

Annex 3

Guidelines for the production and control of inactivated oral cholera vaccines

This document provides information and guidance to national regulatory authorities and vaccine manufacturers concerning the characteristics, production and control of inactivated oral cholera vaccines intended to facilitate progress towards their international licensure and use. The text is presented in the form of Guidelines instead of Recommendations because further work is still needed to develop and standardize appropriate methods and criteria that will assure the consistent quality, safety and stability of these vaccines. Guidelines allow greater flexibility than Recommendations with respect to expected future developments in the field and indicate present deficiencies.



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1. Introduction

A parenterally administered, killed whole-cell cholera vaccine has been widely available for many years. The WHO Requirements for this vaccine were first adopted in 1959 and revised in 1968 (1); an addendum was incorporated in 1973 (2). However, this vaccine offers at best only limited protection of short duration and produces unpleasant side-effects in many vaccinees. In view of these limitations, the vaccine has not been considered satisfactory for general public health use, and in 1973 the twenty-sixth World Health Assembly abolished the requirement in the International Health Regulations for a certificate of vaccination against cholera.

Considerable progress has been made during the past decade in the development of a new generation of oral vaccines against cholera. These have already been licensed in some countries and are now being considered for wider public health application (3). Two distinct types of oral cholera vaccine have been developed; those consisting of live attenuated bacteria and those consisting of killed (inactivated) bacterial cells. In some cases, the latter are combined with the purified recombinant DNA-derived B-subunit of the cholera toxin. These positive developments have led to a need for international guidance to assure the quality and safety of this new generation of cholera vaccines. The present guidelines apply only to inactivated oral cholera vaccines.



Because the WHO Requirements (1) for the production and control of the killed whole cell parenteral cholera vaccine may not be relevant to the production and control of the new generation of cholera vaccines, and because such a vaccine is no longer recommended for general public health use (although it is still produced in some countries), as well as the potential for confusion with guidelines relating specifically to the new vaccines, the Expert Committee for Biological Standardization, decided at its fiftieth meeting to discontinue those requirements (4).

2. General considerations

2.1 The pathogen and the disease

Throughout history, the highly pathogenic waterborne bacterium *Vibrio cholerae* has caused devastating outbreaks of diarrhoeal disease in most parts of the world. Altogether seven cholera pandemics have been recorded, the latest of which started in 1961, and is still continuing. An estimated 120 000 deaths worldwide are caused by cholera each year. Humans are the only known natural host for *V. cholerae* and the disease is closely linked to poor sanitation. Despite the availability of oral rehydration treatment, small children and the elderly are particularly susceptible to the extreme dehydration that results from severe cholera. Although oral rehydration therapy may often save lives it has no effect on the course of the disease or on dissemination of the infection.

V. cholerae is a Gram-negative, rod-shaped bacterium that carries a single polar flagellum. It is a non-invasive pathogen that colonizes the epithelium of the small intestine after penetrating the mucus layer. The organism causes diarrhoea through the secretion of cholera toxin, the toxic action of which depends on a specific host receptor, the monosialosyl ganglioside GM1.

Strains of *V. cholerae* are characterized by serogrouping based on the polysaccharides of the somatic O antigen. Epidemics have almost invariably been caused by *V. Cholerae* of the O1 serogroup. Three serotypes (Ogawa, Inaba and Hikojima) and two biotypes (classical and El Tor) have been described, although there is some debate as to whether Hikojima is truly a separate serotype. Until recently, *V. cholerae* of the O1 serogroup accounted for most cases of cholera, but an additional *V. cholerae* serogroup, O139, has now emerged as a major cause of cholera in India and Bangladesh (5). Serogroup O139 is closely related to the El Tor biotype and has now spread over a large part of Asia. In the 1990s, cholera returned for the first time in



100 years to Central and South America. The causative agent in Latin America is similar, if not identical, to the agent that caused the seventh pandemic in Asia and Africa, i.e. the El Tor biotype of *V. cholerae* serogroup O1.

