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# Production and control of tetanus vaccine

*A training curriculum*

## MODULE III
Principles of tetanus vaccine production

**World Health Organization**
Geneva

*in collaboration with*

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Helsinki

# PRODUCTION AND CONTROL OF TETANUS VACCINE
# A TRAINING CURRICULUM




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# MODULE III

# PRINCIPLES OF TETANUS VACCINE PRODUCTION

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# Module III PRINCIPLES OF TETANUS VACCINE PRODUCTION

## 1. Introduction

Tetanus toxoid (TT) is one of the most immunogenic antigens available for protection against an infectious disease. In the industrialized countries, its use has markedly decreased the incidence of tetanus and the need for tetanus antitoxin. In the developing world much needs to be done to promote and increase its use. This is particularly important in countries with a high incidence of neonatal tetanus, a disease that can be eliminated by the immunization of women before or during pregnancy.

The current WHO requirements call for the product to be purified, since unpurified tetanus toxoid may cause vaccination reactions in humans more often than toxoids purified from medium, other bacterial components, formalin etc. Unpurified toxoid also has more sensitizing properties. However, untoward reactions sometimes occur on revaccination of adults even when purified products are used.

Although purification enables more highly concentrated preparations to be used, it may sometimes reduce the immunizing activity of tetanus vaccine. This is probably due to the removal of substances having an adjuvant effect or the inadequacy of purification techniques employed. Improper purification may destroy the immunogenicity of the toxoid or, if the purification is continued to the extreme, the resulting protein solution becomes so diluted that the toxoid can be absorbed to the vessels and other materials used during the subsequent steps of the production process.

For optimal immunogenicity in final vaccine, purified TT must be combined with an adjuvant. As the final vaccine is a mixture of several components the *antigen content* and the actual *immunogenicity* of the vaccine are not necessarily directly related. In the present requirements, the maximum number of Lf per single human dose of tetanus vaccine (adsorbed) has been reduced to 25 if more than one dose is used for primary immunization. The limit has been adopted to prevent the manufacturers from increasing the amount of toxoid (i.e. the antigen) per dose in order to pass the potency test with less immunogenic toxoid preparations.

### 1.1. Description of the vaccine

Tetanus vaccine contains tetanus toxoid as an antigen (immunogen). The toxoid is prepared by treating tetanus toxin chemically (usually by

formaldehyde, another possibility is glutaraldehyde) to render it nontoxic without losing its immunogenic potency. The toxoid is concentrated, purified and adsorbed on to a suitable adjuvant. It is supplied as a sterile solution in physiological buffer usually containing a preservative.

The international name of the vaccine is *Vaccinum tetani adsorbatum*. The proper name should be the equivalent of the international name in the language of the country of use. The use of the international name should be limited to vaccines that satisfy the requirements formulated by WHO (1).

### 1.2. Method of production

Suitable methods for the production of tetanus vaccine are given in the Manual for the production and control of vaccines: Tetanus toxoid (3). The production of tetanus toxin shall always be based on a seed lot system (Table 1). Cultures of the working seed shall have the same characteristics as those of the strain from which the parent seed lot was derived.

Written descriptions of procedures for the preparation and testing of tetanus vaccine adopted by the manufacturer shall be submitted to the National Control Authority (NCA) for approval. This must be accompanied by appropriate evidence that the most critical steps of the production method have been validated and consistency of the production process and control have been established. Proposals for modifications of the manufacturing and/or control methods shall also be submitted to the NCA for approval before such modifications are implemented.

## 2. Requirements for premises, equipment and staff

The general requirements for manufacturing establishments contained in the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) apply to establishments manufacturing tetanus vaccine with the addition of the following:

All manufacturing processes up to and including the completion of detoxification shall be carried out in completely isolated areas and by means of equipment specially reserved for the purpose. This is because tetanus toxin is highly toxic (lethal dose 50 ng) and because *C. tetani* can form spores which are very resistant to disinfection. Also, all materials have to be disinfected before leaving the production unit and persons entering or leaving the unit have to change clothing.

