Annex 5

Recommendations to assure the quality, safety and efficacy of tetanus vaccines (adsorbed)

Replacement of Annex 2 of WHO Technical Report Series, No. 800, and Annex 5 of WHO Technical Report Series, No. 927

Recommendations published by WHO are intended to be scientific and advisory in nature. Each of the following sections constitutes recommendations for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these WHO Recommendations may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these Recommendations be made only on condition that modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the Recommendations set out below. The parts of each section printed in small type are comments or examples intended to provide additional guidance to manufacturers and NRAs.

Introduction

Tetanus vaccines are among the most frequently used vaccines worldwide and have been remarkably successful products. Their widespread use in routine immunization programmes has resulted in a significant decrease in the incidence of tetanus in both developed and developing countries. Ensuring good coverage of childhood vaccinations and appropriate booster immunization of adults are essential to maintaining protection against tetanus (*1*).

Single-antigen tetanus toxoid vaccine (i.e. containing only tetanus toxoid) is almost never used in infants and children. Instead, tetanus toxoid is delivered as part of a primary immunization series in which it is generally presented in combination with diphtheria toxoid alone, or with diphtheria and whole-cell pertussis (DTP), or with diphtheria and acellular pertussis antigens (DTaP). Tetanus toxoid is also present in other combined vaccines that may contain, in addition to diphtheria and pertussis antigens, inactivated poliovirus, hepatitis B surface antigen or *Haemophilus influenzae* type b capsular polysaccharide conjugates, or some combination of these. Monovalent tetanus toxoid may be used more frequently for immunizations in adults and adolescents to reinforce immunity, which is essential because immunity to tetanus is induced only by vaccination. However, even for booster immunizations in adults, tetanus vaccines are often combined with other vaccines, and are generally formulated with a lower amount of antigens, particularly of diphtheria and acellular pertussis, when compared with vaccines intended for primary immunization (*1*). The potent immunogenicity of tetanus toxoid has also led to its use as a carrier protein in polysaccharide-protein conjugate vaccines such as *Haemophilus influenzae* type b conjugate vaccines (*2*).

The need for and use of tetanus vaccine will continue since immunity to tetanus is induced only by immunization because the pathogenic dose is lower than the immunogenic dose, and recovery from clinical tetanus does not result in protection against further episodes. The widespread use of tetanus vaccine, combined with improved standards of hygiene, has significantly reduced the incidence of tetanus disease in developed countries. However, neonatal tetanus remains a problem in developing countries, with infections occurring primarily through the unhealed umbilicus, and the mortality rate is high even with hospital care (*3*). The impact of neonatal tetanus is substantial and accounts for the most deaths from vaccine-preventable diseases, with an estimated 59 000 babies dying from tetanus in 2008 (*4, 5*).

Widespread coverage has not been achieved in low-income countries owing to the prohibitive cost of vaccination, which results from the requirements for multiple doses, parenteral administration by trained medical staff, and the need for a cold chain. Efforts are thus needed to improve the vaccine in terms of lowering the number of doses – e.g. by developing slow-release products (*6, 7*),

delivering the vaccine without needles (*8*) and improving the formulation and stability to eliminate the need for cold-chain storage. Subunit vaccines produced by recombinant technology that include a nontoxic receptor binding domain, the Hc fragment of tetanus toxin, are in various stages of development, and have shown promising results during preclinical evaluations (*9–11*). However, there are no data available from clinical trials in humans.

History of WHO Requirements and Recommendations, and standardization

The first WHO Requirements for tetanus toxoid vaccines were published in 1964 (*12*). These were revised in 1978 (*13*), with addenda in 1986 (*14*) and 1989 (*15*), and with further amendments made to the potency section during a consultation in 2003 (*16*).

The development of tetanus toxoid vaccines, and the publication of requirements for their manufacture and quality control, was helped considerably by the availability of international standards and international reference preparations.

The first milestone in the global standardization of tetanus toxoid was the establishment of the International Standard for tetanus antitoxin, equine in 1928 (*17*), which was replaced in 1969 (*18*). The availability and use of this preparation enabled toxoids to be assessed in terms of their ability to produce tetanus antitoxin in humans, and allowed protective units for antitoxin to be defined in International Units (IUs).

The first standard for tetanus toxoid established by WHO was the First International Standard for tetanus toxoid, plain (established in 1951). The IU was defined as the immunizing activity of 0.03 mg of the international standard, and was approximately equivalent to the existing German protective unit, the *Schutzeinheit* (*19*). This unit was defined on the basis of results obtained in guinea-pig challenge assays. At the Expert Committee meeting in 1958, it was noted that in mice, adsorbed preparations of tetanus toxoid could not be assayed with validity against the nonadsorbed international standard, and a recommendation was made that a separate International Standard for tetanus toxoid, adsorbed was needed (*20*). Thus the First International Standard for tetanus toxoid, adsorbed was prepared for determining the potencies of vaccines containing tetanus toxoid (adsorbed), and was established in 1966 (*21*). This standard was assigned an activity of 120 IU per ampoule (where 1 mg was determined to be equivalent to 1.5 IU of a previously established international standard (*22*), based on the results obtained in guinea-pig challenge assays). The International Standard for tetanus toxoid, adsorbed has been replaced at 10–20-year intervals with IUs assigned by WHO that have been based solely on calibration in guinea-pig challenge assays relative to the existing standard (*23–27*).

The Requirements published in 1964 specified assays against an international standard vaccine but required no minimum potency. A study published in 1970 provided evidence of a positive correlation between the amount of adsorbed tetanus toxoid in IUs (determined in guinea-pigs) and the antitoxin response in infants immunized with different DTP vaccines (*28*).

It has been recognized that there are difficulties in providing evidence of a direct correlation between the estimated potency of a vaccine (in a biological assay) and the level and duration of protective immunity in humans. Despite this lack of direct evidence, the minimum requirement for tetanus potency – 40 IU per single human dose (SHD) – which was introduced into WHO guidance in 1978 (*13*), has helped to ensure the production and release of safe and effective tetanus vaccines, based on the satisfactory performance of the vaccines in clinical studies and on the low incidence of tetanus in populations with good immunization coverage. The recommendation of 40 IU per SHD as a minimum requirement for tetanus potency for primary immunization is therefore retained in this latest revision of the Recommendations. However, the use of product-specific minimum requirements for potency may be justified, provided they are based on the results of clinical and laboratory studies, and approved by the national regulatory authority (NRA).

At the time of the 1990 revision, it was internationally agreed that the potency of tetanus vaccines could be measured by an active challenge test, and that either guinea-pigs or mice could be used as well as either a lethal challenge or a paralytic challenge dose. It was further stated that in vitro methods could be used for the determination of antibody levels instead of an in vivo challenge method, provided that appropriate validation studies had been done using the vaccine being tested. The 1990 Requirements emphasized explicitly that it was important for countries to adopt the principle of expressing the potency of tetanus vaccines in IUs, but failed to clarify the use of IUs across diverse methods and with increasingly diverse formulations. However, it was noted that when whole-cell pertussis vaccine is mixed with tetanus toxoid, and when the potency assay is carried out in mice, there is a significant adjuvant effect due to the whole-cell pertussis component. Subsequently, an increase in tetanus potency was also noted with combination vaccines containing *Haemophilus influenzae* type b and tetanus toxoid (*29*).

A number of international consultations convened during the late 1990s identified the need to clarify WHO guidance on the introduction and use of alternative potency assays for the purpose of routine lot release, and the transferability of IUs when different methods are used. The main revisions in the 2003 amendments, published in 2005 (*16*), thus included a division of the section on potency testing to clearly distinguish the recommendations for licensing from those for routine lot release. However, as with previous versions of the Recommendations, it was acknowledged that difficulties remained in the global

harmonization of potency testing procedures, even when international standards were used, and that different approaches were taken by different countries. The approach taken by WHO, as well as by the European Pharmacopoeia (*30*), is based on determining the immunizing potency of each final bulk by comparing it with an appropriate reference material that has been calibrated in IUs against the International Standard for tetanus toxoid, adsorbed. In some countries, potency is determined using the United States National Institutes of Health assay. In this test, the vaccine is assessed according to its ability to induce a protective or functional antibody response in guinea-pigs that reaches a minimum threshold of 2 Units per ml, as measured by an in vivo toxin neutralization test (TNT) against a standard antitoxin preparation (*31*). Although data are available demonstrating that vaccines meeting such requirements can induce significant levels of antitoxin response in recipients, the limitations of assays performed in the absence of a reference vaccine have been well documented (*32*). The expression of tetanus potency in IUs defined by the reference vaccine thus remains the approach recommended by WHO. However, there is still no universally accepted method for potency testing, and the formulation of global requirements remains a challenge.

A number of studies have shown that different results may be obtained when potency tests are carried out in mice instead of guinea-pigs (*33–35*). It was acknowledged in the 2003 amendments that mouse-challenge assays could be used for the potency testing of tetanus vaccines provided that the transferability of IUs had been demonstrated. However, this is not an entirely satisfactory procedure in view of the decreasing number of laboratories with experience in performing challenge assays in guinea-pigs (*27*). In addition, such an approach is particularly resource-intensive since a demonstration of transferability (through calibration in IUs) may be required for each product or product type.

It has been noted that many laboratories, particularly in the WHO European Region, rely largely on mouse-protection assays, and have adopted IUs for use in mouse assays from mouse-challenge data, despite the lack of traceability back to the first International Standard for tetanus toxoid, plain (*27*). Further, it has been noted that different laboratories calibrate in-house working standards using diverse methods, some using guinea-pigs and some using mice (with IUs from guinea-pigs or with IUs from mice), and some even use mean values determined in the two assay models (*36*). At its 2010 meeting, during adoption of the Fourth WHO International Standard for tetanus toxoid, adsorbed, the Expert Committee recommended that a working group should review the issue of transferability among different assay models, and the use of mouse-potency assays for expressing tetanus vaccine potency in IUs. The working group noted that a number of studies had highlighted the lack of agreement among guinea-pig assays and mouse assays in terms of tetanus vaccine potency expressed in IUs (*33–35*), and also noted that (according to the

results of a survey by WHO of vaccine manufacturers and control laboratories) in many cases mouse assays are widely used for the routine quality control of tetanus vaccines and for calibrating secondary standards. The logistical and practical difficulties of performing studies using guinea-pigs in order to demonstrate transferability were also recognized. The working group proposed that tetanus vaccine standards (including international standards, and regional, national and other secondary standards) could be calibrated using a mouse-challenge assay, and assigned IU. It is therefore recommended that the Fourth International Standard for tetanus toxoid, adsorbed (NIBSC code 08/218) should be assigned a potency of 260 IU (on the basis of the results obtained in the international collaborative study) for use in mouse-potency assays (*26*, *27*).

The working group acknowledged that the WHO minimum requirement for tetanus potency was originally based on results obtained in guinea-pig challenge assays, using standards calibrated in IUs in guinea-pigs. However, the working group also acknowledged that vaccines with demonstrated clinical safety and efficacy have been licensed and released in which in vivo potency has been determined using the mouse-challenge model, including assays for which the reference preparation has been calibrated using a mouse-challenge assay. The group therefore proposed that the specifications for the minimum requirements for potency assays should be retained. It is recommended that the impact of expressing vaccine potency relative to a reference preparation that has been calibrated in mice should be closely monitored.

The discussion about requirements for potency assays provided an opportunity to question the relevance of animal models for predicting an effective protective response in humans. The minimum specification for potency, as recommended by WHO, has served well over many years, and there is a long history of the successful use of tetanus vaccines. Whereas some clinical studies have confirmed comparable functional antibody levels resulting from different doses of tetanus vaccine in humans and guinea-pigs (*37*, *38*), there have been other examples of clinical studies (39) in which both mice and guinea-pigs were reported to provide comparable information for the type of product investigated. However, the influences of mouse strains on tetanus potency have been well documented (*40–42*), and there is evidence that different ratios of functional to nonfunctional antibodies are induced in different species (*43*). Therefore, the potency assays for new vaccines should be able to detect functional antibodies at an early evaluation stage, and should ultimately be approved on the basis of their effective performance in clinical trials. This is of particular relevance for newer combinations and when additional specifications for indicating consistency are under consideration.

During the past decade, there has been much activity aimed at simplifying the multiple-dilution direct-challenge potency tests by reducing the number of animals used or refining the end-points (such as paralysis), or by using validated

serology assays. Some studies have also considered the possibility of using the same animals to test the potency of several antigens (*44*). The 2003 amendments (published in 2005) emphasized that methods other than challenge tests may be preferred for evaluating the potency of tetanus vaccines on a routine basis, but the amendments also indicated that potencies calculated by methods other than the challenge test should not be assumed to be transferable without validation. Suitable alternative methods and technical advice on validating such methods are given in the revised WHO *Manual for quality control of diphtheria, tetanus and pertussis vaccines* (*45*).

Amendments adopted in 2003 noted that, despite many attempts to harmonize potency requirements globally, there are still no universally accepted methods, and recognized that this leads to problems in the international exchange of these vaccines due to difficulties in the mutual recognition of the results of testing. Recommendations made by the working group on IUs and transferability aim to simplify and harmonize approaches, but it has been recognized that global acceptance and adoption of these remain a challenge.

During the revision process for these Recommendations, WHO held a scientific consultation in Beijing, China, in November 2011. At that consultation, the option of harmonizing minimum potency requirements for tetanus vaccine with those recommended in the *European Pharmacopoeia* was discussed. It was acknowledged that amending the WHO minimum requirement for potency could improve harmonization and the international exchange of vaccines. As a result, the minimum requirement for the potency of tetanus vaccine, tested according to the methods described in these Recommendations, was amended such that the specification (dependent on product type and method – i.e. 40 IU/ SHD for vaccines intended for primary immunization in the absence of a whole-cell pertussis component) now applies to the lower 95% confidence limit, thus demonstrating that the vaccine potency significantly exceeds the defined minimum specification. Because the minimum potency requirement now applies to the lower limit of the 95% confidence interval, there is no requirement to achieve a 95% confidence interval narrower than 50–200%. However, the revised section on potency testing in Part A of these Recommendations includes information on criteria that should be met in order for the potency estimate to be statistically valid.