2.2 Protection against the disease

The available evidence suggests that protection against cholera is best acquired through oral immunization, either through natural infection, or by use of an oral vaccine. Data from studies in Bangladesh indicate that natural cholera infection is about 90% effective in eliciting protection against subsequent attacks for up to 3 years. Infection with the classical biotype of *V. cholerae* (Inaba or Ogawa) appears to stimulate a more potent, or longer-lasting immunity than infection with the El Tor biotype (6–8). The traditional killed parenteral cholera vaccine induces only up to 50% protection for 3–6 months. The limited protection afforded by this vaccine seems to be due mainly to the route of administration. Injected cholera vaccine gives rise to little or no local immune response in the gut where both the pathogen and the toxin it produces exert their action during infection. The pathogenesis of *V. cholerae* involves both the colonization of the intestine and the production of the enterotoxin, cholera toxin (CT), which acts locally to stimulate excessive electrolyte and fluid secretion, primarily from the crypt cells of the small intestine. Cholera toxin acts by inducing increased formation of cyclic adenosine monophosphate (cAMP) and/or cyclic guanosine monophosphate (cGMP) in the epithelial cells resulting in the secretion of chloride and bicarbonate into the lumen of the small intestine. Other enterotoxins, such as zonula occludens toxin (ZOT) and accessory cholera enterotoxin (ACE) may also contribute to pathogenesis, but probably play only a minor role. Protection against cholera may therefore be expected to be provided by immune mechanisms that block colonization and multiplication of the pathogen in the intestine inhibit the toxic activity of the toxin, or both. The ability to stimulate local intestinal immunity is therefore now considered critical if a cholera vaccine is to offer protection against infection and the disease (3). In addition, antibodies to *V. cholerae* have been found in breast milk and saliva and may be an indirect measure of intestinal immunity (9).

2.3 Candidate antigens

Cholera toxin consists of five identical B-subunit peptides that spontaneously associate to form a ring structure into which the enzymatically active A-subunit peptide is non-covalently inserted. The toxic activity resides in the A-subunit while the five B-subunits mediate



binding of the toxin to specific GM1 receptors on intestinal epithelial cells and are primarily responsible for the immunogenicity of the toxin.

The cholera toxin B-subunit elicits an effective antitoxin response that also offers short-lived protection against disease due to the heat-labile toxin (LT) of *Escherichia coli* (10–12). Furthermore, the cholera toxin B-subunit appears to be well-suited as an oral immunogen because it is stable in the intestines and is capable of binding to the intestinal epithelium, including the M-cells of the Peyer's patches, which is important for stimulating mucosal immunity, including local immunological memory (13). It is believed that this is important for protection because studies in animals have shown a direct correlation between protection against cholera toxin-induced fluid secretion and intestinal synthesis of secretory immunoglobulin A (sIgA) antibodies, and also between protection and the number of antitoxin-producing cells in the intestines. Thus, locally produced sIgA antibodies are considered important for providing antitoxic immunity in the gut (14).

There are, however, other cellular components of *V. cholerae* that induce potentially protective immune responses. The killed whole cells themselves elicit an antibacterial response that is directed mainly against the lipopolysaccharide (LPS) of the pathogen; LPS is the predominant antigen producing immunity to cholera in an experimental setting (14). There is also evidence to suggest that an immune response to toxin-coregulated pili (TCP) may also play a role in host protection. In classical *V. cholerae* O1 organisms, TCP have been shown to play an important role in the colonization of the small intestines (15). These pili are rarely found on the El Tor vibrios, although an El Tor-specific type of TCP has been reported to be expressed (16, 17). The El Tor organisms, however, express another type of pili called mannose-sensitive haemagglutinin (MSHA) fimbriae; these are poorly expressed on the surface of the classical vibrios (16). There is no evidence to suggest that the MSHA fimbriae enhance the immunogenicity of the killed oral vaccines. However, it has been proposed that TCP, while not an important antigen in itself, may enhance immunity by mediating the attachment of the bacteria to the intestinal cells. The relative importance of TCP and LPS as components of inactivated vaccines is unclear.

