World Health Organization

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FINAL

Recommendations to Assure the Quality, Safety and Efficacy of Recombinant Hepatitis B Vaccines

Proposed replacement of: TRS 786, Annex 2 and TRS 889, Annex 4.

Adopted by the 61st meeting of the WHO Expert Committee on Biological Standardization, 18 to 22 October 2010. A definitive version of this document, which will differ from this version in editorial but not scientific details, will be published in the WHO Technical Report Series.

Recommendations published by the WHO are intended to be scientific and advisory. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products. If a NRA so desires, these 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 for additional guidance intended for manufacturers and NRAs, which may benefit from those details.

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Introduction

These Recommendations are intended to provide national regulatory authorities (NRAs) and vaccine manufacturers with background and guidance on the production, quality control and evaluation of the safety and efficacy of recombinant hepatitis B vaccines for prophylactic use.

The first document outlining the requirements for the production and control of hepatitis B vaccines containing hepatitis B surface antigen (HBsAg) purified from the plasma of chronically infected individuals was adopted by the Expert Committee on Biological Standardization (ECBS) in 1980 (1) and later revised in 1987 (2).

Following the development of hepatitis B vaccines containing HBsAg produced by recombinant DNA techniques in yeast, a new set of requirements were developed following a meeting of experts in 1985 (3) and adopted by the ECBS in 1986 (4). These were revised to include vaccines produced by recombinant techniques in mammalian cells as well as yeast cells in 1988 (5).

With the development and implementation of new *in vitro* assays to determine antigen content, an amendment was published to include the use of the *in vitro* assay in the quality control of recombinant hepatitis B vaccines (6).

This present document applies to vaccines containing HBsAg only and will replace the WHO *Requirements for hepatitis B vaccine made by recombinant DNA techniques* in Technical Report

Series No. 786, Annex 2 and the corresponding amendment TRS No. 889, Annex 4 and should be read in conjunction with all other relevant WHO guidelines including those on nonclinical (7) and clinical evaluation (8*)* of vaccines.

General considerations

Hepatitis B virus has several characteristics that distinguish it from the other families of DNA viruses. It has an outer coat (more substantial than a membrane or envelope) consisting of protein, lipid, and carbohydrate and bearing a unique antigen complex, HBsAg. Its nucleic acid consists of a circular DNA genome of relative molecular mass about 2 million, part of which is double-stranded and part single-stranded, an unusual feature among viruses. Virus recovered from the plasma of a hepatitis B carrier was used to clone the HBsAg gene.

The HBsAg gene has been inserted into yeast and mammalian cells by means of appropriate expression vectors. Antigen expressed in several species of yeast, namely *Saccharomyces cerevisiae, Pichia pastoris* and *Hansenula polymorpha,* and Chinese hamster ovary (CHO) cells has been used to produce hepatitis B vaccines for over 20 years. Electron microscopy revealed that purified HBsAg obtained from transfected cultures exists as particles 15-30 nm in diameter, with the morphological characteristics of free surface antigen in plasma. Purified antigen has been shown to induce antibodies in mice and guinea-pigs and to protect chimpanzees from infection with hepatitis B virus.

All hepatitis B vaccines currently in the market require formulation with adjuvants. Preservatives are used for multidose presentations but there are some single dose presentations available without preservative. Recombinant hepatitis B vaccines are available as monovalent products or included in combination vaccines together with other antigens such as hepatitis A virus, diphtheria toxoid, tetanus toxoid, whole-cell or acellular pertussis components, *Haemophilus influenzae* type b conjugated antigen and inactivated poliomyelitis viruses.

The requirements that follow apply to the manufacture, quality control, nonclinical and clinical evaluation of hepatitis B vaccines containing HBsAg made by recombinant DNA methods. It is expected that new or significantly modified recombinant hepatitis B vaccine formulations will be characterized according to the recommendations made in Part A and B of this document and assessed in clinical studies as described in Part C.

Particular emphasis is placed on the introduction of "in-process'' controls to monitor consistency of production, in addition to the tests on the final product. Certain tests will be required on every batch of vaccine, whereas others will be required only to support licensure or significant manufacturing changes.

The vaccine lots used in clinical trials should be adequately representative of the formulation and manufacturing scale intended for marketing.