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

**Single harvest:** The homogenous toxic filtrate or toxoid solution obtained from one batch of cultures inoculated, harvested and processed together.

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

**Final bulk (vaccine):** The final homogeneous vaccine present in a single container from which the final containers are filled either directly or through one or more intermediate containers (sub-bulks).

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

Table 1. Terms related to the production process of tetanus vaccine.

Personnel employed in production and quality control must be adequately trained. Basic knowledge in microbiology and aseptic techniques are of course an asset, but there are really no formal requirements for the technicians employed in the production of tetanus toxoid. It is very important, however, that their training be precise and it has been assured that they are fully confident in the practices of the process before allowing them to take independent responsibility for the work.

To prevent the contamination of the product, personnel required to work in clean and aseptic areas should be selected with care to ensure that they may be relied upon to observe the appropriate discipline and are not subject to any disease which would present an abnormal microbiological hazard to the product. High standards of personal hygiene and cleanliness are essential. Staff should be instructed to report any condition (diarrhoea, coughs, colds, infected skin, wounds etc.) which may cause the shedding of abnormal numbers of microbes. Periodic health checks are desirable.

To protect the staff, it is self-evident that all staff need to be fully immunized against tetanus. The serum anti-tetanus levels of all staff should also be monitored regularly (every second year).

## 3. Vaccine production

The production of tetanus vaccine consists of:
1) cultivation of *C. tetani* bacteria,
2) inactivation (detoxification) of tetanus toxin,
3) purification of tetanus toxoid,
4) mixing of bulk vaccine, and
5) aseptic filling.

The production does not result in acceptable vaccine if it is not complemented with extensive in-process quality control testing. From country to country, the number and methods of tests can vary, but as a general rule it can be stated that the more complete the testing, the higher the quality of the final product. Certainly, there are minimum requirements for the panel of tests, as sterility, innocuity and potency testing could never be omitted (Figure 1).

Both the production method, including its validation results, and the quality control methods should be approved by the NCA in the connection of the initial marketing authorization of the product.

### 3.1. Flow diagram

The flow of the production process is summarized in Figure 1.

### 3.2. Media

It is particularly important to ensure that the final product is free from substances likely to cause toxic or allergic reactions in humans. Therefore it is essential to use only high quality starting materials, especially including the media. Müller-Miller medium is a commonly used medium for cultivation of tetanus bacteria. One hundred liters of this medium contains the ingredients given in Table 2.

When the media are prepared from a protein digest, such as casein hydrolysate or digested muscle, precautions should be taken to ensure that digestion has proceeded sufficiently. Established limits, if any, for mammalian protein and human blood-group substances in the final vaccine should not be exceeded. There is a strong trend in vaccine industry to introduce completely artificial growth media into all production processes.

| MINIMUM CONTROLS | PROCESS |
|---|---|
| Bacterial purity | Inoculum Media |
| Bacterial purity, Lf, pH | Cultivation |
| | Filtration |
| Sterility, Lf, Kf, pH | **SINGLE HARVEST** |
| | Detoxification |
| | Concentration<br>Standardization |
| | Purification |
| | Sterile filtration |
| Sterility, irreversibility, specific toxicity, antigen content, purity | **BULK PURIFIED TOXOID**<br>Aseptic mixing with adjuvant and preservative |
| Sterility, specific toxicity, potency, residual free formaldehyde, stability, pH | **FINAL BULK**<br>Aseptic filling into final containers |
| Identity, sterility, potency, innocuity, adjuvant content, preservative content | **FINAL LOT** |

Figure 1. Flow diagram of tetanus toxoid production and the related quality control tests that are required as a *minimum* (more complete list in text).

### 3.3. Strains of *Clostridium tetani*

Strains of *C. tetani* used in preparing tetanus toxoid shall be identified by a record of their history and of all tests made periodically to verify strain characteristics. A highly toxicogenic strain of *C. tetani* should be used. A strain that has proved satisfactory in many laboratories is the Harvard strain. Strains can be obtained from several international institutes.