The growth conditions required for maximum expression of *V. cholerae* antigens in the laboratory need to be carefully determined and may differ significantly from those expected in vivo; for example, the conditions needed for the production of cholera toxin and TCP



(18). Furthermore, studies have shown that *V. cholerae*, like other pathogenic bacteria, express a number of antigens during growth in vivo that are not readily produced by the organism when grown under various conditions in vitro (19, 20). With the development of sophisticated genomic-based technologies, including in vivo expression systems to probe host environments, significant new insights into the complexities of host–pathogen interactions are being gained. These may lead to better control of the expression in vitro of antigens that may be important for vaccine production and host protection. Recent studies using in vivo expression technology have shown cholera toxin and TCP to be expressed sequentially during infection and that full toxin expression occurs only after, and is dependent upon, colonization (21). There is a possibility that a quorum-dependent signal is involved in the process. Quorum sensing is a process whereby cell–cell communications are mediated by the synthesis, secretion and detection of small extracellular signal molecules (22). Cell density is likely to play a part in this process.

2.4 Inactivated oral vaccines

Two killed (inactivated) oral cholera vaccines have been developed and clinically tested. One vaccine, developed in Sweden, consists of inactivated whole cells of *V. cholerae* in combination with a purified recombinant DNA derived B-subunit (rCTB) of the cholera toxin. In early clinical trials of this vaccine a native B-subunit (CTB) was used. The second vaccine, developed in Vietnam following technology transfer from the Swedish manufacturer, consists of whole inactivated *V. cholerae* cells alone. Large-scale field trials in Bangladesh and Peru (3, 23–25) have shown that a whole-cell killed vaccine containing the B-subunit, and a killed whole-cell preparation alone, both produced by a Swedish company, conferred significant protection on recipients for up to 3–5 years depending on age of the vaccines. In the field trial in Bangladesh, three doses of the vaccine containing the B-subunit resulted in 85% and 50% protection when assessed after 6 months and 3 years, respectively, in all age groups, including children aged less than 5 years. However, protection declined rapidly after the first 6 months of follow-up in children aged 2–5 years and disappeared during the third year after vaccination. In contrast, the vaccine from the Swedish manufacturer lacking the B-subunit, that was assessed in Bangladesh, did not confer significant protection against El Tor cholera in young children. In adults, the oral vaccine lacking the B-subunit gave a somewhat lower initial level of protection than that given by the vaccine containing the B-subunit, but after 6 months the protection afforded by the two vaccines was similar. The protective efficacy



of the inactivated whole-cell vaccine containing the rCTB was reproduced in Peru in military recruits in whom two doses gave 86% short-term protective efficacy (25). The second vaccine for which clinical trial results are available was produced in Vietnam. Two oral doses of this killed whole-cell oral vaccine lacking the B-subunit were reported to have an efficacy of 66% 8 months after immunization in all age groups (26). A second-generation bivalent vaccine, containing the serogroup O139 in addition to O1, but with no B-subunit component, is being developed and evaluated (3).

2.5 Correlates of protection

A problem in the evaluation of cholera vaccines is the identification of appropriate markers of protection. Oral vaccination promotes anti-LPS secretory IgA responses similar to those for infection itself (14, 27) whereas parenteral immunization does not. Similarly, the B-subunit of cholera toxin also elicits high antitoxin secretory IgA responses when given orally (14, 28). To be efficacious, cholera vaccine must stimulate a local immune response in the gut mucosa. Intestinal biopsies have shown that there is an increase in antibody-secreting cells specific to the B-subunit of cholera toxin and to whole cells following oral immunization (29). However, serum vibriocidal antibodies may offer an indirect measure of the protective immune response. Vibriocidal antibodies are measured by the degree of bacterial lysis that occurs when serial dilutions of serum are incubated with a large standardized inoculum of *V. cholerae* in the presence of complement. Following natural infection of humans, there is a many-fold rise in titre of serum vibriocidal antibodies. Elevated titres of serum antibodies are correlated with protection if immunization was by the oral route (30, 31). The killed whole-cell parenteral vaccine is also capable of eliciting a high vibriocidal titre in immunized individuals, but this vaccine confers only limited protection for a short time. Vibriocidal titre must therefore be seen only as a marker of the stimulation of an appropriate intestinal immune response and not a goal in itself. Serum vibriocidal antibody responses that occur following the ingestion of live oral antigens, delivered by wild type or attenuated *V. cholerae* have been shown to serve as markers for the stimulation of a potential intestinal immunity that endures long after the serum vibriocidal antibody titres have returned to baseline levels (3, 8). In regions where cholera is endemic, vibriocidal antibody titres are relatively high before vaccination, and rises in titre following oral vaccination are modest in comparison with those obtained by vaccinating people in non-endemic area. The only direct predictor of protection to cholera is the local secretory IgA response in the small



intestine, which is clearly not a practical indicator to measure in the context of a large clinical trial. The serum vibriocidal titre is therefore the most useful marker presently available for indicating an appropriate immune response in humans.