Part A. Manufacturing recommendations

A.1. Definitions

A.1.1 International name and proper name

The international name should be "Recombinant hepatitis B vaccine". The proper name should be the equivalent of the international name in the language of the country of origin. The use of the international name should be limited to vaccines that satisfy the requirements elaborated below.

A.1.2 Descriptive definition

The recombinant hepatitis B vaccine is a preparation of purified HBsAg that has been produced by recombinant DNA techniques. The antigen may be formulated with a suitable adjuvant.

A.1.3 International reference materials

International standards and reference reagents for the control of hepatitis B vaccine potency are not available. Therefore, product-specific reference preparations may be used.

> The Second International Standard (IS) for Hepatitis B Surface Antigen (non-adjuvanted HBsAg) subtype adw2, genotype A contains 33 IU per vial. This material is intended as a quantitative reference standard for HBsAg subtype adw2, genotype A and the use of this standard will give an indication of the analytical sensitivity of an assay for the detection of HBsAg. IS for HBsAg should not be used as a vaccine reference.

An international standard for hepatitis B immunoglobulin is available for use in assays designed to quantify antibody to HBsAg (anti-HBs) in human serum. Antibody responses to hepatitis B vaccines should be expressed in IU. The second International Standard for Hepatitis B Immunoglobulin (2008) was prepared from fractionated human plasma and freeze dried in ampoules. It has an assigned potency of 100 IU/ampoule. This preparation is in the custody of the NIBSC, Potters Bar, UK.

A.1.4 Terminology

Adjuvant: A vaccine adjuvant is a component that potentiates the immune response to an antigen and/or modulates it towards the desired immune responses.

Adventitious agents: Contaminating microorganisms of the cell substrate or source materials used in their cultures, that may include bacteria, fungi, mycoplasmas, and endogenous and exogenous viruses that have been unintentionally introduced.

Aqueous bulk: Purified antigen bulk before the addition of an adjuvant.

Anti-HBs: Antibodies to hepatitis B surface antigen

Cell bank: A collection of containers (e.g. ampoules, vials) containing aliquots of a suspension of cells from a single pool of cells of uniform composition, stored frozen under defined conditions (typically <–60 °C for yeast, and in liquid nitrogen for mammalian cell lines).

End of production cells: A cell suspension containing the cells harvested at the end of culture/fermentation.

Final vaccine bulk: The formulated bulk prepared from one or more batches of aqueous bulk (purified antigen) to which adjuvant has been added, present in the container from which the final containers are filled.

Final vaccine lot: A collection of sealed final containers of vaccine that is homogeneous with respect to the risk of contamination during the filling process. A final vaccine lot must therefore have been filled from a single vessel of final bulk in one working session.

Hepatitis B virus: a 42-nm double-shelled virus particle, originally known as the Dane particle, which contains the DNA genome of the virus.

HBsAg: hepatitis B surface antigen, comprising a complex of antigens associated with the virus envelope and subviral forms (22-nm spherical and tubular particles). Native HBsAg is encoded by envelope gene sequences (S plus pre-S) in the viral DNA. Recombinant DNA-derived hepatitis B vaccines may contain the S gene product or products of the S/pre-S combination.

Master cell bank (MCB): A collection of containers containing aliquots of a suspension of cells from a single pool of cells of uniform composition, stored frozen under defined conditions (typically < −60 °C for yeast, and in liquid nitrogen for mammalian cell lines). The MCB is used to derive all working cell banks for the anticipated lifetime of the vaccine production.

Production cell culture: A cell culture derived from one or more containers of the WCB used for the production of vaccines.

Single harvest: the biological material prepared from a single production run.

Working cell bank (WCB): A collection of containers containing aliquots of a suspension of cells from a single pool of cells of uniform composition, derived from the MCB, stored frozen under defined conditions (typically <–60 °C for yeast, and in liquid nitrogen for mammalian cell lines). One or more aliquots of the WCB are used for routine production of the vaccine. Multiple WCBs are made and used during the lifetime of the vaccine production.

A.2 General manufacturing recommendations

The general manufacturing requirements contained in the *WHO Guidelines on good manufacturing practices for pharmaceuticals* (9) *and biological products* (10) should apply to the establishment of manufacturing facilities manufacturing hepatitis B vaccine, with the addition of the following:

- Production areas should be decontaminated before they are used for the manufacture of hepatitis B vaccine.
- Hepatitis B vaccine should be produced by staff who have not handled animals or infectious microorganisms in the same working day. The staff should consist of persons who have been examined medically and have been found to be healthy.
- No cultures of microorganisms or eukaryotic cells other than those approved by the NRA should be introduced into or handled in the production area at any time during manufacture of the vaccine.