The strain shall be maintained as a freeze-dried culture. For lyophilization the strain is grown on freshly prepared glucose beef heart broth, centrifuged and resuspended in skimmed milk. After lyophilization the culture can be kept at room temperature, but it is best stored refrigerated or at freezer temperature.

The microbial purity of the seed lot must be assured both by cultivation and staining. A Gram smear shows gram-positive rods of the same thickness, but different in length. They usually form no spores in Müller-Miller medium and glucose beef heart broth, but in less rich media spores may be formed. Furthermore, the capacity of the seed to produce tetanus toxin in the selected medium must be demonstrated, in addition to Lf determination, by a direct method such as minimum lethal dose (MLD, see Module VI).

### 3.4. Inoculum

It is essential that the production of tetanus toxin is always based on a seed lot system. It must be ascertained that cultures of the working seed have the same characteristics as those of the strain from which the parent seed lot was derived.

A lyophilized culture is transferred into tubes with proper medium (thioglycolate medium or glucose beef heart broth) and incubated at +35 °C for 24-48 hours in anaerobic conditions. The inoculum for a 150 l culture is made by inoculation of 300 ml production medium in a 500 ml bottle with 10 ml *C. tetani* culture, followed by incubation at +35 °C overnight. The microbial purity of the inoculum must be controlled.

### 3.5. Cultivation

Cultivation can be performed either in fermentors or regular culture vessels (static method). Prior to inoculation samples are drawn from the medium for inspection, pH measurement, and sterility control.

**Müller-Miller medium:**
Add the following ingredients to 80 l distilled water in the order given, at a temperature of about 55 °C (p.a. = pro analysis quality):

| | |
|---|---|
| Glucose | 1100.0 g |
| NaCl (p.a.) | 250.0 g |
| $Na_2HPO_4$ (p.a.) | 100.0 g |
| $KH_2PO_4$ (p.a.) | 15.0 g |
| $MgSO_4.7H20$ (p.a.) | 15.0 g |
| $FeSO_4.7H20$ (1% sol. in distilled water) | 4.0 g |
| Cystine-HCl (10%) | 250.0 ml |
| Tyrosine-HCl (10%) | 500.0 ml |
| Uracil-HCl (2.5%) | 1000.0 ml |
| Ca-pantothenate in ethanol 25% | 100.0 mg |
| Thiamine in ethanol 25% | 25.0 mg |
| Pyridoxin-HCl in ethanol 25% | 25.0 mg |
| Riboflavin in ethanol 25% | 25.0 mg |
| Biotin in ethanol 25% | 250.0 mg |
| 5 M NaOH | 400.0 ml |
| Beef heart infusion*) | 5.0 ml |
| Casein solution**) | 15025.0 ml |

After adjusting the volume to 100 liters the pH is adjusted with 5 M NaOH to pH 7.3. Sterilization is performed at +120 °C for 20 minutes.

*) **Preparation of the beef heart infusion:** Suspend 7.5 kg minced, de-fatted beef heart in 7.5 l distilled water. Bring rapidly to boiling and boil two minutes. Filter through filter paper, autoclave and store at +4 °C.

**) **Preparation of casein solution:** Several commercial preparations are available, e.g. Tryptone T pancreatic enzyme digest of casein (Oxoid) or N-Z-case (Humko Sheffield). Dissolve the amount of powder needed (the final volume should be 5% more than calculated, to account for filtration losses) in distilled water, to obtain a 10% w/v solution by heating in a stainless steel container. Cool down to +40 °C, dilute to the final volume and add 1.25 g charcoal per liter. Stir the solution for 20 minutes. Filter the solution through filter paper. Recycle the first filtrate because some charcoal may have passed through the filters. Autoclave and store at +4 °C.

To determine the optimal concentration of N-Z-case in the Müller-Miller medium (normally between 15 and 25 g/l) each new batch is tested for toxin production on a 0.5 liter scale.