2.6 Production and control of inactivated oral cholera vaccines

The vaccines currently produced typically contain $25\text{--}50 \times 10^9$ cells per dose of each of the strains of *V. cholerae* representing both Inaba and Ogawa serotypes, as well as classical and El Tor biotypes. Some formulations also contain inactivated *V. cholerae* O139 (50×10^9 cells). The vaccine from Sweden also contains 1 mg per dose of purified rDNA derived B-subunit of the cholera toxin.

The whole-cell components of the vaccines are inactivated individually, before or after washing, either by treatment with formaldehyde or by heating. Inactivated bacterial cultures are then harvested by centrifugation or ultrafiltration, washed, resuspended in buffer and mixed with the B-subunit of cholera toxin, if used, to produce the final bulk from which the final lots are produced.

There is no precedent for controlling this new type of vaccine (i.e. an inactivated killed oral vaccine), and there is as yet no internationally accepted direct method for measuring the potencies of such products that guarantees that protective immunity will be elicited in the target population. At present, there is no animal model that can meaningfully be used to measure or predict the potency of these vaccines in humans. It is not known whether animal potency tests using parenteral administration of vaccine would be a reliable indicator of the protective effect of the same vaccine when administered orally. Additionally, the available evidence on tests using the parenteral administration of vaccine to rabbits suggests that the immunological response does not follow a dose-response relationship; in mice parenteral administration results in a large variability in antibody titres that would necessitate the use of a large number of animals. For this reason an animal potency assay has been omitted from these Guidelines. Research to identify appropriate assays that better predict protective efficacy in humans is strongly encouraged. Such assays should be able to detect sub-potent batches of vaccines.

In the light of these difficulties, it is suggested that emphasis should be placed on the characterization and quantification in vitro of the critical vaccine antigens and components. The characteristics of the various antigens and components claimed to contribute to vaccine efficacy, together with data on vaccine composition and dosage, consistency of production, and conformity with specifications, of the



vaccine used in clinical trials, will give some indication, though not definitive proof, of the ability of a vaccine lot to elicit protective immunity. These antigens and components might include LPS, TCP, which it is suggested could act to enhance the immune response rather than as an antigen in itself, and, where indicated, the B-subunit of the cholera toxin. Thus the immunological, biological and biochemical characterization of the individual components claimed to contribute to vaccine efficacy is critical for demonstrating their structural and/or functional integrity in vaccine production lots. Relevant tests should be performed before any procedure such as detoxification, chemical or heat treatment (which may modify the immunological or biological characteristics of the component), is carried out. This would apply to any component considered to be important to the performance of the vaccine, but that may not easily be tested for following inactivation. Other tests, such as that for residual activity of cholera toxin should be undertaken routinely after detoxification, chemical or heat treatment of vaccine lots, or as part of process validation.

Residual cholera toxin is a possible contaminant of inactivated whole-cell oral vaccines. Rigorous washing of the culture and inactivation using heat or formaldehyde treatment are features of the production process. However, a toxicity test to confirm freedom from toxicity will be necessary, and acceptable limits of cholera toxin activity should be set to confirm consistency of manufacture. The amount of active cholera toxin in a new production lot should not exceed that present in lots shown to be safe in clinical studies. The mouse weight-gain test currently in use to monitor the toxicity of vaccine lots is considered to be insufficiently sensitive and of questionable relevance. A more relevant and validated test should be sought. The potential use of the Y-1 adrenal cell assay for cholera toxin as a more specific test for residual toxicity should be investigated. Such a specific test could be used on a lot-to-lot basis or to validate the production process.

Should the use of vaccine involve administration in extra buffer to protect against acid conditions in the stomach (as for the vaccine containing the B-subunit) the buffer should be similar to that used in the clinical studies and compatible with the vaccine.