The main changes included in this latest revision comprise:

- a change of title from Requirements to Recommendations;
- an update of the section on international standards and reference preparations, which has been moved to the General considerations section;
- an update of the section on general manufacturing recommendations and control tests;

- amendment of the minimum requirement for the potency of tetanus vaccine, which now applies to the lower limit of the 95% confidence interval;
- provision for using the mouse-challenge assay for calibration of standard preparations for tetanus toxoid (adsorbed);
- inclusion of new sections to provide guidance on the clinical and nonclinical evaluations of tetanus vaccines to assess safety, quality and efficacy.

In order to facilitate the release process of vaccines made in accordance with these Recommendations, a model protocol is provided in Appendix 1.

Certain issues, such as recommendations for labelling and lot release, are covered in more detail by other WHO documents (46).

Scope of the Recommendations

These Recommendations apply to the production and quality control of adsorbed tetanus vaccines, and have been updated from the 1989 revision of the Requirements for diphtheria, tetanus, pertussis and combined vaccines (15) and the 2003 Amendments to those Requirements (16). The current Recommendations highlight advances made in the production and testing of tetanus vaccines and related intermediates. The recommendations for the quality control of tetanus vaccines included in this document are based on currently licensed vaccines. Other products (such as those containing a new type of antigen or produced using novel technology) may require additional considerations.

Although these Recommendations apply to the production and quality control of tetanus vaccines, most tetanus vaccines are presented in their final formulation with at least one other vaccine. Therefore, in addition to monovalent tetanus vaccine, these Recommendations also apply to tetanus vaccine used in combination vaccines, and the tests recommended for the final bulk or final fill also apply to combined vaccines where appropriate.

In some cases, one or more of the component vaccines may be presented in separate containers that are intended to be mixed prior to administration. In such instances, tests may not need to be performed on the final combination during routine testing, but any effect on assays would normally be confirmed during nonclinical evaluation and as part of the licensing process.

General considerations

The supply of effective tetanus vaccines depends on the use of well characterized and standardized production processes, together with extensive in-process quality control tests and monitoring of the product and its related intermediates

using suitable and validated methods. A written description of detailed and clearly defined standard operating procedures used for the production and testing of the vaccine, together with evidence of appropriate validation for each critical production step and relevant control tests, should be submitted by vaccine manufacturers to the NRA for approval as part of the licensing application. Proposals for any variation to the manufacturing or quality control methods should be submitted to the NRA for approval before implementation and according to national regulatory requirements.

The production of tetanus toxoid vaccine by chemical inactivation of tetanus toxin with formaldehyde has remained virtually unchanged since it was first introduced in the 1920s, although methods to monitor the production process, and characterize key intermediates and the final product, have improved significantly.

The production of tetanus toxin, from which the toxoid is prepared, requires the cultivation of a highly toxigenic strain of *Clostridium tetani* (e.g. the Harvard strain) with a known origin and history. Seed cultures are managed in a defined seed lot system in which toxigenicity is conserved. The approach adopted by most manufacturers is to obtain the greatest possible quantity of toxin during the growth phase of the microorganism and to convert the toxin into stable toxoid by the most effective method. Formaldehyde is most commonly used for the toxin detoxification process.

The purity and yield of toxin is checked to monitor consistency. Generally, toxin is purified prior to detoxification with formaldehyde in order to remove components that are likely to cause adverse reactions during use. The inactivation method must be validated to ensure that the toxoid does not revert to toxicity on exposure to heat but retains its immunogenic properties. Some manufacturers prefer to inactivate the toxin before purification in order to reduce the risk of reversion to toxicity. In view of the risk of reversion to toxicity, especially when a toxin is detoxified after purification, the present Recommendations have been formulated to address this risk by retaining the recommended 6-week incubation period for diluted, purified toxoid stored at elevated temperatures during the irreversibility test.

The demonstration of safety and the confirmation of vaccine potency are the fundamental requirements for the production of tetanus toxoid vaccines. These Recommendations call for the production of purified toxoid to minimize adverse reactions to vaccination in humans. The antigenic strength and purity of tetanus toxoid, defined in flocculation units (Lf units), is an essential quality indicator, and the minimum requirements remain set at 1000 Lf units per mg of protein (nondialysable) nitrogen. In addition to the traditional Ramon flocculation method used to determine the purity of tetanus toxoid, additional physicochemical methods – e.g. high-performance liquid chromatography

(HPLC), circular dichroism (CD) spectroscopy or sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) – should be considered for characterization of the product (and intermediates), and for monitoring the production process.

The measurement of antigen content and the degree of adsorption in the final bulk or final fill are important quality tests, and useful indicators of consistency. The antigen content measured in Lf per dose in products recommended for primary immunization may be higher than in vaccines recommended for reinforcing immunity in adults and adolescents.

Several studies conducted during the last decade have provided useful information on the value and potential use of in vitro immunochemical assays for measurement of the toxoid antigen content and degree of adsorption in the final bulk or final fill of tetanus vaccines (*47, 48*). The results obtained using these methods will not necessarily correlate with measurements of vaccine potency that have been determined in vivo, particularly for complex combinations of vaccines, but the value of these in vitro methods for monitoring trends (*47*) and stability (*27*) has been well documented.

When a new bulk lot of vaccine is made, it is essential to confirm its safety (i.e. the absence of toxin and reversion to toxicity) and potency. Potency is measured using an in vivo challenge test, or a validated alternative, and results are expressed in IUs by comparison with a suitable reference preparation that has been calibrated in IUs. The minimum requirements for tetanus potency depend on the animal model used and the composition of the vaccine being tested. In some countries, the minimum requirement for the potency of vaccines intended for boosting immunity in adults and adolescents is lower than that recommended for vaccines intended for primary immunization because of the reduced antigen content in these products relative to vaccines intended for primary immunization. In addition to the minimum requirements stated in these Recommendations, it is also recommended that manufacturers set consistency limits for the potency of the tetanus vaccine being produced. Such limits may be useful in supporting the evaluation of consistency.

Clinical studies should be performed to support the licensure of new tetanus vaccines. Because new tetanus vaccines are expected to be evaluated in populations with a low incidence of the disease, it is not possible to evaluate efficacy. Instead, the ability to induce levels of tetanus antibodies that are considered to be protective is assessed. It may also be feasible to conduct population-based surveys of the prevalence of antibody in a given population to guide recommendations regarding the need for and timing of booster doses, although these surveys are not necessarily a prelicensure requirement. Further details on clinical evaluation and on the determination of antibody response in humans are included in Part C of these Recommendations.

The stability evaluation of tetanus vaccines is addressed in section A.10.1 of these Recommendations, and emphasizes the importance of real-time stability studies conducted on the final product and under intended storage conditions.

In addition to these Recommendations, the general manufacturing requirements contained in Good manufacturing practices: main principles for pharmaceutical products (*49*) and Good manufacturing practices for biological products (*50*) apply to the production of tetanus vaccines.

Terminology

Definitions for some common terms used throughout this document are given below. They may have different meanings in other contexts.

Bulk purified toxoid: the processed, purified material that has been prepared from either a single harvest or a pool of single harvests. It is the parent material from which the final bulk is prepared.

Final bulk: the homogeneous final vaccine present in a single container 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 is homogeneous in all respects. In principle, a final lot must have been filled from a single final bulk container and processed further (e.g. freeze-dried) in one continuous working session. Different final lots may be filled or processed from the same final bulk in different working sessions. These related final lots are sometimes referred to as sub-lots, filling lots or freeze-drying lots, and should be identifiable by a distinctive final lot number.

Master seed lot: a quantity of bacterial suspension that has been derived from a single strain, has been processed as a single lot, and has a uniform composition. It is used to inoculate media for preparation of the working seed lot. The master seed lot should be stored as frozen stock or as lyophilized stock at a temperature known to ensure stability.

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

Single harvest: the toxic filtrate or toxoid obtained from one batch of cultures that have been inoculated, harvested and processed together.

Working seed lot: a bacterial culture consisting of a single substrain derived from the master seed lot. Working seed lots are stored in aliquots under the conditions described above for master seed lots. The working seed lot should be prepared from the master seed lot using as few cultural passages as possible; it should have the same characteristics as the master seed lot. It is used to inoculate media for the preparation of single harvests.

International reference materials

Subsequent sections of this document refer to WHO reference materials that may be used in laboratory or clinical evaluations. Key standards used in the control of tetanus vaccines include the following.

- The Fourth WHO International Standard for Tetanus Toxoid, Adsorbed – this material (NIBSC code 08/218) was established by the WHO Expert Committee on Biological Standardization in 2010, and was assigned a value of 490 IU per ampoule against the Third WHO International Standard for Tetanus Toxoid, Adsorbed on the basis of challenge assays in guinea-pigs (*26*); in 2012 the Expert Committee assigned a value of 260 IU per ampoule on the basis of challenge assays in mice (*27*). This standard is intended for use as a reference vaccine in tetanus vaccine potency assays.

- The Second WHO International Standard for tetanus toxoid for use in flocculation test – this material (NIBSC code 04/150) was established by the Expert Committee in 2007 (*51*, *52*), and was assigned an activity of 690 Lf per ampoule, replacing the First International Reference Reagent for tetanus toxoid for flocculation test. This standard is intended for use in flocculation tests to determine the specific antigen content of tetanus toxoid in Lf.

- The First WHO International Standard for tetanus immunoglobulin, human – this material (NIBSC code TE-3) was established in 1992 (*53*, *54*) with an assigned unitage of 120 IU per ampoule, replacing the Second International Standard for tetanus antitoxin, equine, which had been in use since 1969. The standard was assigned activity from in vivo TNT assays, and is intended for use as a reference preparation in TNT in vivo.

The material is also used as a reference preparation for measurements of tetanus antitoxin in human serum by in vitro methods.

The above-mentioned international standards and reference materials are held by the National Institute for Biological Standards and Control, Medicines and Healthcare Products Regulatory Agency, Potters Bar, Hertfordshire, EN6 3QG, England.[1] As reference materials may be superseded by replacement standards, the WHO catalogue of international reference preparations should be consulted for the latest list of established standards.[2] International reference materials

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[1] See: http://www.nibsc.org/

[2] See: http://www.who.int/bloodproducts/catalogue/en/index.html

are intended for use in the calibration of national, regional or other secondary standards (*55*) that are used for the production and quality control of tetanus vaccines. They may also be suitable for use as a primary reference preparation for some assays.

Part A. Manufacturing recommendations

A.1 Definitions

A.1.1 International name and proper name

The international name should be tetanus vaccine (adsorbed). 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.

> In some countries the proper name used to refer to a tetanus toxoid-containing vaccine is tetanus toxoid adsorbed.

A.1.2 Descriptive definition

Tetanus vaccine (adsorbed) is a preparation of tetanus toxoid prepared by treating tetanus toxin using chemical means to render it nontoxic without losing its immunogenic potency. The toxoid is adsorbed on to, or precipitated with, a suitable adjuvant. The preparation should satisfy the requirements formulated below.

> The most common method of preparing toxoid from toxins is by using formaldehyde.

A.2 General manufacturing recommendations

The general manufacturing recommendations contained in Good manufacturing practices: main principles for pharmaceutical products (*49*) and Good manufacturing practices for biological products (*50*) apply to the production of tetanus vaccine. These practices include demonstrating the purity and quality of the production strain and seed lots, implementing in-process quality control testing, testing for process additives and process intermediates, and developing and establishing lot-release tests.

A written description of procedures used in the preparation and testing of the tetanus vaccine, together with appropriate evidence that each production step has been validated, should be submitted to the NRA for approval. Proposals for modifying the manufacturing process or quality control methods should also be submitted to the NRA for approval before such modifications are implemented.

A.3   **Production, processing and control**

A.3.l   **Production precautions**

The general production precautions, as formulated in Good manufacturing practices for biological products (*50*), apply to the production of tetanus vaccines and to establishments manufacturing tetanus vaccine.

> Suitable methods for the production of tetanus vaccine are given in the WHO *Manual for the production and control of vaccines: tetanus toxoid* (*56*).

Personnel employed in production and quality control should be adequately trained, should have completed a course of immunization against tetanus, and should receive appropriate booster immunization(s). Appropriate health surveillance should also be carried out.

A.3.2   **Production strain and seed lots**

A.3.2.1   **Strains of *Clostridium tetani***

In order to verify strain characteristics, strains of *C. tetani* used in preparing tetanus toxoid should be identified by using a record of their history and of all tests made in accordance with NRA recommendations. The strain should be maintained as a freeze-dried culture or as a frozen-liquid seed stock.

A highly toxigenic strain of *C. tetani* of known origin, history and verification, should be used. The strain of *C. tetani* used should be approved by the NRA.

> A strain that has proved satisfactory for many manufacturers is the Harvard strain, but other strains have also been used.

A.3.2.2   **Seed-lot system**

The production of tetanus toxin should be based on a well defined and validated seed lot system. The strain used to establish the master seed lot should be chosen for desirable characteristics in which toxigenicity is conserved. Cultures of the working seed should have the same characteristics as those of the strain from which the master seed lot was derived. Detailed records of the origin, passage history, purification and characterization procedures, and storage conditions should be provided to the NRA when new master seeds or working seeds are introduced. Working seeds in use should be characterized at defined intervals that have been approved by the NRA on the basis of prior production history and experience. The preparation of seed lots should comply with the requirements of Part A, section A.3.1. The maximum number of passages of each seed lot used

for production should be specified, should be based on the number shown to result in the production of a safe and effective product, and should be approved by the NRA.

> Where possible, a combination of validated biochemical, molecular and genetic tests should be used for identification and characterization of seed lots. Suitable methods include multilocus enzyme electrophoresis (MEE), matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, pulsed-field gel electrophoresis (PFGE), multilocus sequencing typing (MLST) and restriction fragment length polymorphism (RFLP) analysis.