A.3 Control of source materials

A.3.1 Cell substrates for antigen production

The use of any cell substrate should be based on a cell bank system. Only cells that have been approved and registered with the NRA should be used to produce HBsAg protein. The NRA should be responsible for approving the cell bank. Appropriate history of the cell bank should be provided.

A.3.1.1 Yeast cells

The characteristics of the recombinant production strain (host cell in combination with the expression vector system) should be fully described and information given on the absence of adventitious agents and on gene homogeneity for the master and working cell banks. A full description of the biological characteristics of the host cell and expression vectors should be given. The physiological measures used to promote and control the expression of the cloned gene in the host cell should be described in detail. This should include genetic markers of the host cell, the construction, genetics and structure of the expression vector and the origin and identification of the gene that is being cloned.

The nucleotide sequence of the gene insert and of adjacent segments of the vector and restriction-enzyme mapping of the vector containing the gene insert should be provided as required by the NRA. Characterization of gene product (HBsAg) should be provided in support of licensure (see Part B).

Master and working cell banks should be tested for the absence of adventitious bacteria, fungi according to Part A section of the *WHO General requirements for the sterility of biological substances* (11) or by a method approved by the NRA. Cells must be maintained in a frozen state that allows recovery of viable cells without alteration of genotype. The cells should be recovered from the frozen state, if necessary in selective media such that the genotype and phenotype consistent with the unmodified host and unmodified recombinant DNA vector are maintained and clearly identifiable. Cell banks should be identified and fully characterized by means of appropriate tests.

Where appropriate, plasmid retention in the cell bank should be monitored at regular intervals. Data that demonstrate the stability of the expression system during storage of the recombinant WCB up to or beyond the passage level used for production should be provided and approved by the NRA. Any instability of the expression system occurring in the seed culture, or after a production-scale run (end of production cells), should be documented.

A.3.1.2 Mammalian cells

If mammalian cells are used, the cell substrate and cell banks should conform with the *WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks* (12); other relevant guidelines provide additional information (13). Cell substrates and cell banks should be approved by the NRA.

The maximum population doublings (or number of passages) allowable between the MCB, the WCB and the production cells should be approved by the NRA. The MCB is made in sufficient quantities and stored in a secure environment and is used as the source material to make manufacturers WCB. In normal practice a MCB is expanded by serial subculture up to a population

doubling (or passage number, as appropriate) selected by the manufacturer and approved by the NRA, at which point the cells are combined to give a single pool, distributed into containers (e.g. ampoules, vials) and preserved cryogenically to form the WCB.

Tests on the master and working cell banks are performed in accordance with *Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks* (12) and *WHO guidelines for assuring the quality of pharmaceutical and biological products prepared by recombinant DNA technology for recombinant cells* (14) and should be approved by the NRA.

A.3.2 Cell culture medium

If serum is used for the propagation of mammalian cells, it should be tested to demonstrate freedom from bacteria, fungi and mycoplasmas, according to WHO requirements (11). Suitable tests for detecting viruses in bovine serum are given in *WHO Recommendations for the evaluation of animal cell cultures as substrates for manufacture of biological medicinal products and for the characterization of cell banks* (12).

Validated molecular tests for bovine viruses may replace the cell culture tests of bovine sera. As an additional monitor of quality, sera may be examined for freedom from phage and endotoxin. Gamma-irradiation may be used to inactivate potential contaminant viruses.

The acceptability of the source(s) of any components of bovine, porcine, sheep or goat origin used should be approved by the NRA. These components should comply with current *WHO guidelines in relation to animal transmissible spongiform encephalopathies* (15).

If trypsin is used for preparing cell cultures, it should be tested and found free of bacteria, fungi, mycoplasmas and infectious viruses, especially bovine or porcine parvoviruses, as appropriate. The methods used to ensure this should be approved by the NRA. The trypsin should be gamma irradiated.

Human serum should not be used. However, human serum albumin may be used only if it complies with the *WHO Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives* (16). In addition, human albumin and materials of animal origin should comply with current *WHO guidelines regarding to animal transmissible spongiform encephalopathies* (15).

