in section 2.2.3, *Sterility test.*

2.3.6 *Residual activity of pertussis toxin*

Each final bulk of vaccine should be tested for the presence of active pertussis toxin using a sufficiently sensitive histamine sensitization test (see Appendix). The acceptable amount of active residual pertussis toxin in the final bulk diluted to vaccine strength should meet the specification approved by the national control authority on the basis of vaccine lots shown to be safe in clinical studies.

2.3.7 *Reversion to toxicity*

Manufacturers should demonstrate to the satisfaction of the national control authority that chemically inactivated pertussis toxin present in the final bulk does not revert to its toxic form before the vaccine expiry date. The national control authority may base approval on tests performed on the product (in the final containers and after storage at the recommended temperature for a period at least as long as the validity period) as described in section 2.3.6. In addition, accelerated reversion testing, such as by subjecting the final bulk to the above test after storage for at least 4 weeks at 37 °C, may be useful, and the national control authority may require this test as part of the initial validation of the inactivation process rather than as a test required for the release of individual lots.

2.3.8 *Immunogenicity*

The immunogenicity of each vaccine component claimed to contribute to efficacy should be tested by comparison with a homologous reference vaccine. At present, this test is usually performed in mice (see Appendix). The strain or strains of mouse used should allow a sufficient immune response to be detected for each antigen.

The reference vaccine should preferably be appropriately stabilized to allow for a meaningful comparison between the immunogenic activity of the test vaccine and that of the original lots of vaccine shown to be effective in clinical trials. Careful attention must be given to the demonstration of any effects the stabilizing procedure may have on the immunogenic activity of the reference vaccine. The reference

vaccine should either be drawn from a vaccine lot with proven clinical efficacy, or from a subsequent lot manufactured by the same process used to manufacture the clinical trial lots, and with adequate potency as demonstrated in the immunogenicity assay described in the Appendix.

The immunogenic activity of each antigen claimed to contribute to vaccine efficacy should be within the specification approved by the national control authority on the basis of the immunogenic activity of the corresponding antigen in the reference vaccine.

An alternative method to the immunogenicity test described in the Appendix is a modified intracerebral protection test in mice, which is used for lot release in some countries (see General considerations).

2.4 Control of final lot

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

2.4.1 *Identity*

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

2.4.2 *Sterility*

Each final lot should be tested for sterility as specified in section 2.2.3, *Sterility test.*

2.4.3 *Adjuvant content*

Unless determined for the final bulk, each final lot should be assayed for adjuvant content (where appropriate). The method used and the adjuvant concentration permitted should be approved by the national control authority. The latter should be within the range of values found for vaccine lots shown to be clinically safe and effective.

2.4.4 *Preservative content*

Where appropriate, each final lot should be assayed for preservative content, if this has not been done for the final bulk. The method used and content permitted should be approved by the national control authority.

2.4.5 ***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.

2.5 Stability, storage and expiry date

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 control 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 immunogenicity and lack of specific toxicity of the product up to the expiry date.

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

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

2.6 Reference materials

Manufacturers should set aside, as reference material, a vaccine lot equivalent to the vaccine lots tested in the clinical trials on the basis of which the licence was granted. Manufacturers should also maintain adequate stocks of reference sera.

No formally established international reference materials that would allow standardization of the acellular pertussis component of vaccines are currently available, but their development is under consideration. In the absence of such materials, the following reagents are offered through the courtesy of different manufacturers and national control authorities.

(1) Pertussis toxin for confirmation of the sensitivity of histamine sensitization tests (J-NIH 5).

(2) Serotyping agents for *B. pertussis* strain characterization.
(3) Mouse reference serum for standardization of the immunogenicity test in mice (US Standard Pertussis Antiserum (mouse), Lot 1 (SPAM-1)). SPAM-1 has been calibrated by ELISA against J-NIH 11 (mouse anti-pertussis-FHA serum) and J-NIH 12 (mouse anti-pertussis-toxin serum) to derive anti-FHA and anti-pertussis-toxin unitage, respectively. It has also been assigned anti-pertactin unitage. As supply is limited, a new mouse reference serum is under development.
(4) Human reference sera for standardization of clinical serology (US Reference Pertussis Antiserum (human), Lots 3 and 4).
(5) FHA for quantification of FHA content of vaccine (J-NIH 4).
(6) Pertactin, for quantification of pertactin content of vaccine.

Reagents (1), (2), (5) and (6) are available in limited quantities from: Division of Bacteriology, WHO International Laboratory for Biological Standards, National Institute for Biological Standards and Control, South Mimms, Potters Bar, Herts., England EN6 3QG.

Reagents (3) and (4) are available in limited quantities from: Center for Biologics Evaluation and Research, Food and Drug Administration, 1401 Rockville Pike, Rockville, MD 20852-1448, USA. Additional reagents are under development; details are available upon request.