A.3.2.3 Culture medium for production of toxin

C. tetani should be cultured in a suitable liquid medium known to support the growth of bacteria and ensure a good yield of tetanus toxin. The culture medium should be free from adventitious agents and components that are known to cause toxic or allergic reactions in humans. Human blood products must not be used. Materials or components of animal origin should be identified, and methods for detecting these substances should be approved by the NRA.

Meat-free medium should be used where possible. If the medium is prepared from a protein digest (e.g. casein hydrolysate or digested muscle), precautions should be taken to ensure that digestion has proceeded sufficiently. If any materials of animal origin are used in seed preparation, in the culture medium or in production, they should comply with the WHO *Guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products* (*57*). Where appropriate and relevant, an upper limit should be established for mammalian protein in the final vaccine, and this limit should not be exceeded.

Any change in medium should be submitted for approval to the NRA.

A.3.3 Single harvests

The consistency of production should be demonstrated. This process may involve measurements of culture purity, growth rate, pH, incubation period, temperature range and rate of toxin production. The NRA should establish and approve acceptance specifications with defined limits (and, where relevant, alert limits) that will demonstrate the consistency of production.

Any culture showing anomalous growth characteristics should be investigated and should be shown to be satisfactory before being accepted as a single harvest. Contaminated cultures must be discarded.

Suitable methods for the production of tetanus toxin are given in the WHO *Manual for the production and control of vaccines: tetanus toxoid* (*56*).

Single harvests that meet the acceptance criteria may be pooled to prepare the bulk purified toxoid. Storage times should be supported by data obtained from appropriate stability studies, and should be approved by the NRA.

A.3.3.1 Control of bacterial purity

Samples of individual cultures used for preparing single harvests should be tested for bacterial purity by microscopic examination of stained smears, and by inoculation into appropriate culture media, or by another suitable procedure. Single harvests should be discarded if contamination has occurred at any stage during production.

Toxin-containing culture medium should be collected aseptically or in a way that minimizes the bioburden. Adequate measures and conditions should be in place to minimize the growth of microorganisms while low-bioburden materials are stored.

A.3.3.2 Filtration

After the culture medium has been sampled to control for purity, filtration should be used to separate the medium aseptically from the bacterial mass as soon as possible. A preservative may be added, but phenol should not be used for this purpose.

> To facilitate filtration, cultures may be centrifuged, provided that suitable precautions have been taken to avoid the formation of potentially hazardous aerosols. A filter aid may be added beforehand. A filter that does not shed fibres should be used.

A.3.3.3 Determination of crude toxin concentration

Prior to detoxification, the toxin content of the culture supernatant should be determined using a suitable in vitro method approved by the NRA.

> The flocculation test is suitable for the measurement of toxin content, and is described in the WHO *Manual for the production and control of vaccines: tetanus toxoid* (*56*) and the WHO *Manual for quality control of diphtheria, tetanus and pertussis vaccines* (*45*). A reference material calibrated in Lf against the International Standard for tetanus toxoid for flocculation tests, or an equivalent reference material approved by the NRA, should be included, and the results should be expressed in Lf units.

The measurement of toxin content (defined in Lf per ml) is a good indicator of the consistency of production, and acceptance limits should be defined for monitoring purposes.

It is preferable for culture filtrates to contain at least 40 Lf/ml of toxin, although lower concentrations have been applied by some manufacturers with satisfactory results.

A.3.3.4 Detoxification and purification

Detoxification of tetanus toxin may be performed using crude toxin (culture filtrate) or purified toxin and a well defined and validated process. Purifying the toxin before detoxification results in a purer product and is expected to remove components that are likely to cause adverse reactions in humans, although more care may need to be taken during detoxification because the risk of reversion to toxicity may be increased. The method of purification should be such that no substances are incorporated into the final products that are likely to cause adverse reactions in humans.

The method of purification and the agent used for detoxification should be suitably validated, and should be approved by the NRA. The rate of detoxification may vary, and in-process monitoring of the detoxifying process should be performed.

> Formaldehyde is most commonly used as a detoxifying reagent, and amino acids such as lysine or glycine may be added during detoxification to facilitate cross-linking of toxin molecules, and to prevent reversion. The detoxification conditions should be well defined and controlled with respect to temperature, time, concentration of detoxifying reagent, toxin concentration and any other critical parameter, in order to produce consistent, inactivated toxoid of the desired immunogenicity.

The method used for purification should be approved by the NRA.

> Crude toxoid can be concentrated using ultrafiltration prior to purification by fractionation with ammonium sulfate, dialysis, gel filtration, ion-exchange chromatography, or a combination of these methods.

> Bioburden testing may also be performed after purification to ensure that potential levels of contamination have been minimized for subsequent steps that are not done aseptically.

When measured in the final bulk vaccine, the amount of residual free detoxifying agent remaining after detoxification and purification have been completed should not exceed the limit stated in section A.3.5.2.7.

Harvests should be treated as potentially toxic, and subject to the appropriate safety restrictions until the detoxification has been shown to be complete by performance of a specific toxicity test (as detailed in section A.3.4.4) or any other suitable in vivo method.

The conditions of storage, including shelf-life, should be validated and supported by an appropriate stability programme, and should be approved by the NRA.

A.3.4 Bulk purified toxoid

A.3.4.1 Preparation

The bulk purified toxoid should be prepared from either a single harvest or a pool of single harvests, and should be sterile. A preservative may be added, provided that it has been shown not to adversely affect the safety and immunogenicity of the toxoid; this addition is subject to approval by the NRA. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity.

It is advisable to sterilize the bulk toxoid by filtration.

A.3.4.2 Sterility

Each bulk purified toxoid should be tested for bacterial and mycotic sterility in accordance with the requirements in Part A, section 5, of the General requirements for the sterility of biological substances (*58*) or by a method approved by the NRA. The sterility test is performed using at least 10 ml of each bulk purified toxoid. If a preservative has been added to the purified bulk, appropriate measures must be taken to prevent any interference in the sterility test.

A.3.4.3 Antigenic purity

Each bulk purified toxoid should be tested for antigenic strength and purity by determining the antigen concentration in Lf and the concentration of protein (nondialysable) nitrogen. The antigen concentration should be determined using a suitably standardized binding assay in solution (e.g. the Ramon assay) and compared with a reference material that has been calibrated against the International Standard for tetanus toxoid for flocculation test, or an equivalent reference preparation approved by the NRA. The method of testing should be approved by the NRA. The bulk purified toxoid passes the test if it contains at least 1000 Lf/mg of protein (nondialysable) nitrogen.

The flocculation (Ramon) assay is suitable for measuring antigen content, and is described in the WHO *Manual for the production and control of vaccines: tetanus toxoid* (*56*) and in the *WHO Manual for quality control of diphtheria, tetanus and pertussis vaccines* (*45*).

Physicochemical analysis, using methods such as SDS-PAGE and HPLC, may be used to monitor antigenic purity and to provide additional

information on antigen integrity and the extent of aggregation and proteolysis. These additional characterization tests should be performed whenever a new working seed is introduced.

A.3.4.4 **Specific toxicity**

Each bulk purified toxoid, diluted with the same buffer solution as used in the final vaccine, should be tested for the absence of tetanus toxin in guinea-pigs; the guinea-pigs should each weigh 250–350 g and not previously have been used for experimental purposes. At least five guinea-pigs should be injected subcutaneously with 1 ml of a dilution of purified tetanus toxoid containing at least 500 Lf of toxoid; they must be observed daily for signs of tetanic paralysis over a period of 21 days. A suitable method is outlined in the WHO *Manual for quality control of diphtheria, tetanus and pertussis vaccines* (*45*).

Animals that die, whatever the cause, will need to be examined by necropsy. The bulk purified toxoid passes the test if no guinea-pig shows symptoms of specific paralysis or any other signs of tetanus within 21 days of injection, and if at least 80% (i.e. four fifths) of the animals survive the test period. If more than one animal dies from nonspecific causes, the test must be repeated (using at least five more guinea-pigs).

If more than one animal dies in the second test, the toxoid sample does not comply with the test.

A.3.4.5 **Reversion to toxicity**

Each bulk purified toxoid must be tested to ensure that reversion to toxicity cannot take place during storage. The test employed should be approved by the NRA, and should be sufficiently sensitive to detect very small amounts of toxin. To determine whether reversion to toxicity has occurred, diluted toxoid that has been stored at an elevated temperature for six weeks should be tested. The diluted toxoid is prepared in such a way that the chemical environment is comparable to that found in the final vaccine except for the absence of adjuvant.

> For bulk toxoid that will be used in the preparation of more than one final-bulk formulation, the test should be performed using dilutions of the bulk toxoid that represent the lowest and highest concentrations of toxoid that will be present in the final formulations.

The diluted toxoid sample is incubated at 34–37 °C for a period of six weeks (42 days). At the end of the incubation period, five guinea-pigs are each injected subcutaneously with 5.0 ml (i.e. 10 human doses, using multiple injection sites where necessary) of test sample. The animals are observed for 21 days for signs of ill health.

> Similar dilutions of toxoid held at 2–8 °C during the same period of time as those held at 34–37 °C may be tested as controls.

No toxicity should be detected. The bulk purified toxoid passes the test if no guinea-pig shows symptoms of specific paralysis or any other signs of tetanus within 21 days of injection.

A.3.4.6 Storage of bulk purified toxoid

Storage times for bulk purified toxoid should be supported by data from appropriate stability studies, should comply with WHO Guidelines on stability evaluation of vaccines (*59*), and should be approved by the NRA.

A.3.5 Final bulk

A.3.5.1 Preparation

The final bulk is prepared from purified toxoid by adsorption on to, or precipitation with, a suitable quantity of a mineral carrier, such as hydrated aluminium phosphate or aluminium hydroxide (or other suitable adjuvant). The resulting mixture is approximately isotonic with blood. Suitable antimicrobial preservatives may be added. The final formulation of the vaccine should be based on formulations that have been shown to be safe and effective in clinical use. The number of Lf in a single human dose should be approved by the NRA.

> It is recommended that the tetanus antigen content in vaccines intended for primary immunization should not exceed 25 Lf/SHD. In vaccines intended for reinforcing the immunity of adults and adolescents, the number of Lf per SHD may be reduced, and in some countries it is recommended that it should not exceed 10 Lf/SHD.

A.3.5.2 Control tests

A.3.5.2.1 *Preservative*

If the vaccine is to be filled into multidose containers, a suitable antimicrobial preservative should be added. The amount of preservative in the final bulk should be shown to have no deleterious effect on the toxoid or on other vaccine components with which the toxoid may be combined in the final product; the preservative should also be shown to cause no unexpected adverse reactions in humans. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity of tetanus vaccines. The preservative and its concentration should be shown to be effective, and should be approved by the NRA. WHO Guidelines on regulatory expectations related to the elimination, reduction or replacement of thiomersal in vaccines should be followed (*60*).

Determine the amount of antimicrobial preservative by using a suitable chemical method. The amount should be at least 85% and not more than 115% of the intended amount.

A.3.5.2.2 *Adjuvants*

The nature, purity and concentration of the adjuvant used in the formulation should be determined by methods approved by the NRA. When aluminium compounds are used as adjuvants, the concentration of aluminium should not exceed 1.25 mg/SHD. If other (new) adjuvants are used, quality specifications should be set for the adjuvant used alone and in combination with the antigen, and should be approved by the NRA.

In some countries these recommended limits for adjuvant concentrations are considered too high, and lower limits have been approved and shown to be safe and effective.

A.3.5.2.3 *Degree of adsorption*

The degree of adsorption should be measured and should be shown to be comparable to that measured in vaccine lots used in clinical studies to support licensing. The measurement of antigen content and the degree of adsorption to adjuvant are useful indicators of consistency in production. In-house specifications, with appropriate acceptance limits, can be established once a suitable number of lots have been tested.

Suitable methods for determining antigen content and the degree of adsorption are provided in the WHO *Manual for quality control of diphtheria, tetanus and pertussis vaccines* (*45*).

These tests may be omitted provided they are performed on the final lot.

A.3.5.2.4 *Sterility*

Each final bulk should be tested for bacterial and mycotic sterility in accordance with the requirements in Part A, section 5, of the General requirements for the sterility of biological substances (*58*) or by a method approved by the NRA. The sterility test is performed using at least 10 ml of each final bulk. If a preservative has been added to the final bulk, adequate measures should be taken to prevent it from causing any interference in the sterility test.

A.3.5.2.5 *Specific toxicity*

Each final bulk should be tested for specific toxicity in at least five guinea-pigs; each guinea-pig should weigh 250–350 g and not have been used previously for experimental purposes. Each guinea-pig is given a subcutaneous injection of a quantity equivalent to at least 5 SHD, and is observed daily for a period of 21 days. Animals that die from any cause should undergo necropsy and be

inspected for symptoms of tetanus paralysis. The final bulk passes the test if no guinea-pig shows symptoms of specific paralysis or any other signs of tetanus intoxication within 21 days of injection. If more than one animal dies, the test must be repeated (using at least five more guinea-pigs). If more than one animal dies during the retest, the final bulk does not comply with the test.

> Subject to the approval of the NRA, the specific toxicity test used on the final bulk may be omitted from routine lot-release procedures once consistency in production has been established.

A.3.5.2.6 *Potency*

The potency of each final bulk (or final lot) should be determined by comparison with a suitable reference preparation that has been calibrated in IUs against the International Standard for tetanus toxoid, adsorbed. Appropriate statistical methods should be used to calculate the potency of the final bulk (*44*). The NRA should approve the assay method and the method used for calculating the results.

Details on methods suitable for potency testing of tetanus vaccines can be found in the WHO *Manual for quality control of diphtheria, tetanus and pertussis vaccines* (*45*).