Penicillin and other beta-lactams should not be used at any stage of the manufacture because of their nature as highly sensitizing substances.

Other antibiotics may be used in the manufacture provided that the quantity present in the final product is acceptable to the NRA.

Non-toxic pH indicators may be added, e.g. phenol red at a concentration of 0.002%. Only substances that have been approved by the NRA may be added.

A.4 Fermentation

A.4.1 Production of cell cultures

Only cell cultures derived from the WCB should be used for production. All processing of cells should be done in an area in which no cells or organisms are handled other than those directly required for the process. The medium used should comply with the requirements given in section A.3.2.

A.4.1.1 Control of HBsAg production up to single harvest in yeast expression system
Microbial purity in each fermentation vessel should be monitored at the end of the production run by methods approved by the NRA.

Any agent added to fermentor or bioreactor in purpose to feed cells or to induce /increase cell density should be approved by the NRA.

A.4.1.2 Control of HBsAg production up to single harvest in mammalian cells
Production of cell cultures should be carried out under conditions agreed with the NRA. These conditions should include details of the culture system used, the cell doubling time, the number of subcultures or the duration of the period of subcultivation permitted, and the incubation temperature. Cell culture vessels should be monitored for potential microbial contamination during and at the end of the production runs by methods approved by the NRA.

A.5 Single harvests

A.5.1 Storage and intermediate hold times

During the purification process, all intermediates should be maintained under conditions shown by the manufacturer to retain the desired biological activity. Hold times should be approved by the NRA.

A.5.2 Tests on single harvest

A.5.2.1 Sampling
Samples required for the testing of single harvests should be taken immediately on harvesting prior to further processing. For mammalian cell cultures, if the tests for adventitious agents are not performed immediately, the samples taken for these tests should be kept at a temperature of -60ºC or below and subjected to no more than one freeze-thaw cycle.

A.5.2.2 Test for bacteria, fungi and mycoplasma contamination
Bacterial and fungal contamination in the cell culture vessels should be monitored during and at the end of the production runs by methods approved by the NRA. If mammalian cells are used in production each single harvest or single harvests pool should be shown to be free from bacteria, fungi and mycoplasma contamination by appropriate tests (11).

> Nucleic Acid Amplification Techniques (NAT) alone or in combination with cell culture, with an appropriate detection method, might be used as an alternative to one or both of the compendial mycoplasma detection methods after suitable validation and agreement from NRA as described in *WHO Recommendations for the evaluation of animal cell cultures as substrates for manufacture of biological medicinal products and for the characterization of cell banks* (12).

A.5.3 Consistency of yield

Data on the consistency of yield between runs and during individual production runs should be provided, and the NRA should approve the criteria for an acceptable production run.

A.5.4 Plasmid retention

A sample of cells that are representative of each harvest must be tested to confirm that the recombinant phenotype has been retained. The method used should be approved by the NRA.

When the production method has shown to yield consistently harvests that comply with the requirement for plasmid retention, the test may be omitted on the harvest after approval by the NRA. However, the stability of the vector should be monitored on a regular basis on the working cell bank. Particular attention should be paid to plasmid copy number during conditions of storage and recovery.

Where the plasmid is integrated in the host cell genome, presence of the integrated HBsAg gene insert should be confirmed.

A.5.5 Tests for adventitious agents if mammalian cells are used in production

Single harvest or single harvests pool should be tested for adventitious agents in cell cultures in accordance with *Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks* (12).

Additional testing may be performed using nucleic acid amplification methods.

A.6 Control of aqueous bulk (purified antigen bulk)

The purification procedure can be applied to a single harvest, to a part of a single harvest, or to a pool of single harvests. The maximum number of single harvests that may be pooled should be approved by the NRA. Adequate purification may require several purification steps based on different principles. This will minimize the possibility of co-purification of extraneous cellular materials. The methods used for the purification of the HBsAg should be appropriately validated and approved by the NRA. Any agent added during the purification process, should be documented and its removal adequately validated and tested for as appropriate (see A.6.1.8).

The monovalent purified antigen bulk can be stored under conditions shown by the manufacturer to retain the desired biological activity. Intermediate hold times should be approved by the NRA. Additional tests on intermediates during purification process may be used to monitor the consistency/ yields.