The need for a preservative in multidose presentations of an oral vaccine should be carefully evaluated and consideration given to the use of a non-mercury-based preservative should one be thought necessary. If no preservative is added to multidose containers a time-limit of a maximum of 6 hours should be imposed on the storage of opened containers.



3. Manufacturing recommendations

These Guidelines apply to the production and control of liquid formulations of inactivated cholera vaccine intended for oral administration. The Guidelines emphasize the importance of in-process controls for biologicals and cover the following three areas:

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

The general manufacturing recommendations contained in good manufacturing practices for pharmaceutical (35) and biological products (36) should be applied at establishments manufacturing inactivated oral cholera vaccine.

Production and control of the rDNA-derived B-subunit using a genetically modified strain of *V. cholerae* should be according to the guidelines for assuring the quality of pharmaceutical and biological products prepared by recombinant DNA technology (32) and other relevant recommendations (33, 34). The same guidelines would apply equally to the production of rCTB in any other host organism, such as *Escherichia coli*.

V. cholerae is a class 2 pathogen and represents a particular hazard to health through infection by the oral route. It should be handled under appropriate conditions for this class of organism (37). Standard operating procedures need to be developed for dealing with emergencies arising from the accidental spillage, leakage or other dissemination of cholera organisms. Personnel employed in the production and control facilities should be adequately trained. Appropriate protective measures including vaccination should be implemented. Adherence to current good manufacturing practice and appropriate biosafety measures are important to the integrity of the product, to protect workers and to protect the environment.

Details of standard operating procedures for the preparation and testing of inactivated oral cholera vaccines adopted by a manufacturer, together with evidence of appropriate validation of each production step, should be submitted for approval to the national regulatory authority. All assay procedures used for quality control of the vaccine and vaccine intermediates should also be validated (38). Proposals for modifications of the manufacturing process or control methods should be submitted for approval to the national regulatory authority before they are implemented.

The general recommendations for control laboratories contained in the guidelines for national regulatory authorities on quality assurance



for biological products (39) should be applied. A vaccine lot should be released using a batch release procedure and only if it fulfils national requirements.

3.1 **Control of starting materials**

3.1.1 ***Strains of V. cholerae***

The current vaccines consist of classical and El Tor biotypes of Inaba and Ogawa serotype and, in some cases, the O139 serotype may be included. The strains used should have the appropriate morphological, cultural, biochemical, serological and other properties appropriate to the strain. A strain of *V. cholerae* that has been genetically modified to delete cholera toxin A-subunit genes is currently used to produce the rDNA derived B-subunit when this is included in the vaccine.

3.1.2 ***Seed-bank system***

The production of *V. cholerae*, including strains containing the plasmid encoding the recombinant B-subunit should be based on a master and working seed lot system. Cultures derived from the working seed lot should have the same characteristics as the cultures of the strain from which the master seed lot was derived. If materials of animal origin are used in the medium for seed production, preservation of strain viability for freeze-drying, or for frozen storage, they should comply with the guidance given in the report of a WHO consultation on medical and other products in relation to human and animal transmissible spongiform encephalopathies (40) and should be approved by the national control authorities.

3.1.3 ***Culture media for growth of organisms***

Where possible, materials of non-animal origin should be used. If materials of animal origin are used, they should comply with the guidance given in the report of a WHO consultation on medical and other products in relation to human and animal transmissible spongiform encephalopathies (40) and should be approved by the national regulatory authorities. Human blood or reagents derived from human blood must not be used in either the culture media used for the production of seed banks or of vaccine. If human albumin is used in any part of the production process, it should meet the requirements for the collection, processing and quality control of blood, blood components and plasma derivatives (41) as well as current guidelines in relation to human transmissible encephalopathies.



3.2 Control of the manufacturing process

3.2.1 Control of production cultures

Production cultures should be shown to be consistent in respect of growth rate, pH and yield of cells or cell products. Acceptance specifications should be established.

Cultures should be checked at different stages of fermentation for purity, identity and cell density. Unsatisfactory cultures must be discarded. Where a plasmid-containing strain (see section 3.2.2) is used for the production of the recombinant B-subunit, the cultures should be checked for the presence and identity of appropriate genetic markers. Numbers of plasmid copies should be checked routinely at lot release or confirmed during process validation.