The minimum potency specifications introduced into WHO guidance in 1978 have helped to ensure the production and use of safe and effective tetanus vaccines, as evidenced by the satisfactory performance of these products in clinical studies and the low incidence of tetanus in populations with good immunization coverage. Therefore, it is recommended that the potency of a tetanus vaccine used for the primary immunization of children should significantly exceed 40 IU/SHD (based on data showing that the lower 95% confidence limit of the estimated potency is at least 40 IU/SHD). Where the test is performed in mice for a vaccine containing a whole-cell pertussis component, the minimum requirement is 60 IU/SHD (based on data showing that the lower 95% confidence limit of the estimated potency is at least 60 IU/SHD). The minimum potency specification for tetanus vaccine intended for booster immunization in older children and adults may be lower and should be approved by the NRA.

Product-specific minimum requirements for tetanus potency are acceptable, provided they have been justified and are based on potency values obtained for the vaccine in question. A suitable number of lots should be tested in order to define the minimum requirement for potency. Vaccine lots used for the establishment of the potency specification should include lots that have been shown to be safe and effective in clinical studies. Product-specific minimum requirements should be approved by the NRA. Once defined and approved, the potency of the vaccine should be shown to exceed the minimum requirement significantly (based on data showing that the lower 95% confidence limit of the estimated potency is at least that of the minimum requirement).

The following criteria should be met in order for the potency estimate to be statistically valid:

- the statistical analysis should show a significant regression ($P < 0.05$) of the log dose–response curves without significant deviation from linearity and parallelism ($P > 0.05$);
- the 50% protective dose should lie between the smallest and largest vaccine doses.

When more than one assay is performed, the results of all statistically valid tests should be combined into a geometric mean estimate, and the confidence limits calculated.

Manufacturers are also encouraged to monitor the potency of different vaccine bulks and lots by setting minimum and maximum alert criteria once a suitable number of lots have been tested.

Calibration of reference preparations

The potency of a tetanus vaccine is determined in comparison with a reference preparation that has been calibrated in IUs against the International Standard for tetanus toxoid, adsorbed. Secondary reference preparations (regional, national, working or product-specific standards) should be calibrated with a multiple-dilution protection assay (of at least three dilutions), using either guinea-pigs or mice. Standards calibrated in IUs in guinea-pigs are considered to be suitable for use in guinea-pig potency assays, and standards calibrated using mouse assays are considered to be suitable for use only in mouse-potency tests. Adequate controls should be in place to ensure and monitor the stability of all secondary standards and, where possible, subsequent replacement batches of secondary or working standards should be calibrated against the primary international standard rather than against the previous working standard (*55*).

Potency test for routine lot release

For routine testing, the potency of every new bulk of tetanus vaccine is determined by the immunization of guinea-pigs or mice with appropriate dilutions of the calibrated reference preparation and test vaccine. Care should be taken to ensure that dilutions are inert (phosphate may interfere with the adsorption of toxoid) and not pyrogenic. Between four and six weeks after immunization, animals may be directly challenged with tetanus toxin by the subcutaneous route, or they may be bled for titration of immune serum.

Titration of immune serum samples may be performed using an in vivo TNT or by using a suitably validated in vitro method, such as an enzyme-linked immunosorbent assay (ELISA), the toxin-binding inhibition (ToBI) test or the particle agglutination test (*30*, *54*, *61*, *62*). If in vitro serological assays are used,

they should show that the product induces an appropriate antibody response in animals when compared with the reference preparation.

> The ELISA assay or another suitable in vitro method may be used to measure the antibody response to tetanus, provided that these assays have been validated against the functional assays (challenge or TNT) using the particular product in question. A minimum of three assays with a suitable dose–response range is likely to be required for validation for each product (*30*, *63*). These methods require precise definition of the characteristics of reagents (such as the coating antigen, and positive and negative control sera) that are critical for the successful performance of the testing method.

Potency assay modifications: reduced dilution schemes

Consistency limits (upper and lower) for tetanus potency should be established in house once a suitable number of lots has been tested using a multiple-dilution assay. Production consistency has been demonstrated if the vaccine potency expressed in IUs obtained for at least 10 consecutive vaccine lots produced from different toxoid bulks is within the defined consistency limits, and if the expectations of linearity and parallelism have been consistently satisfied. Once consistency in production has been demonstrated for the vaccine, the potency assay (using the challenge or serology model) may, with the approval of the NRA, be performed using a reduced number of animals or doses, or both. Once approved, fewer doses of the test and reference vaccines may be used, and the assumptions of linearity and parallelism need not be tested for each assay. When particular vaccine lots consistently give the lower limit of the 95% confidence intervals for the estimated potency values (well in excess of the minimum requirement), one-dilution assays may offer an advantage. If one-dilution assays are not advantageous, a reduction in animal usage may nevertheless be achieved by using two-dilution assays or another suitable design modification.

> A one-dilution assay is based on the same principles for evaluating the response as three-dilution assays. The assay involves the selection of a dose of the reference vaccine, expressed as a fraction of 40 IU (or the minimum requirement for the product expressed as an SHD), that elicits a minimum protective effect (or antibody response) in immunized animals; the effect of the reference vaccine is compared with the response elicited by the same fraction of a human dose of the test vaccine. If the response to the test vaccine is significantly greater than the response to the reference vaccine ($P \leq 0.05$), the potency of the test vaccine is satisfactory.

One-dilution assays provide assurances that the lower limit of the estimated potency exceeds the minimum requirement. A disadvantage of such an approach is that a strictly quantitative estimate of vaccine potency will not be obtained.

Therefore, in order to ensure the overall consistency of production, there is a need to support the data generated by a simplified potency assay with data from other suitable in vitro assays. When a one-dilution assay is used with serological methods of analysis, measurements of a geometric mean antibody response in the group of animals immunized with the test vaccine can provide some information about production consistency on a continual basis, provided that the in vitro assay used to measure antibody titres contains suitable internal controls.

Lot release based on the use of a simplified approach will require periodic review to ensure that the validity of all procedures (including assumptions of linearity and parallelism) is maintained. The timing of the review should be decided on a case by case basis, depending on the number of lots of vaccine produced annually or by time schedule (at least every 2 years), and should be approved by the NRA. It is important to note that testing should revert to multiple-dilution assays if there is a significant change in the production process, and production consistency should be reconfirmed before the reduction scheme is reintroduced.

A.3.5.2.7 *Amount of residual free detoxifying agent*

The amount of residual free detoxifying agent in each final bulk should be determined. The methods used and acceptable limits should be approved by the NRA.

> If formaldehyde has been used as detoxifying agent, the residual content should not exceed 0.2 g/l. The colorimetric determination of the reaction product of formaldehyde and fuchsin–sulfurous acid is a suitable method for detecting residual free formaldehyde.

> Where applicable, appropriate tests for the detection and quantification of other detoxifying agents should be performed. The tests used and the maximum permissible concentrations of such chemicals should be approved by the NRA.

A.3.5.2.8 *pH*

The pH of the final bulk should be measured and should be within the range of values measured in vaccine lots shown to be safe and effective in clinical use.

A.3.5.3 Storage of final bulk

The final bulk may be stored in a single container or in multiple containers. When multiple storage containers are used, the contents must be pooled into a single container for filling into the final containers. Storage times for the final bulk should be supported by stability studies, and approved by the NRA.

Filling and containers

The requirements concerning filling and containers given in Good manufacturing practices: main principles for pharmaceutical products (*49*) and Good manufacturing practices for biological products (*50*) apply to vaccine filled in the final presentation. Single-dose or multiple-dose containers may be used. Vaccines in multidose containers should contain a suitable antimicrobial preservative (see section A.3.5.2.1).

The filling process should be suitably validated by comparing key parameters measured in the final bulk and in the final lot. Such studies should include measurement of the degree of adsorption.

Control of final product

Quality control procedures and tests should be validated and approved by the NRA to ensure that the final containers hold the appropriate amounts of each of the intended vaccine constituents (active components and excipients) in a formulation suitable for the intended use of the final product.

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

Identity

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

> The method used should be based on the specific interaction between the tetanus antigen in the vaccine and tetanus antitoxin. Suitable detection methods include flocculation (Ramon and light-scattering methods), immunoprecipitation assays and ELISA (*44*, *56*). Tests on toxoids adsorbed on to an aluminium carrier should be performed after the carrier has been dissolved or the adsorbed toxoid has been wholly or partially eluted by sodium citrate or ethylenediaminetetraacetic acid (EDTA) (*44*, *56*).

Sterility

Final containers should be tested for bacterial and mycotic sterility using a method approved by the NRA. Many countries have regulations governing the sterility testing of the final product. Where such regulations do not exist, the criteria published by WHO for the sterility of biological and pharmaceutical products (*58*) should apply. If a preservative has been added to the vaccine, appropriate measures should be taken to prevent it from causing any interference in the sterility test.

A.5.3   Potency

A potency test should be carried out on each final lot as outlined in Part A, section A.3.5.2.6, if such a test has not been performed on the final bulk.

A.5.4   Innocuity

Each final lot should be tested for innocuity. This test is also referred to as the abnormal toxicity test or the general safety test. One human dose, but not more than 1 ml, of the final lot is injected by the intraperitoneal route into each of five mice (weighing 17–22 g) and each of two guinea-pigs (weighing 250–350 g). The test should be approved by the NRA. The final product is considered to be innocuous if the animals survive for at least seven days without showing significant signs of toxicity.

> If the NRA approves, the innocuity test on the final lot may be omitted from routine lot release once the consistency of production has been demonstrated.

A.5.5   Adjuvant content

The adjuvant content of each final lot should be determined using a method approved by the NRA and as described in Part A, section A.3.5.2.2. The formulation should be such that after shaking, the vaccine remains suspended as a homogeneous solution for a defined period of time (to allow sufficient time for administration).

A.5.6   Degree of adsorption

A test for the degree of adsorption should be carried out on each final lot as described in Part A, section A.3.5.2.3.

A.5.7   Preservative content

The content of the preservative(s) in each final lot should be determined as described in Part A, section A.3.5.2.1. The methods used should be approved by the NRA.

> In some cases, if the NRA approves, this test may be performed only on the final bulk.

A.5.8   pH

The pH of each final lot should be measured and should be within the range of values measured in vaccine lots shown to be safe and effective in clinical use. The test may be omitted during the final fill if it has been performed on the final bulk.

> In some cases, determination of osmolality may also be required.

A.5.9 **Extractable volume**

For vaccines filled into single-dose containers, the extractable volume should be checked and should be shown to be not less than the intended dose.

For vaccines filled into multidose containers, the extractable volume should be checked and should be shown to be sufficient for the intended number of doses.

A.5.10 **Inspection of final containers**

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

A.6 **Records**

The requirements given in Good manufacturing practices: main principles for pharmaceutical products (*49*) and Good manufacturing practices for biological products (*50*) apply. Written records should be kept of all tests, irrespective of their results. The records should be of a type from which annual trends can be determined.

A model of a suitable summary protocol for tetanus vaccines is given in Appendix 1.

A.7 **Retained samples**

Vaccine samples should be retained, as recommended in WHO good manufacturing practices for pharmaceutical products: main principles (*49*) and Good manufacturing practices for biological products (*50*).

A.8 **Labelling**

The label printed on or affixed to each container, and the label on the carton enclosing one or more containers, should be approved by the NRA. The labels should be easily readable and should show as a minimum:

- the words "tetanus vaccine (adsorbed)" or the proper name of the product, or both
- the licence number of the product
- the name of the manufacturer
- the number of the final lot
- the identity of any preservative or adjuvant
- the amount of antigen in Lf/ml or the minimum potency in IU/SHD, or both

- the recommended storage temperature and the expiry date if kept at that temperature

- the recommended SHD and the route of administration.

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

- a statement that the vaccine satisfies the requirements of this document;

- the address of the manufacturer;

- the recommended temperature for transport;

- a warning that the adsorbed vaccine should not be frozen;

- a warning that the adsorbed vaccine should be shaken before use;

- instructions for the use of the vaccine, and information on contraindications and the reactions that may follow vaccination.

A.9 Distribution and transport

The requirements given in Good manufacturing practices: main principles for pharmaceutical products (*49*) and Good manufacturing practices for biological products (*50*) apply.

A.10 Stability, storage and expiry date

A.10.1 Stability

Stability evaluations form a critical part of quality assessment. The purpose of stability studies is to ensure that the vaccine at the end of its shelf-life, and during the storage period (or other defined storage period for intermediates) or period of use, retains the required characteristics to support its quality, safety and efficacy. Current recommendations on the evaluation of vaccine stability, as provided in the WHO Guidelines on stability evaluation of vaccines, apply (*59*).

The stability of the vaccine in final containers maintained at the recommended storage temperature should be demonstrated to the satisfaction of the NRA. The vaccine should be manufactured in such a way that reversion to toxicity does not occur during the defined period for shelf-life, provided that the vaccine is stored under the conditions recommended on the label. Typically, containers from at least three consecutive final lots (each derived from different toxoid bulk intermediates) should be tested and included in real-time stability studies that are supported by evidence of potency and lack of specific toxicity at the expiry date. To confirm that the vaccine does not revert to toxicity during

storage, the specific toxicity test described in Part A, section A.3.5.2.5, should be scheduled up to the expiry date as part of the stability studies. In addition, at the time of the expiry date, the vaccine should meet the requirements for the final product in terms of sterility, potency, adjuvant content, degree of adsorption, preservative content, pH and extractable volume, where applicable (as described in Part A, sections A.5.2, A.5.3 and A.5.5–A.5.9), provided that the vaccine has been stored at the recommended temperature. The frequency of testing should be approved by the NRA.

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

> Stability studies performed at temperatures other than those recommended for storing the vaccine may be useful in providing information about transporting the vaccine at different temperatures for a limited time. Accelerated stability studies may provide additional evidence of product stability, but cannot replace real-time stability studies.

A.10.2 Storage conditions

The recommended storage conditions and the defined maximum duration of storage should be based on stability studies, as described in section A.10.1, and should be approved by the NRA. For tetanus vaccine (adsorbed), storage at a temperature of 2–8 °C is generally considered to be satisfactory and should ensure that the minimum potency specified on the label of the container or package will be maintained after release and until the end of the proposed shelf-life, provided that the vaccine is stored under the recommended conditions and in accordance with instructions on the label.