A.6.1 Tests on the aqueous bulk (purified antigen)

The aqueous bulk should be tested according to the tests listed below. All quality control release tests and specifications for aqueous bulk, should be validated and approved by the NRA.

A.6.1.1 Purity

The degree of purity of each aqueous bulk should be assessed by suitable methods. Examples of suitable methods of analysing the proportion of potential contaminating proteins in the total protein of the preparation are polyacrylamide gel electrophoresis (PAGE), optionally followed by densitometric analysis, or high-performance liquid chromatography (HPLC). Other methods include automated electrophoresis systems. The aqueous bulk should be not less than 95% pure.

A.6.1.2 Protein content
The protein content should be determined using a suitable method such as the micro-Kjeldahl method, the Lowry technique or another suitable method.

A.6.1.3 HBsAg content
The HBsAg content of the aqueous bulk should be determined by an appropriate immunochemical method. An appropriate reference material should be included in these assays so that consistency of production is monitored. This reference material could be a representative bulk of known HBsAg and protein content or a highly purified preparation of HBsAg of known HBsAg and protein content with acceptable stability profile, it should be stored in single use aliquots. It is important to note that the reference materials based on adjuvanted product is not suitable for use in assays of non-adjuvanted intermediate bulks of HBsAg.

The ratio of HBsAg content to protein content should be determined. The antigen/protein ratio should be within the limits approved by the NRA.

A.6.1.4 Identity
The test for antigen content will generally serve as confirmation of the identity of the protein in the bulk. Alternatively, immunoblots using HBsAg specific antibodies in the assessment of purity could also serve to confirm the molecular identity of the product. Such tests should be approved by the NRA.

A.6.1.5 Lipids
The lipid content of each aqueous bulk should be determined by an appropriate method. The methods used and the permitted concentrations of lipid should be approved by the NRA. This test may be omitted for routine lot release upon demonstration of consistency of the purification process to the satisfaction of the NRA.

A.6.1.6 Carbohydrates
The carbohydrate content of each aqueous bulk should be determined by an appropriate method. The methods used and the permitted concentrations of carbohydrates should be approved by the NRA. This test may be omitted for routine lot release upon demonstration of consistency of the purification process to the satisfaction of the NRA.

A.6.1.7 Sterility tests for bacteria and fungi
Each aqueous bulk should be tested for freedom from bacteria and fungi according to WHO Requirements (11), or by a method approved by the NRA.

A.6.1.8 Tests for agents used during purification or other phases of manufacture
The aqueous bulk should be tested for the presence of any potentially hazardous agents used during manufacture. The method used and the concentration limits should be approved by the NRA. This test may be omitted for routine lot release upon demonstration that the purification process consistently eliminates the agent from the purified bulks.

Where a monoclonal antibody is used in vaccine preparation, for example for immunological affinity chromatography to purify HBsAg, the antibody used should be characterized and its purity determined. The product should be tested for residual antibody. The methods used and the permitted concentrations of antibody should be approved by the NRA.

Several NRAs have drafted guidelines for the control of monoclonal antibody preparations used for the manufacture of biological products for human use.

If the HBsAg has been treated with formaldehyde and/or other agents, then the material should be tested for the presence of free formaldehyde and/or the other agents. The method used and the permitted concentration should be approved by the NRA.

A.6.1.9 Tests for residuals derived from the antigen expression system

The amount of residuals derived from the antigen expression system (e.g. DNA or host cell proteins) should be determined in each monovalent antigen purified bulk by sensitive methods. In the case of yeast-derived products, these tests may be omitted for routine lot release upon demonstration that the purification process consistently eliminates the residual components from the monovalent bulks to the satisfaction of the NRA.

In general, for mammalian cells acceptable limits of residual cellular DNA for specific products should be set in consultation with the NRA/NCL. The characteristics of the cell substrate, the intended use of the vaccine, and most importantly the effect of the manufacturing process on the size, quantity and biological activity of the residual cellular DNA fragments should be considered as outlined in the *WHO Recommendations on animal cells as in vitro substrates for the production of biologicals* (12).

One licensed vaccine produced in mammalian cells contains less than 100 pg DNA per dose.

For products produced in yeast, residual cellular DNA is considered an impurity.

One licensed vaccine produced in yeasts contains less than 10 pg cellular DNA per dose.