Authors

The first draft of these guidelines was prepared at an informal consultation held at the Istituto Superiore di Sanità, Rome (2–3 November 1995), attended by the following participants:

Dr J.L. Arciniega, Center for Biologics Evaluation and Research, Rockville, MD, USA

Dr M. Corbel, National Institute for Biological Standards and Control, Potters Bar, Herts., England

Dr N. Dellepiane, National Microbiology Institute, Buenos Aires, Argentina

Dr R. Dobbelaer, Institute of Hygiene and Epidemiology, Brussels, Belgium

Dr E. Griffiths, Chief, Biologicals, World Health Organization, Geneva, Switzerland

Dr I. Heron, Statens Seruminstitut, Copenhagen, Denmark

Dr B. Ivanoff, Vaccine Research and Development, World Health Organization, Geneva, Switzerland

Dr H. Kreeftenberg, National Institute of Public Health and Environmental Protection, Bilthoven, Netherlands (*Chairman*)

Dr P. Mastrantonio, Istituto Superiore di Sanità, Rome, Italy

Dr B. Meade, Center for Biologics Evaluation and Research, Rockville, MD, USA

Dr J. Milstien, Scientist, Vaccine Supply and Quality, World Health Organization, Geneva, Switzerland

Dr S. Robertson, Vaccine Research and Development, World Health Organization, Geneva, Switzerland

Dr A. Robinson, Centre for Applied Microbiology and Research, Salisbury, England (*Rapporteur*)

Dr H. Sato, National Institute of Health, Tokyo, Japan

Dr Y. Sato, National Institute of Health, Tokyo, Japan

Dr M. Schwanig, Paul Ehrlich Institute, Langen, Germany

Dr M. Tiru, National Bacteriological Laboratory, Stockholm, Sweden

Acknowledgements

The draft guidelines were discussed and received valuable comments at the International Meeting on the Control and Standardization of Acellular Pertussis Vaccines, co-sponsored by WHO, the International Association of Biological Standardization and the National Institute for Biological Standards and Control, Potters Bar, held in Potters Bar, Herts., England, 26–27 September 1996.

Acknowledgements are also due to the following experts for their comments and advice:

Dr I.R. Clement, Director, Regulatory Affairs, Connaught Laboratories, North York, Ontario, Canada; Dr R.K. Gupta, Chiron Vaccines, Emeryville, CA, USA; Mr J. Holst, Head of Control, National Institute of Public Health, Oslo, Norway; Dr R. Mignolet, Director, Regulatory Affairs Europe, Wyeth-Lederle Vaccines and Pediatrics, Louvain-la-Neuve, Belgium; Dr B. Montagnon, Pasteur–Mérieux–Connaught, Marcy l'Etoile, France; Dr H. Sato, Vaccine and Therapeutic Development, National Institutes of Health, Bethesda, MD, USA; Dr G. Schild, Director, National Institute for Biological Standards and Control, Potters Bar, Herts., England; Dr P. Parkman, Parkman Associates, Kensington, MD, USA; Dr J.R. Vose, Director, Scientific and Regulatory Affairs, Pasteur–Mérieux–Merck Sharp and Dome, Lyon, France.

References

1. Good manufacturing practices for pharmaceutical products. In: *WHO Expert Committee on Specifications for Pharmaceutical Preparations. Thirty-second Report.* Geneva, World Health Organization, 1992, Annex 1 (WHO Technical Report Series, No. 823).

2. Good manufacturing practices for biological products. In: *WHO Expert Committee on Biological Standardization. Forty-second Report.* Geneva, World Health Organization, 1992, Annex 1 (WHO Technical Report Series, No. 822).

3. *Report of a WHO Consultation on Medical and other Products in relation to Human and Animal Transmissible Spongiform Encephalopathies.* Geneva, World Health Organization, 1997 (unpublished document WHO/BLG/97.2; available on request from Biologicals, World Health Organization, 1211 Geneva 27, Switzerland).

4. General Requirements for the Sterility of Biological Substances (Requirements for Biological Substances No. 6, revised 1973). In: *WHO Expert Committee on Biological Standardization. Twenty-fifth Report.* Geneva, World Health Organization, 1973, Annex 4 (WHO Technical Report Series, No. 530).

Appendix

Methods currently used in some countries for quality control of acellular pertussis vaccines

In this Appendix, immunogenicity and histamine-sensitization tests in mice are described. These methods are currently widely used for the lot release of final bulk pertussis vaccine. However, this should not be interpreted to imply that the immunogenicity test in mice possesses all the desirable characteristics of a potency assay. Further research will be necessary to develop improved assays for predicting the protective efficacy of pertussis vaccine in humans.