Adsorbed vaccines must not be frozen.

A.10.3 Expiry date

The statement concerning the expiry date that appears on the label, as required in Part A, section A.8, should be based on experimental evidence, and approved by the NRA on the basis of the data obtained during the stability studies referred to in section A.10.1. The date of manufacture (i.e. blending or filling) or the start date of the last satisfactory potency determination, as performed in accordance with section A.5.3 or section A.3.5.2.6 – i.e. the date on which the test animals were immunized with the vaccine – is taken as the start date for the shelf-life.

> In some cases, the date of the first satisfactory potency determination is used as the start date for the shelf-life.

Part B. Nonclinical evaluation of tetanus vaccines

B.1 Introduction

The nonclinical testing of tetanus vaccines and their related intermediates is an essential part of the development of candidate vaccines, and is a prerequisite for the initiation of clinical trials in humans and for licensure. Studies are aimed at defining the in vitro and in vivo characteristics of candidate vaccines and, within the scope of this document, nonclinical evaluation means all in vitro and in vivo testing performed before and during the clinical development of the vaccine. The recommendations included in this document follow a sequential approach and include the nonclinical evaluations that may need to be considered at different points in the manufacturing process – including during the production of tetanus toxin, purification and detoxification of the toxin, blending with the adjuvant in the final bulk and formulation of the final product. The recommendations are intended for new manufacturers of tetanus vaccine, and should also be relevant if a significant change to the production process or product formulation is made by a manufacturer already producing tetanus vaccine.

The extent to which nonclinical studies will be required depends on existing clinical experience with similar product types or with products containing the same antigen in a different formulation or combination. These Recommendations refer only to products based on those that are currently licensed and in clinical use – i.e. vaccines based on the use of chemically detoxified tetanus toxin (toxoid) as the antigen adsorbed on to, or precipitated with, an aluminium-based adjuvant. Tetanus vaccines based on novel antigens or formulations that have not previously been evaluated for safety and efficacy in clinical trials are likely to require more extensive nonclinical characterization, which is beyond the scope of this document.

The nonclinical evaluation of tetanus vaccines should be based on WHO guidelines on nonclinical evaluation of vaccines (*64*) which also contain definitions of commonly used terms related to nonclinical evaluations. Studies related to the nonclinical evaluation of vaccine intermediates and the final product should be performed in accordance with the principles of good laboratory practice (GLP). Adhering to the principles of GLP promotes the development of high-quality test data, and provides a tool for ensuring that a sound approach is taken to the management of laboratory studies, including how they are conducted, and how their data are reported and archived (*65*).

The nonclinical characterization of vaccine intermediates and in-process materials should be based on the use of adequately characterized, homogenous starting materials of defined origin and acceptable quality. Demonstrating consistency in production may not be applicable during the early stages of

nonclinical evaluation, but adequate validation of the production process is required to demonstrate that the manufacturing conditions are reproducible.

The vaccine lots used in preclinical studies should be adequately representative of the formulation intended for clinical investigation, and, ideally, preclinical testing should be performed using the same lot(s) as those proposed for clinical trial. Manufacturers should make every effort to keep some of this characterized material for future reference. Early communications between the vaccine manufacturer and the NRA are recommended in order to agree on the requirements for, and type of, nonclinical testing.

B.2 Nonclinical testing and characterization of intermediates and in-process materials

Intermediates and in-process materials must be tested and characterized to confirm that they meet the requirements in Part A of this document. The source and quality of all starting materials should be documented and should include detailed descriptions of the characterization of the strain, master seed lot and working seed lot. Defined procedures should also be shown for the preparation of new working seeds from the master seed. Working seeds must be shown to produce an adequate yield of active toxin under defined culture conditions. Characterization studies should include measurements of viability, growth rate, rate of toxin production, confirmation of toxin activity (using the specific toxicity test, and defined as the minimum lethal or paralytic dose per ml of culture medium) and standard microbiological identification techniques. Cultures of the working seed should have the same characteristics as those of the strain from which the master seed lot was derived. Tests that are performed as part of the characterization of seed lots should include a combination of validated biochemical, molecular and genetic tests. Methods such as MEE, MALDI-TOF mass spectrometry, PFGE, MLST, RFLP or N-terminal sequencing could be considered. The maximum number of passages of each seed lot used for production should be specified, based on those shown to result in the production of a safe and effective product, and should be approved by the NRA.

The culture medium used for toxin production should be well defined, and all animal components, if present in the medium, should be identified and documented. Protein contaminants derived from the bacterium or from components of the culture medium may increase the potential for adverse reactions to immunization with the toxoid, and the detoxification and purification processes used should minimize the presence of any substances likely to cause adverse reactions in humans. The methods used for the detoxification and purification of crude toxin should be adequately described and supported by appropriate validation data.

B.2.1   **Safety evaluation**

The production process should be validated to confirm that the detoxification of tetanus toxin has been completed without reversion to toxicity, particularly when the toxoid is exposed to heat. Both the specific toxicity test (section A.3.4.4) and the reversion-to-toxicity test (A.3.4.5) should be performed on the bulk purified toxoid.

Aside from studies to confirm the absence of specific toxicity and reversion to toxicity, additional toxicology studies may need to be undertaken. The nonclinical toxicology studies should be such that reasonable assurance is obtained that it is safe to proceed to clinical evaluation. The potential toxic effects of the purified, inactivated toxoid should be evaluated in at least one animal species; this assessment should include the histopathology of important organs. The study should investigate the potential for local inflammatory reactions, systemic toxicity and effects on the immune system. The animal species used should be sensitive to the biological effects of the vaccine and to the toxin. Where feasible, the highest dose to be used in the proposed clinical trial should be evaluated in an animal model. Reproductive toxicity studies may be considered, particularly for novel tetanus antigens, since tetanus vaccine is likely to be used in women of childbearing potential as well as in pregnant women.

Information on endotoxin content may be obtained during validation of the production process as part of the nonclinical evaluation. A bioburden test may be used to ensure that potential levels of contamination have been minimized during steps that are not performed aseptically.

B.2.2   **Characterization**

The purity of the inactivated toxin should be established by determining the Lf concentration in relation to the concentration of total protein (nondialysable) nitrogen. A range of protein purity-indicating methods – such as HPLC, SDS-PAGE and Western blotting – are useful in providing full characterization and for evaluating the integrity of tetanus toxoid vaccine antigen prior to further formulation with adjuvant.

B.2.3   **Immunogenicity and/or potency**

An assessment of the ability of purified tetanus toxoid or antigen to induce functional antibody responses may be performed as part of preclinical studies, but most studies will be performed on the adsorbed bulk vaccine, which should be evaluated as described in section B.3.1.

B.2.4   **Stability**

Stability studies should be based on the Guidelines on stability evaluation of vaccines (59). The stability of all intermediates not used within a short

period of time should be evaluated and demonstrated using suitable methods. Manufacturers are encouraged to assign a shelf-life to all materials during the vaccine-production process, and in particular to key intermediates, such as single harvests and bulk purified toxoid. The choice of stability-indicating parameters and the frequency of testing should be justified to and approved by the NRA. Storage periods proposed for intermediates produced during the manufacturing process should be based on data obtained from the stability studies.

B.2.5 Adjuvants

Where appropriate, the adjuvant should be characterized in terms of chemical composition, physical form, adsorption capacity, purity, endotoxin content and sterility. The interaction between the adjuvant and antigen should also be evaluated; this evaluation should include measurement of the degree of adsorption. This should be shown to be consistent from lot to lot, and quality specifications should be established once a sufficient number of lots have been produced. The stability of the adjuvant alone, as well as in combination with the antigen, should be established during development, and should be shown to remain stable throughout the intended storage period.

B.3 Nonclinical characterization of formulated vaccine

Lots of the final formulated vaccine used in nonclinical studies should be adequately representative of those intended for clinical investigation, and, ideally, should be the same lots as those intended for clinical use. Manufacturers should make every effort to keep some of this characterized material for future reference. As a minimum, the candidate vaccine should be prepared under conditions of good manufacturing practice (GMP) for clinical trial material (66), and full implementation of the principles of GMP will be required during the later stages of clinical development (49, 50).

     The final formulated vaccine should be evaluated using a combination of immunological and physicochemical approaches to determine key product characteristics including sterility, pH, antigen content, degree of adsorption, potency/immunogenicity, and safety, as described in Part A of this document. Particular attention should be paid to the assessment of safety, toxicology, potency and stability. In some cases, comparability testing should be performed (e.g. after a significant change in the manufacturing process or at the time of scale-up following licensure). The requirement for and extent of comparative studies, and the choice of the comparator vaccine, should be approved by the NRA.

B.3.1 Immunogenicity and/or potency

Immunogenicity and/or potency studies should be performed in appropriate animal models, and may include more than one animal model. These studies

should include a potency assay consisting of multiple dilutions (consisting of at least three dilutions of each test vaccine and the suitable reference preparation), which should be performed using guinea-pigs or mice, followed by challenge with tetanus toxin or by titration of immune serum samples to determine functional (i.e. toxin neutralizing) antibody responses. The potency of the vaccine should be determined and should meet the requirements of Part A, section A.3.5.2.6. More details on the methods used for potency testing of tetanus vaccines can be found in the WHO *Manual for quality control of diphtheria, tetanus and pertussis vaccines* (*45*).

Multiple-dilution potency assays should also be used to demonstrate consistency in the production of the vaccine, and for stability testing for the purpose of establishing shelf-life.

B.3.2   Safety and toxicology

Aside from the specific toxicity test and innocuity test described in Part A, additional toxicology studies to address reproductive toxicity may be needed in certain cases (e.g. if a new production process or new tetanus antigen is introduced) since tetanus vaccine will probably be used in women of childbearing potential as well as in pregnant women (to prevent neonatal tetanus). Toxicological studies should be undertaken in accordance with the WHO guidelines on nonclinical evaluation of vaccines (*64*). Nonclinical toxicology studies should be such that reasonable assurance is obtained that it is safe to proceed to clinical evaluation. The potential toxic effects of the final formulated vaccine should be evaluated in at least one animal species; this evaluation should include histopathology of important organs. The study should investigate the potential for local inflammatory reactions, systemic toxicity and effects on the immune system. The animal species used should be sensitive to the biological effects of the vaccine and to the toxin. Where feasible, the highest dose to be used in the proposed clinical trial should be evaluated in an animal model. Further information on considerations related to dose, controls and parameters to be monitored can be found in the WHO guidelines on nonclinical evaluation of vaccines (*64*).

B.3.3   Stability

Stability studies should be based on WHO Guidelines on stability evaluation of vaccines (*59*), and should be seen as a continual process occurring from the development of the vaccine through licensing and on to post-licensure monitoring. Stability studies should be performed as real-time studies under conditions intended for storing the final product. During the preclinical phase, the amount of real-time stability data may be limited, but sufficient data should be generated to support the stability of the vaccine for the intended duration of the trial. Tests must include those for potency and specific toxicity, and should

also include physical and chemical characterization. Immunogenicity assessment (including measurement of functional antibody responses) and antigenicity assessment (as part of evaluating the degree of adsorption of the antigen) may provide valuable information in support of potency testing. Accelerated stability studies of products stored for limited periods at temperatures that may affect stability could support preliminary data from continuing real-time stability studies but should not replace them. Final containers from at least three lots of vaccine derived from different bulks should be tested on their expiry date to demonstrate stability during storage.

Any modification to the duration of shelf-life requires additional stability data to support the proposed modification, and should be approved by the NRA. Following licensure, stability should be monitored throughout the proposed shelf-life.

Part C. Clinical evaluation of tetanus vaccines

C.1   Introduction

This section addresses issues that are relevant during the clinical development of tetanus vaccines. Progression through the phases of clinical development should follow the principles outlined in WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (*67*). Only those vaccines with an adequate nonclinical evaluation, as described in Part B, should be considered for clinical evaluation, with the NRA taking responsibility for evaluating the adequacy of the nonclinical information.

Clinical evaluation is required for any new tetanus vaccines, and may also be required for existing vaccines if a significant change to the manufacturing process has been proposed. The content and extent of the clinical programme will vary according to each possible scenario. It is strongly recommended that early dialogue is established between the vaccine manufacturer and the NRA in order to clarify requirements for carrying out clinical trials as well as for marketing approval.

C.1.1   General considerations for clinical studies

All clinical trials on pharmaceutical products should adhere to the standards of good clinical practice set out by WHO (*68*). However, tetanus vaccines are special in some respects and so demand careful consideration during clinical evaluation because the vaccines are given to healthy people, mostly in the paediatric population, to prevent rather than cure disease, and this limits the tolerance to adverse events. Tetanus vaccine for booster immunization is also given to adults, including pregnant women.

It is expected that at least some of the clinical studies, including those in the primary target population, will be conducted with different lots of vaccine manufactured using the same process as the vaccine intended for marketing. Consistency in the manufacturing of the vaccine lots used in clinical trials should be demonstrated and well documented. Although a formal clinical trial to evaluate lot consistency may not always be needed, in some instances clinical data may be required to provide evidence to support manufacturing consistency (e.g. if there is a particular concern about the consistency of the product). Vaccine lots used in clinical studies should ideally be the same lots that have been evaluated in nonclinical studies, and should be adequately representative of the formulation intended for marketing. Where this is not feasible, the lots used in clinical studies should be comparable to those lots used in the nonclinical studies in terms of their manufacturing process, immunogenicity and potency, safety, stability and other relevant characteristics of quality. The number of different vaccine lots evaluated as part of the clinical studies should be approved by the NRA but should be more than one. It is important to note that clinical data used to provide evidence of production consistency do not replace the need to demonstrate consistency in the manufacturing process during nonclinical evaluations.