At the time of harvest and prior to detoxification, whole cell bulks should be checked for purity, identity, opacity, pH and relevant biochemical and antigenic characteristics. For assessing purity, samples of the culture should be examined by microscopy of Gram-stained smears, by inoculation of appropriate culture media or by another suitable procedure.

Following killing by heat or formaldehyde treatment, the cultures should be checked for viability, purity, opacity, identity and pH. The inactivation process may affect cell morphology or integrity, and opacity measurements may not be a reliable indicator of bacterial numbers. Assays for specific antigen content should be used to determine the concentrations of the monovalent bulks used for formulating vaccines based on killed cells only. Assays for each specific LPS should be employed.

3.2.2 Control of production of purified rDNA derived B-subunit

3.2.2.1 Strategy for cloning and expressing the gene

A full description of the host cell and expression vectors used in production should be given. This should include:

- the source, genetic characteristics and details of maintenance of the host strain or strains;
- the construction, genetics and structure of the expression vector;
- the origin and identification of the gene that is being cloned.

The cultural conditions used to promote and control the expression of the cloned gene in the host cell should be described in detail. Agents known to provoke sensitivity reactions in certain individuals, such as penicillin or other beta-lactam antibiotics, should not be used in the fermentation process.



The stability of the expression system during storage and beyond the passage level used in production should be documented and specifications set for plasmid retention during storage of seed and during production. The stability of the host-vector system should either be confirmed during process validation or checked routinely at the end of fermentation. Unstable systems should not be used. The expression system should be approved by the national regulatory authority.

3.2.2.2 Characterization of the recombinant vector

The nucleotide sequence of the gene insert and of adjacent flanking segments of the vector, together with restriction enzyme mapping and/or full sequencing of the vector containing the gene insert should be provided to the national regulatory authority.

3.2.2.3 Purification procedures

The methods used to purify the rDNA B-subunit from culture harvests should be described in detail; the capacity of each stage of the purification procedure to remove or inactivate substances other than the B-subunit should also be determined. In particular, the capacity of the purification process to assure the absence of significant quantities of any holotoxin or other *V. cholerae* toxins, such as zonula occludens toxin or accessory cholera enterotoxin, should be assessed, unless it has been demonstrated that the cloning and expression procedures eliminate all possibility of production of such factors. Limits should be established for the quantities of impurities detected in the purified B-subunit preparation and these impurities should be identified and characterized as appropriate.

3.2.3 Characterization of rDNA derived B-subunit

Rigorous characterization of the rDNA derived B-subunit product should be undertaken using a variety of analytical techniques exploiting several different properties of the molecule, including size, charge and amino acid composition. Techniques suitable for such purposes include SDS-polyacrylamide gel electrophoresis (SDS-PAGE), size-exclusion and reverse-phase chromatography. Sufficient sequence information should be obtained by direct sequencing and by peptide mapping, or another appropriate molecular technique, for example, mass spectrometry, in comparison with the natural material. The identity of the product should be confirmed by at least partial *N*-terminal and *C*-terminal amino acid sequencing. Several lots of the product should be as fully characterized as possible. Several appropriate methods should then be selected for use in routine lot release.



Data should be provided on the consistency of yield in terms of both quantity and quality of product for sequential production runs. The effects of freeze-drying should also be investigated.

The rDNA derived B-subunit should be shown to elicit antibody responses in humans, with the antibodies shown to be functional (e.g. toxin-neutralizing) in a suitable assay.

3.3 **Control of final bulk**

3.3.1 **Preparation**

For vaccine formulated from killed cells only, the final bulk is prepared by mixing suitable quantities of each monovalent bulk suspended in the appropriate buffer. For vaccines containing the rDNA B-subunit, this component is dissolved in buffer to an appropriate concentration and then mixed with the cell suspension final bulk to achieve a mixture containing each component at the required concentration. Preservative, if used, may be added either to individual monovalent bulks or at the final bulk stage.

3.3.2 **Antigen content**

The concentration of each specific antigen (i.e. total O1 or O139 LPS, TCP as appropriate) that is considered to play a part in protection should be assayed in the final bulk by a suitable immunoassay approved by the national regulatory authority. Similarly, for formulations containing the B-subunit, its concentration in the final bulk should be assayed by an approved method, for example, single radial diffusion. The final concentration of each active component should be within limits that are consistent with those of lots shown to be safe and efficacious in clinical trials.