A.6.1.10 Bacterial endotoxins

Each final aqueous bulk should be tested for bacterial endotoxins. Endotoxin content should be consistent with levels found to be acceptable in vaccine lots used in clinical trials and the limits should be approved by the NRA.

A.6.1.11 Albumin content (when mammalian cells are used)

If animal serum is used in mammalian cell cultures, or at any stage in the manufacturing process, testing should be performed to assess the residual serum in the purified bulk. The concentration of animal serum in the vaccine should be not more than 50ng per human dose of vaccine.

A.6.2 Adjuvant bulk

A.6.2.1 Tests on adjuvants

Hepatitis B vaccines may contain the immunostimulant monophosphoryl lipid A (MPL) adsorbed onto aluminium compounds (e.g. aluminium phosphate), when the adsorption of the MPL is performed prior to the final formulation step, the degree of adsorption of MPL should be determined using a suitable method (e.g. gas chromatography). The test for completeness of adsorption of the MPL may be omitted upon demonstration of process consistency and/or if performed on the final vaccine lot.

A.6.2.2 Sterility tests

Each adjuvant bulk should be tested for bacterial and fungal sterility according to the WHO Requirements (11).

A.6.2.3 pH
The pH of each adjuvant bulk should be tested and shown to be within the range of values approved by the NRA.

A.7 Final vaccine bulk

The final vaccine bulk may consist of one or more purified aqueous bulks. Only aqueous bulks that have satisfied the requirements outlined in previous sections should be formulated into the final vaccine bulk. The antigen concentration in the final formulation should be sufficient to ensure the dose which is consistent with that shown to be safe and effective in human clinical trials. Formulation is generally based on protein content, but HBsAg content may be used.

> It should be noted that formulation based on HBsAg may be affected by changes in the kits and/or reagents used to determine antigen content. This should be considered and included in validation/bridging studies when kit changes occur (see Appendix 1).

The operations necessary for preparing the final vaccine bulk should be conducted in such a manner as to avoid contamination of the product. In preparing the final vaccine bulk, any substances such as diluents, stabilizers or adjuvants that are added to the product should have been shown to the satisfaction of the NRA not to impair the safety and efficacy of the vaccine in the concentration used. Until the bulk is filled into containers, the final vaccine bulk suspension should be stored under conditions shown by the manufacturer to retain the desired biological activity.

A.7.1 Tests on the final vaccine bulk

All tests and specifications for the final vaccine bulk, unless otherwise specified, should be approved by the NRA.

The HBsAg may be formulated with other vaccine antigens into a combined vaccine (e.g. HAV, DTwP-HBsAg, DTaP-HBsAg etc). Specific issues related to the formulation and quality control of final vaccine bulk of combination vaccines will be addressed in a separate document.

A.7.1.1 Sterility tests
Each final vaccine bulk should be tested for bacterial and fungal sterility according to the WHO Requirements (11).

A.7.1.2 Adjuvants
Each final vaccine bulk should be assayed for the content of adjuvants. This test may be omitted if performed on the final vaccine lot. Where aluminium compounds are used, the content of aluminium should not be greater than 1.25 mg per single human dose

Should an immunostimulant (e.g. monophosphoryl lipid A) be present, each final vaccine lot should be assayed for the immunostimulant content. This test may be omitted if performed on the final vaccine bulk.

A.7.1.3 Degree of adsorption

The degree of adsorption of HBsAg antigen present in the final vaccine bulk should be assessed. This test may be omitted upon demonstration of the process consistency to the satisfaction of the NRA or if performed on the final vaccine lot.

A.7.1.4 Preservative content

The final vaccine bulk should be assayed for preservative content, if added. This test may be omitted if performed on the final vaccine lot.

A.7.1.5 Potency

If an *in vivo* potency test (i.e. immunogenicity) is used, this test may be performed on the final vaccine bulk. The method for detection of antibodies to HBsAg and the analysis of data should be approved by the NRA. The vaccine potency should be compared with that of a reference preparation and the NRA should determine limits of potency and approve the reference preparation used. If an *in vitro* potency test is performed, it should be performed on every lot of final vaccine lot. Methodological considerations regarding potency assays are outlined in Appendix 1.

A.8 Filling and containers

The requirements concerning filling and containers given in the *WHO Guidelines on good manufacturing practices for biological products* (10) should apply to vaccine filled in the final form.