C.1.2 Scope of the studies

The size and design of the studies and the selection of end-points for evaluation require justification, and should be such that they provide reasonable assurance of the clinical benefit and safety of the candidate vaccine. Because of the widespread use of tetanus toxoid and the low incidence of disease, it is unlikely that clinical end-point efficacy trials would be feasible for tetanus vaccines. In the case of tetanus, for which there are generally accepted serological correlates of protection, studies are based on the evaluation of antitetanus antibody responses in addition to the evaluation of safety. The primary immunogenicity end-point(s) should be the most relevant for the target population, and will differ for the evaluation of priming and booster doses of tetanus vaccine. Generally, studies of new tetanus vaccines are expected to make comparisons with licensed vaccines, and to include a noninferiority evaluation of the immune response to the investigational vaccine compared with the control vaccine, as well as an evaluation of safety.

Studies should evaluate the safety and immunogenicity of the investigational vaccine when co-administered with other routinely recommended vaccines given on the same schedule to the target population. Some studies should include an evaluation of the immune response to concomitantly administered vaccines in order to ensure that the investigational vaccine does not interfere with responses to concomitantly administered vaccines. Of particular interest in the evaluation of tetanus vaccines are any effects on the safety and immunogenicity found during co-administration with conjugate vaccines containing tetanus toxoid as a carrier protein.

Clinical studies may be needed when substantial manufacturing changes are made to an existing, licensed vaccine. Any change in the formulation of a vaccine should be considered carefully, both by manufacturers and the NRA.

C.1.3 Comparator vaccine

Immunological correlates of protection are well established for tetanus vaccines (see section C.2.2), and it should be acknowledged that comparison studies do not bridge to efficacy but to serological correlates. In some cases, it may be decided to perform immunogenicity analyses that are not comparative, although in most cases comparative studies are recommended. The inclusion of a comparator aids interpretation of the results of the trial, particularly if the expected seroprotection rate in the investigational group is not observed (e.g. if the study is conducted in a population where high levels of maternal tetanus antibody suppress in infants the response to immunization). Decisions about whether to include a comparator vaccine, and the selection of a comparator, should be justified by the manufacturer, and approved by the NRA. In studies performed to support major changes to the manufacturing process for a licensed vaccine (including a change in formulation or antigen dose), the candidate vaccine should be compared with the existing product (i.e. one manufactured according to the licensed production process). In this case, a comparative study is particularly useful for directly evaluating the effect of the change in the process or formulation. In studies of a new vaccine, the comparator is typically a licensed vaccine.

C.2 Assessment of immunogenicity in humans

C.2.1 Assays to assess antibody responses

Assays to measure the antibody response to tetanus vaccine can be divided into functional assays and nonfunctional binding assays (which demonstrate the capacity of tetanus antibody in a serum sample to bind directly to, or compete with, tetanus toxin or toxoid). Fully functional assays include the in vivo TNT performed in guinea-pigs or mice. The in vivo assay is a moderately severe procedure involving the injection of toxin/antitoxin mixtures into animals by the subcutaneous route. The procedure requires specialized facilities, is expensive and requires relatively large volumes of serum. As a result, it is not typically used in vaccine clinical trials.

Validated in vitro serological assays are preferred and are more suited to screening large numbers of serum samples because they are faster, use lower sample volumes, are easy to use and can be automated. There is no suitably validated in vitro assay with the capacity to detect functional antibodies to tetanus and that can be used as a TNT. However, several in vitro serological assays have been developed and validated against the in vivo TNT and show excellent correlation, particularly when the level of antibody is high (*54*). Examples of

these include ELISA (*69*), the ToBI test (*70*) and the particle agglutination test (*61*, *62*). Other methods include double-antigen ELISAs or dual double-antigen time-resolved fluorescence immunoassays, which are also used for assessing antibody responses to tetanus and at least one other antigen (*71*, *72*) delivered at the same time. The passive haemagglutination assay has also been used by some laboratories, but it is considered to be more variable and shows a poorer correlation with the in vivo TNT (*1*, *54*).

The method chosen for antibody assessment should be validated for the intended purpose, using relevant samples, and should be approved by the NRA. Where feasible, an assay that measures functional antibody responses may need to be used at some stages during the clinical evaluation (e.g. to analyse a subset of samples from the clinical trial).

C.2.2 Criteria for evaluation of immune responses

Clinical protection against tetanus correlates well with the presence and level of circulating tetanus antitoxin. The end-points and criteria used for the evaluation of tetanus antibody response require a justification that takes into account the assay used to measure antibody levels and the intended use of the vaccine (i.e. for primary or booster immunization). It is generally accepted that, when measured by TNT, an antitetanus antibody level of 0.01 IU/ml is the minimum protective level. Thus, subjects with antitetanus antibody levels greater than 0.01 IU/ml (as measured by TNT) are considered to be protected (*1*) and those with antitetanus antibody levels below 0.01 IU/ml are considered susceptible to disease. When a validated ELISA is used, the minimum level of antibody needed to provide protection against tetanus is usually considered to be 0.1 IU/ml. When other in vitro assays are used, these criteria for evaluation of immune responses to tetanus vaccination may not apply because they are specific to each assay. In some settings (e.g. in booster immunization studies), a high proportion of subjects may have a level of tetanus antibody that is higher than the minimum protective level prior to vaccination with the investigational vaccine. In such cases, the minimum protective level of antibody would not be a meaningful end-point for evaluation of the immune response to the investigational vaccine.

Information from phase II studies in the target population may help guide the determination of appropriate primary end-points for phase III studies. The end-points and evaluation criteria proposed by the manufacturer should be approved by the NRA.

C.2.2.1 Primary immunization of infants

The proportion of subjects with a tetanus antibody level above the minimum protective level, or above a prespecified threshold, should be determined approximately one month after the last primary dose. For primary immunization,

a typical end-point uses a validated ELISA to assess the proportion of subjects with an antitetanus antibody level of 0.1 IU/ml or higher post-vaccination.

Where noninferiority of the investigational vaccine relative to a comparator vaccine is evaluated, noninferiority is shown if the upper limit of the 95% confidence interval for the difference in the seroprotection rates (i.e. the comparator vaccine minus the investigational vaccine) is less than a prespecified margin (usually 10%, although a margin of 5% may be recommended if the expected rates of seroprotection are very high, as may be the case for tetanus). The specified threshold antibody concentration, noninferiority margin and bleeding times should be approved by the NRA.

If the studies are performed in the absence of a comparator vaccine, an acceptance criterion should be used for the proportion of subjects – usually 90% or 95% – that reach the prespecified threshold antibody level (e.g. 0.1 IU/ml or higher using a validated ELISA), measured approximately one month after the last primary dose. The acceptance criterion should be based on the 95% confidence interval for the proportion of subjects achieving the prespecified antibody level (e.g. the lower limit of the 95% confidence interval for the observed proportion should be greater than 90% or 95%). The NRA should approve the specified antibody threshold, acceptance criterion and bleeding time.

In addition to the level of tetanus antibody specified as the primary end-point, it is recommended that the geometric mean titre (GMT) of antitetanus antibody should be evaluated as a secondary end-point. In comparative studies, the GMT ratio of the investigational vaccine to the comparator vaccine may be evaluated using a predetermined margin of noninferiority (e.g. the lower limit of the 95% confidence interval of the observed ratio of the investigational vaccine to the comparator vaccine should be greater than 0.67). The presentation of reverse cumulative distribution curves, which show the accumulated proportion of subjects with an antibody concentration greater than or equal to a given level, may also provide useful information for the purposes of comparison.

C.2.2.2 Primary immunization of adolescents or adults

In some countries it may be desirable to evaluate a tetanus vaccine to be used for primary immunization in adults and adolescents, including women of childbearing age, who have no prior history of vaccination against tetanus. Pre-enrolment screening criteria to identify previously unvaccinated (naive) subjects may include a tetanus antibody level of less than 0.1 IU/ml when measured by validated ELISA (or less than 0.01 IU/ml if using TNT) prior to and seven days after receipt of the first dose of tetanus vaccine. In some cases, it may be difficult to identify sufficient numbers of naive individuals, and the choice of study design (i.e. comparative or noncomparative) may therefore be dependent on the number of naive subjects that can be identified in the target

population. If sufficient numbers of naive subjects in older age groups cannot be identified, consideration may be given to extrapolating the effectiveness of primary immunization from infants to older age groups.

The end-points and noninferiority or acceptance criteria for evaluation of the primary immune response to a tetanus vaccine in adolescents or adults are the same as those recommended for the primary immunization of infants (section C.2.2.1).

C.2.2.3 Booster immunization of children, adolescents and adults

For the evaluation of tetanus vaccines intended for booster immunization, the age of the subjects (e.g. preschool age, school age, adults, elderly) and the interval since the last dose of tetanus vaccine should be considered when designing the clinical trial, since these factors may influence the baseline level of antibody and may have a significant impact on the response to a booster dose. In designing tetanus-booster immunization studies, appropriate enrolment criteria should be developed regarding factors that may affect immunogenicity outcomes (e.g. the time since previous dose). Enrolment procedures should also be designed to ensure adequate representation across the age range for which the vaccine is intended.

Criteria for evaluation should reflect the prevaccination immunity level in the study population. Specifically, if a substantial proportion of subjects is expected to have tetanus antibody levels above the minimum protective level prior to booster immunization, other primary end-points (e.g. the booster response rate) should be used. Information from phase II studies in the target population may help in determining appropriate primary end-points. The assessment of antibody levels both prior to and after vaccination is recommended in order to facilitate the interpretation of the data.

Typically, for tetanus-booster immunization studies, the proportion of subjects with a booster response is considered to be the primary end-point. If booster response is used as a primary end-point, the definition of booster response (e.g. a four-fold rise in antibody concentration from prevaccination to post-vaccination) should be prespecified and should include a provision that subjects attain at least four times the protective level – e.g. in subjects with a prevaccination antibody concentration of < 0.1 IU/ml (by validated ELISA), a post-vaccination concentration of ≥ 0.4 IU/ml would be acceptable or in subjects with a prevaccination concentration ≥ 0.1 IU/ml, an increase of at least four times the prevaccination concentration would be acceptable. Alternative definitions for booster response (e.g. a two-fold rise for subjects with prespecified high levels of antibody prior to vaccination) may be considered if well justified. As for the comparative evaluation of seroprotection rates, booster response rates should be

compared between groups using an appropriate predefined noninferiority limit – e.g. the upper limit of the two-sided 95% confidence interval of the observed difference (i.e. the comparator vaccine minus investigational vaccine) should be less than a prespecified margin (usually 10%). In noncomparative studies, the acceptability criterion for the proportion of subjects who achieve a booster response (e.g. 80%) should be based on the 95% confidence interval of the observed proportion.

In booster immunization studies, secondary end-points may include antitetanus antibody GMTs and the proportion of subjects with antibody levels greater than or equal to the minimum protective level.

The choice of end-points and criteria for evaluation (including the need for a comparator vaccine) should be justified by the manufacturer, and approved by the NRA.

C.2.3 Antibody persistence

Where possible, it is recommended that subsets of subjects should be identified for longer-term follow-up of the persistence of immunity in order to determine the need for, and appropriate timing of, booster doses. Population surveillance studies conducted to determine the prevalence of tetanus antibody in different age groups can help guide recommendations on the need for booster doses.

C.3 Safety evaluation

The assessment of safety should follow the general principles outlined in WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (*67*). The size of the prelicensure safety database required for a tetanus-toxoid-containing vaccine will depend on many factors including other vaccine components, the age group for which use is being sought, and manufacturing methods (e.g. the use of novel components versus common components). For vaccines in which efficacy is inferred from immunogenicity data without larger clinical end-point efficacy trials, the number of subjects that need to be evaluated for safety may exceed the number of subjects required for the end-points evaluating immunogenicity. The size of the prelicensure safety database should be justified by the manufacturer, and approved by the NRA.

The frequency and severity of adverse reactions following receipt of tetanus vaccine may vary by vaccine formulation (e.g. according to the amount of tetanus toxoid), subjects' characteristics (e.g. prior vaccination history, time since previous dose, age, prevaccination level of tetanus antibody) and use with concomitantly administered vaccines. Theoretically, there is the potential for increased reactogenicity when tetanus toxoid vaccines are administered at the same time as (or shortly after) polysaccharide conjugate vaccines containing

tetanus toxoid as the carrier protein. Higher rates of some local adverse reactions have been observed following booster immunization with tetanus vaccine when compared with primary immunization. In designing tetanus-booster immunization studies, appropriate enrolment criteria should be developed regarding factors that may affect safety outcomes (e.g. the time since previous dose). Enrolment procedures should also be designed to ensure adequate representation across the age range for which the vaccine is intended.

Safety data should be collected throughout the duration of clinical development. Prelicensure clinical safety assessment will generally include safety data from comparisons of the investigational vaccine with the licensed control vaccine(s). Subjects should be carefully monitored for commonly occurring adverse events as well as less common, serious adverse events. Commonly occurring adverse reactions expected after tetanus immunization include pain, redness and swelling at the injection site. Post-vaccination fever may also occur. Although serious adverse events should be monitored during prelicensure clinical trials, serious adverse events that have been associated with tetanus toxoid (e.g. arthus reactions, Guillain–Barré syndrome) occur too infrequently to be reliably evaluated in most clinical trials, and post-marketing surveillance must also be performed to monitor serious adverse events.

C.4 Post-marketing studies and surveillance

Monitoring the efficacy, safety and quality of licensed vaccines consists of post-marketing surveillance and post-marketing studies (phase IV studies). The purpose of post-licensure monitoring is to assess the performance of a vaccine in the target population under conditions of routine use, and to monitor rare adverse events. Post-marketing studies may also be useful for assessing antibody persistence and the need for booster doses. Marketing authorization holders should be committed to presenting a post-marketing surveillance programme at the time of licensure. The programme should be based on criteria for assessing the quality, safety and effectiveness of a particular vaccine to gain marketing approval. In many cases, comprehensive post-marketing safety and effectiveness data cannot be collected by manufacturers alone, and close cooperation between manufacturers and public-health authorities is required.

Post-marketing surveillance may be the only means of detecting rare adverse events that occur too infrequently to have been detected during clinical trials. For the collection of safety data, surveillance may be conducted by active or passive processes. Voluntary reporting of serious adverse events (passive surveillance) is most commonly used. All data collected should be submitted to the NRA at regular intervals so that action can be taken if there are implications for the marketing authorization.