Histamine-sensitization test for active pertussis toxin

A histamine sensitization test for the detection of active pertussis toxin is as follows. Groups of between 10 and 20 mice each, defined with respect to strain,[1] sex and age, should be randomly allocated to the different treatment protocols. One such group (the positive control group) should be injected with three or more serial dilutions of a preparation of pure pertussis toxin. Another group should be injected with one or more dilutions of the vaccine final bulk. Dilution factors should be chosen so as to obtain a graded response; however, dilution factors should be no greater than five. The dilution scheme should be optimized for each preparation. An additional group of mice (the negative control group) should be injected with diluent. The positions of the cages on the storage shelves during the testing period should be allocated at random. All mice should be challenged by injection with a defined dose of histamine between 4 and 5 days after sensitization or injection with diluent. Histamine challenge should follow the place-order of the cages on the shelves. Deaths within 24 hours of histamine injection should be recorded. For the assay to be considered valid, mice injected with diluent must not show substantial sensitization to the lethal effect of histamine. In addition, the susceptibility to sensitization of the mice used in each test should be validated and the median histamine-sensitizing dose (HSD_{50}) of pertussis toxin should be shown to be acceptable. The HSD_{50} should be calculated by using a suitable statistical method, such as probit analysis. Departure from linearity of the log dose–response relation for the positive control

[1] Several strains of mouse (all with Swiss-Webster ancestry) are highly responsive to histamine sensitization, but a number of Swiss-Webster strains, both inbred and outbred, are weakly responsive. A limit for the strain chosen should be established on a statistical basis. Strains considered acceptable show a median histamine-sensitizing dose (HSD_{50}) (point estimate) for pure pertussis toxin below 50 ng. When validating mice used in the assay, variability of the estimate of HSD_{50} should be taken into account.

group should not be demonstrable (with 99% confidence). Once linearity has been established by repeated experiments, the assay may be simplified so as to include in each test only a single positive control group.

An estimate of the amount of residual active pertussis toxin in the final bulk can be expressed in terms of an HSD_{50} dose. Alternatively, the amount of residual active pertussis toxin can be expressed as the proportion of animals that die upon sensitization with a single dose of pertussis vaccine (usually a single human dose) and subsequent histamine challenge. The residual pertussis toxin activity in the final bulk should not exceed that of vaccine lots shown to be safe in clinical trials. If a vaccine lot fails in a single test, it should pass two additional consecutive and independent assays to be considered suitable for release.

Immunogenicity test in mice

The immunogenicity test for acellular pertussis vaccine is a standardized assay designed to demonstrate consistent immunogenicity in mice from lot to lot for each antigen in the vaccine. Immunogenicity can be measured as either the geometric-mean amount of antibody produced in mice injected with a test dose of vaccine, or as the minimal dose of each antigen inducing a measurable antibody response in a certain proportion of mice (e.g. the median effective immunizing dose (ED_{50})).

In the first method, a group of mice is injected with a pre-selected dose of vaccine that is within the linear-response region of the dose–response curve (vaccine dose versus antibody production) for a given antigen. After an appropriate length of time, another test dose of vaccine may be required for preparations containing multiple antigens, because of the differential immunogenicity of the antigens in mice. In the second method, groups of mice are injected with serial dilutions of vaccine. After consistency in manufacturing and testing has been demonstrated to the satisfaction of the national control authority, the serial-dose method may be simplified to a 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 and standardized 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 a group of mice injected with homologous reference vaccine. The

reference vaccine ensures that the test mice respond in a way consistent with previous testing. It is therefore essential that the reference vaccine be appropriately stabilized, preferably by lyophilization.

The results of the mouse immunogenicity test for new lots of acellular pertussis vaccine are compared with those from lots for which efficacy was directly demonstrated in human clinical trials, or with lots shown in human immunogenicity studies to be equivalent to such lots. Immunogenicity of vaccine as measured in this test is not an index of clinical efficacy, but rather a means of showing that newly manufactured lots have an equivalent immunogenicity to clinically efficacious vaccine lots when tested with a standardized assay in mice.

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.

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. Studies for reference serum standardization should include an evaluation of the parallelism of the dose–response 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 should be studied. Two parameters of the assay system that define its performance are the minimal amount of antibody that can be accurately distinguished from background (the limit of detection, LOD), and the minimal amount of antibody that can be measured with a conventional precision (the limit of quantification, LOQ). These limits are necessary for evaluating the capacity of the assay to discriminate between a mouse that has responded to a given antigen and one that has not.

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 LOD or 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. The number of mice responding to each antigen should be used to calculate 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 strain of mouse used in the assay was validated. If the criteria are met, the test vaccine should be examined. If the criteria 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. Such limits should be determined by performing several tests on all available lots shown to be clinically efficacious. If geometric mean antibody levels are below the established limit or the ED_{50} is above the established limit, as determined in valid ELISAs, immunizations and ELISAs should be repeated for those antigens that fail the test. After a second test (if

valid), the geometric mean antibody levels or ED_{50} should be calculated and results of the two tests should be combined by appropriate statistical methods. The limit values 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 potency test. If any antigen does not satisfy its adjusted limit after two assays, the vaccine fails the potency test.

The method used to calculate antibody response, as well as the treatment of non-responder mice in the calculation of vaccine potency, should be approved by the national control authority.