3.3.3 **Detoxifying agents**

If formaldehyde or another detoxifying agent is used in the preparation of killed cells, its residual concentration should be determined in the final bulk by a method approved by the national regulatory authority. The final concentration should not exceed the limits established for clinical trial lots that have been shown to be safe and efficacious.

3.3.4 **Sterility**

Each final bulk should be tested for bacterial and fungal sterility in accordance with the requirements of Part A, sections 5.1 and 5.2 of the revised requirement for biological substances (41) or by a method approved by the national regulatory authority. If a preservative has



been added to the product, appropriate measures should be taken to prevent it from interfering with the test.

3.3.5 **Preservative**

If a preservative has been added, its concentration may be determined at the bulk stage by a method approved by the national regulatory authority. The preservative, its concentration and its limits should be approved by the national regulatory authority.

3.3.6 **Potency/immunogenicity**

At present there is no animal potency or immunogenicity assay that can be recommended for use as a reliable indicator of the protective efficacy of inactivated oral cholera vaccines in humans or for the detection of sub-potent batches (see section 2.6).

3.3.7 **Residual toxin activity**

Cholera toxin should be assayed by a method approved by the national regulatory authority. Alternatively, the production process should be validated to show that the quantities of clinically active cholera toxin present in the product are insignificant. The inactivation process should also be validated to assure the absence of significant quantities of holotoxin or other *V. cholerae* toxins.

3.4 **Control of final lot**

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

3.4.1 **Appearance**

The final containers should be inspected visually (manually or with automatic inspection systems). After shaking, the vaccine should form a uniform, turbid, white or brownish suspension free of aggregates and extraneous particles. Containers showing abnormalities must be discarded.

3.4.2 **Identity**

An identity test should be performed on at least one labelled container from each final lot. The test used should identify the type of vaccine formulated. For preparations formulated from killed cells alone, a serological test that detects *V. cholerae* O1 and O139 (if present) antigens will suffice. For preparations formulated from killed cells and rDNA B-subunit, the identity test must be able to detect the presence of both types of component. The procedures used should be



approved by the national regulatory authority. The antigen-content assays (see below) could also serve as an identity test.

3.4.3 **Antigen content**

The concentration of each specific antigen (i.e. total O1 or O139 LPS, TCP as appropriate), that is considered to play a part in protection, should be assayed by a suitable immunoassay approved by the national regulatory authority. Similarly, for formulations containing the B-subunit, its concentration should be assayed by an approved method, for example, single radial diffusion. The final concentration of each active component should be within limits that are consistent with those of lots shown to be safe and efficacious in clinical trials.

3.4.4 **Sterility**

Each final lot should be tested for bacterial and fungal sterility as indicated in section 3.3.4.

3.4.5 **Preservative content**

If a preservative is included, each final lot should be assayed for preservative content unless this was done on the final bulk. The assay method used and the preservative content permitted should be approved by the national regulatory authority.

3.4.6 **pH**

The pH should be tested and shown to be within the range of values found suitable for vaccine lots that have been shown to be safe and effective in clinical trials and in stability studies.

3.4.7 **General safety (innocuity)**

No such test is recommended for an oral preparation.

3.5 **Stability, storage and expiry date**

The stability of the vaccine in its final container, when maintained at the recommended temperature, should be established using real-time studies. These should be conducted on at least three consecutive final lots, derived from separate antigen-production lots.

The content of *V. cholerae* LPS and other specified antigens should remain within specified limits for the duration of the shelf-life. If the formulation contains the B-subunit, its content must also remain within specified limits for the duration of the shelf-life. Accelerated stability studies at elevated temperatures may provide additional evidence of vaccine stability, but cannot replace real-time studies.



When any changes that may affect the stability of the product are made in the production process, the stability of the vaccine produced by the new procedure should be demonstrated by additional studies.

If monovalent bulks or final bulk products are to be stored, stability studies should be performed and an appropriate shelf-life assigned on the basis of the data obtained.