Part D. Recommendations for NRAs

D.1 General

The general recommendations for NRAs and national control laboratories (NCLs) given in Guidelines for national authorities on quality assurance for biological products (*73*) and Guidelines for independent lot release of vaccines by regulatory authorities (*46*) apply.

The details of production and quality control procedures, as well as any significant changes in them that may affect the quality, safety and efficacy of tetanus vaccines, should be discussed with and approved by the NRA. For control purposes, the international standards currently in use (see the section on General considerations) should be obtained for the purpose of calibrating national, regional and working standards (*55*). The NRA may obtain the product-specific or working references from the manufacturer to be used for lot release until an international or national standard preparation has been established.

Consistency in production has been recognized as an essential component in the quality assurance of tetanus vaccines. In particular, NRAs should carefully monitor production records and the results of quality-control tests on clinical lots as well as results from tests on a series of consecutive lots of the vaccine.

D.2 Release and certification by the NRA

A vaccine should be released only if it fulfils all national requirements or satisfies Part A of these Recommendations, or both (*46*).

A protocol based on the model given in Appendix 1, signed by the responsible official of the manufacturing establishment, should be prepared and submitted to the NRA in support of a request for the release of a vaccine for use.

A statement signed by the appropriate official of the NRA should be provided to the manufacturing establishment, and should certify that the lot of vaccine in question meets all national requirements as well as Part A of the present Recommendations. The certificate should provide sufficient information about the vaccine lot. A model certificate is given in Appendix 2. The official national release certificate should be provided to importers of the vaccine. The purpose of the certificate is to facilitate the exchange of vaccines between countries.

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References

1. *The immunological basis for immunization series. Module 3: Tetanus update 2006.* Geneva, World Health Organization, 2007 (http://whqlibdoc.who.int/publications/2007/9789241595551_eng. pdf, accessed 24 June 2013).

2. Recommendations for the production and control of *Haemophilus influenzae* type b conjugate vaccines. In: *WHO Expert Committee on Biological Standardization. Forty-ninth report*. Geneva, World Health Organization, 2000 (WHO Technical Report Series, No. 897), Annex 1.

3. Roper MH, Vandelaer JH, Gasse FL. Maternal and neonatal tetanus. *Lancet*, 2007, 370:1947–1959.

4. Black RE et al. Global, regional, and national causes of child mortality in 2009: a systematic analysis. *Lancet*, 2010, 375:1969–1987.

5. Ganatra HA, Zaidi AKM. Neonatal infections in the developing world 2010. *Seminars in Perinatology*, 2010, 34:416–425.

6. Howard CR, Beale AJ, Aguado MT. The development of controlled release vaccines – the role of the World Health Organization. In: Gander B, Merkle HP, Corradin G, eds. *Antigen delivery systems: immunological and technological issues*. Newark, NJ, Harwood Academic Publishers, 1997:1–15.

7. O'Hagan DT, Singh M, Gupta RK. Poly(lactide-co-glycolide) microparticles for the development of single-dose controlled-release vaccines. *Advanced Drug Delivery Reviews*, 1998, 32:225–246.

8. Tierney R et al. Transcutaneous immunization with tetanus toxoid and mutants of *Escherichia coli* heat-labile enterotoxin as adjuvants elicits strong protective antibody responses. *Journal of Infectious Diseases*, 2003, 188:753–758.

9. Qazi O et al. Reduction of the ganglioside binding activity of the tetanus toxin HC fragment destroys immunogenicity: implications for development of novel tetanus vaccines. *Infection and Immunity*, 2006, 4:4884–4891.

10. Johnston L et al. Transcutaneous delivery of tetanus toxin Hc fragment induces superior tetanus toxin neutralizing antibody response compared to tetanus toxoid. *Human Vaccines*, 2009, 5:230–236.

11. Tierney R et al. A single-dose cytomegalovirus-based vaccine encoding tetanus toxin fragment C induces sustained levels of protective tetanus toxin antibodies in mice. *Vaccine*, 2012, 30:3047–3052.

12. Requirements for diphtheria toxoid and tetanus toxoid (Requirements for biological substances No. 10). In: *WHO Expert Committee on Biological Standardization. Seventeenth report*. Geneva, World Health Organization, 1964 (WHO Technical Report Series, No. 293), Annex 1.

13. Requirements for diphtheria toxoid, pertussis vaccines, tetanus toxoid, and combined vaccines. In: *WHO Expert Committee on Biological Standardization. Thirtieth report*. Geneva, World Health Organization, 1979 (WHO Technical Report Series, No. 638), Annex 1.

14. Requirements for diphtheria toxoid, pertussis vaccine, tetanus toxoid, and combined vaccines (Addendum 1986). In: *WHO Expert Committee on Biological Standardization. Thirty-seventh report*. Geneva, World Health Organization, 1987 (WHO Technical Report Series, No. 760), Annex 4.

15. Requirements for diphtheria, tetanus, pertussis and combined vaccines (revised 1989). In: *WHO Expert Committee on Biological Standardization. Fortieth report*. Geneva, World Health Organization, 1990 (WHO Technical Report Series, No. 800), Annex 2.

16. Recommendations for diphtheria, tetanus, pertussis and combined vaccines (Amendments 2003). In: *WHO Expert Committee on Biological Standardization. Fifty-fourth report*. Geneva, World Health Organization, 2005 (WHO Technical Report Series, No. 927), Annex 5.

17. Sokhey S. Therapeutic substances in their international aspects. *BMJ*, 1950, 1:1037–1044.

18. *WHO Expert Committee on Biological Standardization. Twenty-second report*. Geneva, World Health Organization, 1970 (WHO Technical Report Series, No. 444).

19. *WHO Expert Committee on Biological Standardization. Fifth report*. Geneva, World Health Organization, 1952 (WHO Technical Report Series, No. 56).

20. *WHO Expert Committee on Biological Standardization. Twelfth report*. Geneva, World Health Organization, 1959 (WHO Technical Report Series, No. 172).

21. *WHO Expert Committee on Biological Standardization. Eighteenth report*. Geneva, World Health Organization, 1966 (WHO Technical Report Series, No. 329).

22. *WHO Expert Committee on Biological Standardization. Nineteenth report*. Geneva, World Health Organization, 1967 (WHO Technical Report Series, No. 361).

23. *WHO Expert Committee on Biological Standardization. Thirty-second report*. Geneva, World Health Organization, 1982 (WHO Technical Report Series, No. 673).

24. *WHO Expert Committee on Biological Standardization. Fifty-first report*. Geneva, World Health Organization, 2002 (WHO Technical Report Series, No. 910).

25. Sesardic D et al. Calibration of replacement international standard and European Pharmacopoeia biological reference preparation for tetanus toxoid, adsorbed. *Biologicals*, 2002, 30:49–68.

26. *Reference preparations established by the Expert Committee on Biological Standardization, 2010. Biological substances: International standards and reference reagents*. Additions. Geneva, World Health Organization, 2010 (WHO/BS/10.2150) (http://www.who.int/entity/biologicals/Reference_preparations_established_by_ECBS.pdf, accessed 24 June 2013).

27. Tierney R et al. Collaborative study for the calibration of a replacement International Standard for Tetanus Toxoid Adsorbed. *Biologicals*, 2011, 39:404–416.

28. Murata R, Someya S, Kaneko J. Standardization of tetanus toxoid combined with pertussis vaccine. *Japanese Journal of Medical Science and Biology*, 1970, 23:211–216.

29. Redhead K et al. Interaction of *Haemophilus influenzae* type b conjugate vaccines with diphtheria-tetanus-pertussis vaccine in control tests. *Vaccine*, 1994, 12:1460–1466.

30. Assay of tetanus vaccine (adsorbed). In: *European Pharmacopoeia*, 7th ed. Strasbourg, European Directorate for Quality of Medicines & HealthCare, 2011 (01/2011:20708).

31. *Minimum requirements: tetanus toxoid*. Fourth Revision. Bethesda, MD, National Institutes of Health, 1952.

32. Brown JH et al. Precision of potency assay of alum-precipitated tetanus toxoid in mice: an inter-institutional study. *Journal of Immunology*, 1953, 70:171–180.

33. Lyng J, Nyerges G. The second international standard for tetanus toxoid (adsorbed). *Journal of Biological Standardization*, 1984, 12:121–130.

34. Van Ramshorst JD, Sundaresan TK, Outschoorn AS. International collaborative studies on potency assays of diphtheria and tetanus toxoids. *Bulletin of the World Health Organization*, 1972, 46:263–276.

35. Mussett MV, Sheffield F. A collaborative investigation of methods proposed for the potency assay of adsorbed diphtheria and tetanus toxoids in the European Pharmacopoeia. *Journal of Biological Standardization*, 1973, 1:259–283.

36. Tierney R et al. Calibration of the Ph. Eur. Biological Reference Preparation (BRP) for tetanus vaccine (adsorbed) batch 3. *Pharmeuropa Bio and Scientific Notes*, 2011, 2011(1):1–26.

37. Kondo S, Kameyama S, Murata R. Standardization and control of toxoid components in the combined vaccine (DPT). *Japanese Journal of Medical Science and Biology*, 1974, 27:315–319.

38. Ikic D. Immunity in humans and guinea-pigs vaccinated with different doses of tetanus vaccine. *Proceedings of the International Symposium on Microbiological Standardization*, Opatija, 1960:415–422.

39. Ipsen J Jr. Bio-assay of four tetanus toxoids (aluminium precipitated) in mice, guinea-pigs and humans. *Journal of Immunology*, 1953, 70:426–434.

40. Hardegree MC, Pittman M, Maloney CJ. Influence of mouse strain on the assayed potency (unitage) of tetanus toxoid. *Applied Microbiology*, 1972, 24:120–126.

41. Cohen H, Van Ramshorst JD, Tasman A. Consistency in potency assay of tetanus toxoid in mice. *Bulletin of the World Health Organization*, 1959, 20:1133–1150.

42. Hardegree MC. Overview of tetanus toxoid: history and current status. In: *Tetanus vaccines for human use*. Pharmeuropa, October 2000: 5–28. (http://pharmeuropa.edqm.eu/PharmeuropaArchives/, accessed 9 September 2013).

43. Gupta RK et al. Evaluation of a guinea-pig model to assess interference in the immunogenicity of different components of a combination vaccine comprising diphtheria, tetanus and acellular pertussis (DTaP) vaccine and *Haemophilus influenzae* type b capsular polysaccharide conjugate vaccine. *Biologicals*, 1999, 27:167–176.

44. Stickings P et al. Animal refinement and reduction: alternative approaches for potency testing of diphtheria and tetanus vaccines. *Procedia in Vaccinology*, 2011, 5:200–212.

45. *Manual for quality control of diphtheria, tetanus and pertussis vaccines*. Geneva, World Health Organization, 2013 (WHO/IVB/11.11).

46. Guidelines for independent lot release of vaccines by regulatory authorities. In: *WHO Expert Committee on Biological Standardization. Sixty-first report*. Geneva, World Health Organization, 2013 (WHO Technical Report Series, No. 978), Annex 2.

47. Coombes L et al. Development and use of a novel in vitro assay for testing of diphtheria toxoid in combination vaccines. *Journal of Immunological Methods*, 2009, 350:142–149.

48. Prieur S et al. Development of an in vitro potency test for tetanus vaccines: an immunoassay based on Hc fragment determination. *Developments in Biologicals*, 2002, 111:37–46.

49. Good manufacturing practices: main principles for pharmaceutical products. In: *WHO Expert Committee on Specifications for Pharmaceutical Preparations. Forty-fifth report*. Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 961), Annex 3.

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

51. *WHO Expert Committee on Biological Standardization. Fifty-eighth repor*t. Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 963).

52. Preneta-Blanc R et al. Calibration of replacement International Standards of diphtheria and tetanus toxoids for use in flocculation test. *Biologicals*, 2008, 36:315–326.

53. *WHO Expert Committee on Biological Standardization. Forty-third report*. Geneva, World Health Organization, 1994 (WHO Technical Report Series, No. 840).

54. Sesardic D et al. The First International Standard for Antitetanus Immunoglobulin, Human: pharmaceutical evaluation and international collaborative study. *Biologicals*, 1993, 21:67–75.

55. *WHO manual for the establishment of national and other secondary standards for vaccines*. Geneva, World Health Organization, 2011 (WHO/IVB/11.03).

56. *Manual for the production and control of vaccines: tetanus toxoid*. Geneva, World Health Organization, 1977 (BLG/UNDP/77.2) (http://whqlibdoc.who.int/hq/pre-wholis/BLG_UNDP_77.2.pdf, accessed 24 June 2013).

57. *WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products*. Geneva, World Health Organization, 2003 (WHO/BCT/QSD/03.01) (http://www.who.int/biologicals/publications/en/whotse2003.pdf, accessed 22 June 2013).

58. General requirements for the sterility of biological substances. (Requirements for Biological Substances No. 6, revised 1973, amendment 1995). In: *WHO Expert Committee on Biological Standardization. Forty-sixth report*. Geneva, World Health Organization, 1998 (WHO Technical Report Series, No. 872), Annex 3.

59. Guidelines on stability evaluation of vaccines. In: *WHO Expert Committee on Biological Standardization. Fifty-seventh report*. Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 962), Annex 3.

60. Guidelines on regulatory expectations related to the elimination, reduction or replacement of thiomersal in vaccines. In: *WHO Expert Committee on Biological Standardization. Fifty-third report*. Geneva, World Health Organization, 2004 (WHO Technical Report Series, No. 926), Annex 4.

61. Hendriksen C, Winsnes R. Serological methods for potency testing of tetanus toxoid vaccines for human use. *Developments in Biologicals*, 2002, 111:131–140.

62. Coplu N et al. Tetanus antibody assay combining in-house ELISA and particle agglutination test and its serosurvey application in a province in Turkey. *Japanese Journal of Infectious Diseases*, 2004, 57:97–102.