Table 2. Preparation of media

The tetanus toxin is prepared in homogeneous culture usually in volumes from 20 liters up to several hundred liters. Müller-Miller medium is best suited for the cultivation. *C. tetani* is an anaerobic microorganism and the cultivation has to be started under anaerobic conditions. However, during the growth and lysis phase the fermentor culture may be gently mixed and flushed with nitrogen or air.

Throughout the cultivation process it is essential to control the microbial purity of the culture. Samples should be taken initially from the inoculum and daily from the growing culture for routine plate cultures both in aerobic and anaerobic conditions. Also Gram stained slides should be inspected. Contaminated cultures are discarded altogether.

Cell lysis is completed after 5 to 7 days and corresponds with the maximum toxin content (Lf determination) in the culture fluid. The average yield is around 40 to 60 Lf/ml. The cultivation is stopped by either cooling or adding formalin as soon as all cells have lysed.

### 3.6. Filtration

Separation of the lysed cells and toxin containing culture fluid is performed in multiple steps, starting from coarse and continuing to sterilizing filtration.

### 3.7. Detoxification

Detoxification can be started either before or after filtration. One method to detoxify the harvest is to add 0.4% v/v of a 40% w/v formaldehyde solution (=formalin). The pH is adjusted to 7.6 in the beginning and controlled weekly. After four weeks detoxification at +35 °C sterility, specific toxicity and Lf/ml are controlled.

If toxicity is still detectable, the detoxification can be continued by keeping the containers at +35 °C for an additional two weeks after checking that pH and amount of formaldehyde are appropriate. As a safety margin, many producers require that a successful specific toxicity test is *always followed by several days to two weeks of further detoxification*. Subsequently, the quality control tests need to be repeated before release of the crude toxoid. The containers awaiting release are kept at +4 °C.

The above is only one example of the detoxification method and each manufacturer should validate their own method. The amount of formalin needed depends on whether it is added before or after the filtration of bacteria.

### 3.8. Purification

First the crude toxoid is concentrated by ultrafiltration through hollow fibre filter cartridges or in tangential flow apparatus. Then the filtrate is purified by fractional salt precipitation. Salt ions are removed either by dialysis or gel filtration column chromatography. Finally the product (bulk purified toxoid) is processed by sterile filtration with filters of pore size 0.2 $\mu$m. The bulk purified toxoid must be free of tetanus toxin (specific toxicity test, refer to Module VI) and its antigenic purity must be $\geq$ 1000 Lf/mg protein nitrogen.

### 3.9. Further processing

The toxoid is then adsorbed on a suitable adjuvant by mixing aseptically the bulk purified toxoid and the adjuvant in same vessel. Currently aluminum hydroxide and aluminum phosphate are used as adjuvants. The addition of a preservative, such as thiomersal, is always necessary for multi-dose vials but not for single dose ampoules provided that satisfactory aseptic techniques are used during manufacture. The preservative is added simultaneously with the adjuvant. Generally, at least one month is allowed for the adsorption to become complete.

Final vaccine is then filled into ampoules or vials by automated equipment; manual filling implies an unacceptable risk of contamination.

### 3.10. In-process tests

Each step of the production process is monitored by a number of quality control tests. The listing below is rather comprehensive and all are not absolutely necessary. Minimum requirements are shown in Figure 1. Detailed descriptions of the tests are given the Modules IV, V and VI.

**Tests on starting materials.** Quality of water: conductivity, hardness, microbial purity, pyrogenicity. Media: pH, sterility. Inoculum: bacterial purity.

**Tests on single harvest.** Microbial purity, immunodiffusion, Kf, Lf, MLD, pH.

**Tests on crude tetanus toxoid.** Lf, Kf, pH, specific toxicity, sterility.

**Tests on bulk purified toxoid.** Antigen content, irreversibility, Lf, potency, purity, pyrogenicity, specific toxicity, stability, sterility, residual free formaldehyde, pH.