3.6 Reference materials

No formally established international reference materials are currently available for the standardization of oral cholera vaccines, but their development is under consideration. Manufacturers should set aside, as reference material, a vaccine lot identical with, or demonstrated to be equivalent to, a lot shown to give acceptable performance in clinical trials. It is recommended that the reference lot should be stabilized by a validated procedure, such as freeze-drying, to maintain stability over a long period.

Other reference materials should include a stabilized preparation of the rDNA B-subunit and holotoxin.

Manufacturers and national regulatory authorities, should establish reference antisera against O1 and O139 LPS antigens and, monospecific antisera or monoclonal antibodies to Inaba, Ogawa epitopes and B-subunit.

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Acknowledgements

Acknowledgements are due to the following experts for their comments and advice on the issues in the standardization and control of oral cholera vaccines which were first discussed by a WHO Working Group at a meeting held 10–11 May 1999, in Geneva: Dr I. Feavers, National Institute for Biological Standards and Control, Potters Bar, Herts., England; Dr D. Garcia, Agence Francaise de Sécurité Sanitaire des Produits de Santé, Lyon, France; Dr J. Holmgren, Department of Medical



Microbiology and Immunology, Göteborg, Sweden; Dr Huynh Anh Hong, National Centre for Vaccines and Biologicals, Nha Trang, Viet Nam; Dr D. Kopecko, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA; Dr Chung Keel Lee, International Vaccine Institute, Seoul, Republic of Korea; Dr M.M. Levine, University of Maryland, Baltimore, MD, USA; Dr V. Oeppling, Paul Ehrlich Institute, Langen, Germany; Dr S. Rijpkema, National Institute for Biological Standards and Control, Potters Bar, Herts., England; Dr J. Stadler, Federal Office of Public Health, Berne, Switzerland; Dr A.M. Svennerholm, Department of Medical Microbiology and Immunology, Göteborg, Sweden; Dr R. Winsnes, Norwegian Medicines Control Authority, Oslo, Norway; Dr P. Askeloff, SBL Vaccin, Stockholm, Sweden; Dr U. Bjare, SBL Vaccin, Stockholm, Sweden; Dr E. Furer, Swiss Serum and Vaccine Institute, Berne, Switzerland; Dr J. Que, Swiss Serum and Vaccine Institute, Berne, Switzerland; Dr M. Schroeder, Swiss Serum and Vaccine Institute, Berne, Switzerland and Dr J-F Viret, Swiss Serum and Vaccine Institute, Berne, Switzerland. Dr N. Dellepiane, Access to Technologies; Dr E. Griffiths, Coordinator, Quality Assurance and Safety of Biologicals; Dr B. Ivanoff, Vaccine Development; Dr L. Kuppens, Communicable Diseases Surveillance and Response; Dr M. Neira, Director Communicable Diseases Prevention, Control and Eradication.

A draft of these guidelines was reviewed at a WHO informal consultation held in October 2001 at the International Vaccine Institute, Seoul, Republic of Korea. Participants included: Dr Sang-Ja Ban, Division of Bacterial Products, Korea Food and Drug Administration, Seoul, Republic of Korea; Dr Le Van Be, National Centre for Vaccines and Biologicals, Nha Trang, Viet Nam; Dr N. Carlin, SBL Vaccin, Stockholm, Sweden; Dr D. Garcia, Agence française de Sécurité Sanitaire des Produits de Santé, Lyon, France; Dr E. Griffiths, World Health Organization, Geneva, Switzerland; Dr M. Haase, Paul Ehrlich Institute, Langen, Germany; Dr Hasbullah, Bio Farma, Bandung, Indonesia; Dr Lei Dianliang, National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China; Dr L. Slamet, National Agency of Drug and Food Control, Jakarta, Indonesia; Dr Doan Thi Tam, National Centre for Vaccines and Biologicals Control, Hanoi, Viet Nam; Dr Nguyen Thu Van, Vabiotech, National Institute of Hygiene and Epidemiology, Hanoi, Viet Nam; Mr M. Welin, Medical Products Agency, Uppsala, Sweden; Dr R. Winsnes, Norwegian Medicines Control Authority, Oslo, Norway; Dr J. Clemens, International Vaccine Institute, Seoul, Republic of Korea and Dr C.K. Lee, International Vaccine Institute, Seoul, Republic of Korea.

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