63. *A WHO guide to good manufacturing practice (GMP) requirements. Part 2: Validation*. Geneva, World Health Organization, 1997 (WHO/VSQ/97.02).

64. WHO guidelines on nonclinical evaluation of vaccines. In: *WHO Expert Committee on Biological Standardization. Fifty-fourth report*. Geneva, World Health Organization, 2005 (WHO Technical Report Series, No. 927), Annex 1.

65. *Handbook: good laboratory practice (GLP). Quality principles for regulated non-clinical research and development*. 2nd ed. Geneva, World Health Organization, 2009.

66. Good manufacturing practice: supplementary guidelines for the manufacture of investigational pharmaceutical products for clinical trials in humans. In: *WHO Expert Committee on Specifications for Pharmaceutical Preparations. Thirty-fourth report*. Geneva, World Health Organization, 1996 (WHO Technical Report Series, No. 863), Annex 7.

67. Guidelines on clinical evaluation of vaccines: regulatory expectations. In: *WHO Expert Committee on Biological Standardization. Fifty-second report*. Geneva, World Health Organization, 2004 (WHO Technical Report Series, No. 924), Annex 1.

68. Guidelines for good clinical practice (GCP) for trials on pharmaceutical products. In: *The Use of Essential Drugs. Sixth report of the WHO Expert Committee.* Geneva, World Health Organization, 1995 (WHO Technical Report Series, No. 850), Annex 3.

69. Melville Smith ME, Seagroatt VA, Watkins JT. A comparison of enzyme-linked immunosorbent assay (ELISA) with the toxin neutralization test in mice as a method for the estimation of tetanus antitoxin in human sera. *Journal of Biological Standardization*, 1983, 11:137–144.

70. Hendriksen CFM et al. The toxin binding inhibition test as a reliable in vitro alternative to the toxin neutralization test in mice for the estimation of tetanus antitoxin in human sera. *Journal of Biological Standardization*, 1988, 16:287–297.

71. Kristiansen M, Aggerbeck H, Heron I. Improved ELISA for determination of anti-diphtheria and/or anti-tetanus antitoxin antibodies in sera. *Acta Pathologica, Microbiologica et Immunologica Scandinavica*, 1997, 105:843–853.

72. Reder S et al. Measuring immunoglobulin G antibodies to tetanus toxin, diphtheria toxin, and pertussis toxin with single-antigen enzyme-linked immunosorbent assays and a bead-based multiplex assay. *Clinical and Vaccine Immunology*, 2008, 15:744–749.

73. Guidelines for national authorities on quality assurance for biological products. In: *WHO Expert Committee on Biological Standardization. Forty-second report*. Geneva, World Health Organization, 1992 (WHO Technical Report Series, No. 822), Annex 2.

Appendix 1

Model protocol for the manufacturing and control of tetanus vaccines (adsorbed)

The following protocol is intended for guidance. It indicates the information that should be provided as a minimum by a manufacturer to the NRA. Information and tests may be added or omitted as necessary, with the authorization of the NRA.

It is possible that a protocol for a specific product may differ in detail from the model provided. The essential point is that all relevant details demonstrating compliance with the licence and with the relevant WHO Recommendations for a particular product should be given in the protocol submitted.

The section concerning the final product must be accompanied by a sample of the label and a copy of the leaflet that will accompany the vaccine container. If the protocol is being submitted in support of a request to permit importation, it should also be accompanied by a lot-release certificate from the NRA or from the NCL in the country where the vaccine was produced or released stating that the product meets national requirements as well as the recommendations in Part A of this annex.

1. Summary information on finished product (final lot)

International name: _____

Trade name/commercial name: _____

Product licence (marketing authorization) number: _____

Country: _____

Name and address of manufacturer: _____

_____

Name and address of licence holder,
    if different: _____

Final packaging lot number: _____

Type of container: _____

Number of containers in this packaging: _____

Final container lot number: _____

Number of filled containers in this final lot: _____

Date of manufacture: _____

Description of final product (adsorbed): _____

Preservative, and nominal concentration: _____

Volume of each single human dose: _____

Number of doses per final container: _____

Summary of the composition (include a summary of the qualitative and quantitative composition of the vaccine in each human dose, including any adjuvant used and other excipients):

_____

_____

Shelf-life approved (months): _____

Expiry date: _____

Storage conditions: _____

The following sections are intended for recording the results of the tests performed during the production of the vaccine so that the complete document will provide evidence of consistency in production. If any test has to be repeated, this must be indicated. Any abnormal result must be recorded on a separate sheet.

2. Detailed information on manufacture and quality control

Starting materials

Identity of *C. tetani* strain used for
     vaccine production: _____

Reference number of seed lot: _____

Date(s) of reconstitution (or opening) of
     seed lot ampoule(s): _____

Single harvests used for preparing the bulk purified toxoid

Name of the culture medium: _____

Date of inoculation: _____

Temperature of incubation: _____

Control of bacterial purity
     Methods: _____
     Result: _____
     Date: _____

Date of harvest: _____

Volume of harvest: _____

Yield (Lf/ml): _____

Volume after filtration: _____

Toxin purification method: _____

Method of detoxification: _____
     Date started: _____
     Date finished: _____

Volume used for inactivation: _____

Temperature: _____

Concentration of detoxification agent: _____

Confirmation of detoxification (before or after purification)

Method: _____

Dose of inoculation (Lf): _____

Route of inoculation: _____

Date of inoculation: _____

Date of observation: _____

Result: _____

Purification methods used for toxoid: _____

Yield of purified toxoid

Volume: _____

Toxoid content (Lf): _____

Bulk purified toxoid

Reference number: _____

Volume and Lf: _____

Sterility test

Tests for bacteria and fungi

Method: _____

Media: _____

Number of bulks tested: _____

Volume of inoculum per bulk: _____

Volume of medium per bulk: _____

Temperature of incubation: _____

Date of start of test: _____

Date of end of test: _____

Result: _____

Antigenic purity (Lf/mg of protein (nondialysable) nitrogen): _____

Method: _____

Date of test: _____

Specific toxicity test

Method: _____

Dose of inoculation (Lf): _____

Route of inoculation: _____

Date of inoculation: _____

Date of observation: _____

Result: _____

Test of reversion to toxicity

Lf of test toxoid solution: _____

Temperature of incubation of toxoid: _____

Dates of beginning and end of incubation: _____

Method: _____

Dose of inoculation (Lf): _____

Route of inoculation: _____

Date of inoculation: _____

Date of observation: _____

Result: _____

Final bulk

Identification (lot number): _____

Date of manufacture or blending: _____

Volume: _____

Lf: _____

Blending:	Prescription (SHD)	Added
Toxoid (Lf):	_____	_____
Adjuvant:	_____	_____
Preservative (specify):	_____	_____
Others (salt):	_____	_____
Final volume (ml):	_____	_____

Preservative content

Method: _____

Date of test: _____

Result: _____

Adjuvant content

Method: _____

Date of test: _____

Result: _____

Degree of adsorption

Method: _____

Date of test: _____

Result: _____

Sterility test

    Tests for bacteria and fungi

    Method: _____

    Media: _____

    Number of containers tested: _____

    Volume of inoculum per container: _____

    Volume of medium per container: _____

    Temperature of incubation: _____

    Date of start of test: _____

    Date of end of test: _____

    Result: _____

Specific toxicity test (when required)

    Method: _____

    Dose of inoculation (Lf): _____

    Route of inoculation: _____

    Date of inoculation: _____

    Date of observation: _____

    Result: _____

Potency test

Challenge method (multiple-dilution or single-dilution assay)

If single dilution, date of last satisfactory

    multiple-dilution assay: _____

Species, strain and weight range

    of animals: _____

Number of animals per dilution: _____

Reference vaccine used (IU): _____

Date of immunization: _____

Route of injection and volume of

    dilutions administered: _____

Date of challenge: _____

Challenge method used (lethal or

    paralytic challenge): _____

Challenge toxin used: _____

Challenge dose(s) used: _____

Date of end of observation: _____

Results (See Table 5.1 for an example of how to report the results from a lethal method, and see Table 5.2 for an example of reporting a paralytic method)

Table 5.1
Reporting results from a lethal challenge assay

Vaccine	Dilution	Lethal method No. survivors/No. tested	Median effective dose (ED$_{50}$)
Reference vaccine ……………… IU/ml	1	/	
	2	/	……………… ml
	3	/	
Test vaccine lot number ………………………	1	/	
	2	/	……………… ml
	3	/	
Potency of test vaccine in IU/SHD with 95% confidence limits: ………………………………… 95% confidence limits expressed as % of potency estimate: …………………………………			
Single-dilution assays only: *P* value indicating the probability that the test vaccine contains more than the minimum potency requirement per SHD: ……………………………………			

Table 5.2
Reporting results from a paralytic challenge assay

Vaccine	Dilution	Mean score
Reference vaccine ……………… IU/ml	1	
	2	
	3	
Test vaccine lot number ………………………	1	
	2	
	3	
Potency of test vaccine in IU/SHD with 95% confidence limits: ………………………………… 95% confidence limits expressed as % of potency estimate: …………………………………		
Single-dilution assays only: *P* value indicating the probability that the test vaccine contains more than the minimum potency requirement per SHD: ……………………………………		

When a single-dilution assay is performed, only the responses at the single dilution used are shown. For the paralytic challenge assay, the ED$_{50}$ is not applicable.

Serological method

Species, strain and weight range of animals: _____

Number of animals per dilution: _____

Reference vaccine used (IU): _____

Date of immunization: _____

Route of injection and volume of dilutions
administered: _____

Date of bleeding: _____

Method for titration of immune serum samples: _____

Reference serum or antibody: _____

Results (See Table 5.3 for an example of reporting results from a serological method)

Table 5.3
Reporting results from a serology assay

Vaccine	Dilution	Mean scores or response
Reference vaccine	1	
……………… IU/ml	2	
	3	
Test vaccine lot number	1	
	2	
………………………	3	
Positive control		
Negative control		
Potency of test vaccine in IU/SHD with 95% confidence limits: ………………………………… 95% confidence limits expressed as % of potency estimate: …………………………………		
Single-dilution assays only: *P* value indicating the probability that the test vaccine contains more than the minimum potency requirement per SHD: …………………………………		

Test for amount of residual free detoxifying agent

Detoxifying agent: _____

Method: _____

Result (g/1): _____

Date of test: _____

pH

    Result: _____

    Date of test: _____

Final product

Identification: _____

Volume: _____

Identity test

    Method: _____

    Result: _____

    Date of test: _____

Sterility test

    Tests for bacteria and fungi

        Method: _____

        Media: _____

        Number of containers tested: _____

        Volume of inoculum per container: _____

        Volume of medium per container: _____

        Temperature of incubation: _____

        Date of start of test: _____

        Date of end of test: _____

        Result: _____

Potency test

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

Innocuity test (when required)

    Tests in mice

        Date of start of test: _____

        Date of end of test: _____

        Number of animals tested: _____

        Route of injection: _____

        Volume of injection: _____

        Observation period: _____

        Results (give details of deaths): _____

Tests in guinea-pigs

    Date of start of test: _____

    Date of end of test: _____

    Number of animals tested: _____

    Route of injection: _____

    Volume of injection: _____

    Observation period: _____

    Results (give details of deaths): _____

Test for adjuvant content

    Nature and concentration of adjuvant/SHD: _____

    Method of testing: _____

    Result: _____

    Date of test: _____

Test for degree of adsorption (when required)

    Method: _____

    Desorption method and reagent: _____

    Result: _____

    Date of test: _____

Test for preservative

    Nature and concentration of preservative: _____

    Method of testing: _____

    Result: _____

    Date of test: _____

pH

    Method of testing: _____

    Result: _____

    Date of test: _____

Extractable volume

    Result: _____

    Date of test: _____

Inspection of final containers

    Date of inspection: _____

    Organoleptic characteristics: _____

    Number of containers inspected: _____

    % of containers rejected: _____

3. Certification by the manufacturer

Name of the manufacturer _____

Name of head of production (typed) _____

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

I certify that lot no. _____ of tetanus vaccine (adsorbed), whose number appears on the label of the final container, meets all national requirements and/or satisfies Part A[1] of the WHO Recommendations to assure the quality, safety and efficacy of tetanus vaccines (adsorbed) (2014).[2]

Signature _____
Name (typed) _____
Date _____

4. Certification by the NRA

If the vaccine is to be exported, attach a certificate from the NRA (as shown in Appendix 2), a label from a final container and an instruction leaflet for users.

---

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

[2] WHO Technical Report Series, No. 980, Annex 5.

Appendix 2

Model certificate for the release of tetanus vaccines (adsorbed) by NRAs

Lot-release certificate

Certificate no. _____

The following lot(s) of tetanus vaccine (adsorbed) produced by _____[1] in _____,[2] whose numbers appear on the labels of the final containers, complies with the relevant national specifications and provisions for the release of biological products[3] and Part A[4] of the WHO Recommendations to assure the quality, safety and efficacy of tetanus vaccines (adsorbed) (2014),[5] and complies with WHO good manufacturing practices: main principles for pharmaceutical products;[6] Good manufacturing practices for biological products;[7] and Guidelines for independent lot release of vaccines by regulatory authorities.[8]

The release decision is based on _____[9]

The certificate may include the following information:

- name and address of manufacturer
- site(s) of manufacturing
- trade name and common name of product
- marketing authorization number
- lot number(s) (including sub-lot numbers and packaging lot numbers if necessary)

---

[1] Name of manufacturer.

[2] Country of origin.

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

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

[5] WHO Technical Report Series, No. 980, Annex 5.

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

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

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

[9] Evaluation of the summary protocol, independent laboratory testing, or procedures specified in a defined document etc., as appropriate.

- type of container used
- number of doses per container
- number of containers or lot size
- date of start of period of validity (e.g. manufacturing date) and/or expiry date
- storage conditions
- signature and function of the person authorized to issue the certificate
- date of issue of certificate
- certificate number.

The Director of the NRA (or other appropriate authority)

Name (typed) ⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯
Signature ⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯
Date ⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