Care should be taken to ensure that the materials of which the container and, if applicable, transference devices and closure are made do not adversely affect the quality of vaccine. The manufacturers should provide the NRA with adequate data to prove the stability of the product under appropriate conditions of storage and shipping.

A.9 Control tests on final vaccine lot

Samples should be taken from each final vaccine lot to be tested and fulfill requirements of this section. All the tests and specifications including methods used and the permissible limits for the different parameters listed under this section, unless otherwise specified, should be approved by the NRA.

A.9.1 Inspection of containers

Each container of each final vaccine lot should be inspected visually or mechanically and those showing abnormalities should be discarded.

A.9.2 Appearance

Visual inspection of the appearance of the vaccine should be described with respect to the form and color.

A.9.3 Identity

The vaccine should be identified as HBsAg by appropriate methods. The assay use for potency may serve as the identity test. For manufacturers producing both vaccines with or without MPL, the final vaccine lot should also be identified by the checking of the presence or not of MPL.

A.9.4 Sterility tests

Each final vaccine lot should be tested for bacterial and fungal sterility according to the WHO requirements (11), or by acceptable methods.

A.9.5 General safety (innocuity) test

Each final lot should be tested for the absence of abnormal toxicity in mice and guinea pigs using a general safety (innocuity) test approved by the NRA and should pass the test. This test may be omitted for routine lot release once consistency of production has been established to the satisfaction of the NRA.

A.9.6 pH and osmolality

The pH and osmolality of a pool of final containers should be tested. The test for osmolality may be omitted once consistency of production is demonstrated to the satisfaction of the NRA.

A.9.7 Preservatives

Each final vaccine lot should be tested for preservative content, if added. This test may be omitted if performed on the final vaccine bulk.

A.9.8 Pyrogen / endotoxin content

The vaccine in the final container should be tested for either pyrogenic activity by intravenous injection into rabbits or by a Limulus amoebocyte lysate test. Endotoxin content or pyrogenic activity should be consistent with levels found to be acceptable in vaccine lots used in clinical trials and approved by the NRA. The test is conducted until consistency of production is demonstrated to the satisfaction of the NRA.

A.9.9 Assay for adjuvant

Each final vaccine lot should be assayed for adjuvant content. Where aluminium compounds are used, the amount of aluminium should not be greater than 1.25 mg per human dose.

Should an immunostimulant (e.g. monophospohoryl lipid A) be present, each final vaccine lot should be assayed for the immunostimulant content. This test may be omitted if performed on the final vaccine bulk.

A.9.10 Degree of adsorption

The degree of adsorption of the antigen and, where applicable, MPL to the aluminium compounds (aluminium hydroxide or hydrated aluminium phosphate) in each final vaccine lot should be assessed if not performed on the MPL bulk or final bulk. This test may be omitted for routine lot release upon demonstration of the product consistency to the satisfaction of the NRA.

A.9.11 Potency tests

An appropriate quantitative test for potency by an *in vivo* or *in vitro* method should be performed on samples representative of the final vaccine lot. The method and the analysis of data should be approved by the NRA. If an *in vivo* potency test is performed on the final bulk, the test on final container may be omitted. Methodological considerations regarding potency assays are outlined in Appendix 1.

Because of the diversity in the reactivity of vaccines produced by different manufacturing techniques and differences in the adjuvants used for the formulation, it is unlikely that an International Standard will be suitable for the standardization of assays of vaccines from all

manufacturers. Manufacturers should therefore establish a product specific reference preparation which is traceable to a lot of vaccine shown to be efficacious in clinical trials. The NRA should approve the reference preparation used and agree with the potency limits applied. The performance of this reference vaccine should be monitored by trend analysis using relevant test parameters and it should be replaced when necessary.

> The stability of the product-specific reference vaccine may also be monitored by routine assay against a stable HBsAg preparation. The inclusion of such a preparation in assays at regular intervals would monitor stability of the product-specific reference and monitor test/kit performance. However, this preparation would not be intended for use in establishing potency values.

A.10 Records

The requirements given in the *WHO Guidelines on good manufacturing practices for biological products* (10) should apply.

A.11 Retained samples

The requirements given in the *WHO Guidelines on good manufacturing practices for biological products* (10) should apply.