**Tests on adjuvant.** Aluminum content, chloride ion content, pH, sterility.

**Tests on preservative (thiomersal).** Mercury content, pH, sterility.

**Tests on final bulk vaccine.** Aluminum content, antigen content, innocuity, opacity, pH, potency, residual free formaldehyde, specific toxicity, stability, sterility, preservative content.

**Tests on final lot.** Aluminum content, identity, innocuity, opacity, potency (repeat is not necessary if already done on final bulk vaccine), preservative content, pH, sterility, filling volume.

## 4. Stability

Before marketing extensive testing is necessary to determine the loss of potency to be expected during storage. The stability of the vaccine shall be demonstrated to the satisfaction of the NCA. Final containers from at least three lots derived from different lots of purified bulk toxoid shall be tested on the expiry date to demonstrate stability during storage. It is practical to investigate the short term stability of the vaccine at potential exposure temperatures as well.
The vaccine shall meet the requirements for the final product up to the expiry date, provided that it has been stored at the recommended temperature.

When any changes are made in the production procedure that may affect the stability of the product, the vaccine produced by the new method shall be shown to be stable. The statements concerning storage temperature and expiry date appearing on the label shall be based on experimental evidence and shall be submitted for approval to the NCA.

## 5. Storage

Storage at a temperature of +2 to +8 °C has been found to be satisfactory. It is also important to note that adsorbed vaccines shall not be frozen since this may result in a non-homogenous suspension which may reduce potency and increase the reactogenicity of the vaccine.

## 6. Expiry date

The expiry date shall be approved by the NCA based on accepted stability studies. Counting for the date of expiry starts from the date when the last satisfactory potency determination was begun (the date on which the test animals were immunized with the vaccine).

In general terms, toxoids are very stable, and they can be stored up to several years. For tetanus toxoid the approved period of use is three years (WHO) or even five years (Ph. Eur.) from the starting date of the last approved potency test. After piercing of the cap of a multi-dose vial the vaccine should be held under refrigerated conditions. EPI recommends that multi-dose vials should be used only during one immunization session and then discarded.

## 7. Labelling

The label printed on or affixed to each container and the label on the carton enclosing one or more containers shall show as a minimum:

- the words *Vaccinum tetani adsorbatum* and/or the proper name of the product,
- the name and address of the manufacturer,
- the number of the final lot,
- the recommended storage temperature and the expiry date if kept at that temperature, and
- the recommended single human dose and route of administration.

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

- a statement that the vaccine satisfies the national requirements,
- the potency of the vaccine, i.e. amount of tetanus toxoid expressed in IU per single human dose,
- the nature and amount of any preservative present in the vaccine,
- the nature and amount of the adsorbing agent,
- the recommended temperature for storage and transport,
- a warning that the adsorbed vaccine should not be frozen,
- a warning that the adsorbed vaccine should be shaken before use, and
- instructions for the use of the vaccine and information on contraindications and the reactions that may follow vaccination.

## 8. Release

The batches may be released for distribution only after all relevant tests have been performed, results have been found acceptable by the quality control of the manufacturer and the lot formally approved and released by the NCA.

## 9. Retained samples

From each lot samples must be taken in a sufficient amount to satisfy the requirements for samples of the national control laboratory. Additional samples shall be retained throughout the dating period as reference material in a manner that ensures the identity of the lot. Also manufacturers should retain sufficient additional samples of the final product and the bulk purified toxoid to facilitate the repetition of the control tests if necessary.

The number of retained samples must be predetermined so that the repeat of complete panel of quality control testing will be possible. As a guideline for the final product 40 ampoules or 20 injection bottles from each lot will suffice.

## 10. References

1. WHO. WHO Expert Committee on Biological Standardization. Fortieth Report. Technical Report Series no 800. Geneva: WHO, 1990.

2. UNIDO. Model programme for the production of vaccines in developing countries.

3. WHO. Manual for the production and control of vaccines: Tetanus toxoid. 1977, BLG/UNDP/77.2 Rev.1.

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