A.12 Labelling

The requirements given in the *WHO Guidelines on good manufacturing practices for biological products* (10) should apply, with the addition of the following information.

The label on the carton, the container or the leaflet accompanying the container should state:

- the nature of the cells used to produce the antigen;
- the nature of any preservative and amount of adjuvant, present in the vaccine;
- the volume of one recommended human dose, the immunization schedules, and the recommended routes of administration (this information should be given for newborn babies, children, adults, and immunosuppressed individuals, and should be the same for a given vaccine for all regions of the world);
- the amount of HBsAg protein contained in one recommended human dose.

A.13 Distribution and shipping

The requirements given in the *WHO Guidelines on good manufacturing practices for biological products* (10) should apply.

In addition, the conditions of shipping should be such as to ensure that the adjuvanted vaccine does not freeze. Temperature indicators should be packaged with each vaccine shipment to indicate whether freezing occurs, further guidance is provided in the *WHO Model guidance for the storage and transport of time and temperature-sensitive pharmaceutical products* (17) If freezing has occurred, the vaccine should not be used.

A.14 Stability testing, storage and expiry date

A.14.1 Stability testing

Adequate stability studies form an essential part of vaccine development. Current guidance on evaluation of vaccine stability is provided in the *WHO guidelines on stability evaluation of vaccines*

(18). Stability testing should be performed at different stages of production, namely on single harvests, aqueous bulk, final vaccine bulk and final vaccine lot to validate their claimed shelf life. The stability of the vaccine in its final form and at the recommended storage temperatures should be demonstrated to the satisfaction of the NRA on final containers from at least three lots of final product. The formulation of HBsAg antigens and adjuvant must be stable throughout its shelf-life. Acceptable limits for stability should be agreed with national authorities.

A.14.2 Storage conditions

The final container vaccine should be kept at 2-8°C. If other storage conditions are used, they should be fully validated by appropriate stability studies and approved by the NRA. The vaccine should have been shown to maintain its potency for a period equal to that between the date of release and the expiry date. During storage, liquid adsorbed vaccines should not be frozen.

A.14.3 Expiry date

The expiry date should be fixed upon the approval of the NRA, and should take account of the experimental data on stability of the vaccine.

> Some manufacturers base the expiry date on the date of formulation of the final bulk. Others base the expiry date on the date of the last satisfactory potency test, i.e., the date on which the animals were inoculated with the vaccine in an in vivo test, or from the date of the *in vitro* potency test performed on the final container.

Part B. Nonclinical evaluation

Nonclinical evaluation of hepatitis B vaccines should be based on *WHO guidelines on nonclinical evaluation of vaccines* (7). The following specific issues should be considered in the context of the development of new recombinant hepatitis B vaccines. Prior to clinical testing of any new or modified hepatitis B vaccine in humans there should be extensive product characterization, immunogenicity, safety testing and proof of concept studies in animals.

B.1 Strategy for cloning and expressing the gene product

A full description of the biological characteristics of the host cell and expression vectors used in production should be given. This should include details of: (a) the construction, genetics, and structure of the expression vector; and (b) the origin and identification of the gene that is being cloned (c) potential retrovirus-like particles in and genetic markers for mammalian cell based expression systems. The physiological measures used to promote and control the expression of the cloned gene in the host cell should be described in detail.

Data that demonstrate the stability of the expression system during storage of the WCB and beyond the passage level used for production should be provided. Any instability of the expression system occurring in the seed culture or after a production-scale run, for example involving rearrangements, deletions, or insertions of nucleotides, must be documented. The NRA should approve the system used.

B.2 Characterization of purified HBsAg for new vaccines

Rigorous identification and characterization of recombinant DNA-derived vaccines is required as part of the Marketing Authorization application. The ways in which these products differ chemically, structurally, biologically, or immunologically from the naturally occurring antigen must be fully documented. Such differences could arise during processing at the genetic or post-translational level, or during purification.

B.2.1 Characterization of gene products

The molecular size of the expressed protein and its composition should be established by techniques such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining under reducing and non-reducing conditions or N-terminal sequencing by Edman degradation method.

The identity of the protein should be established by peptide mapping and/or terminal amino acid sequence analysis. Following SDS PAGE, the protein bands should be identified in immunoblots using specific antibodies, e.g monoclonal antibodies, to confirm the presence of the expected products of the hepatitis B virus surface antigen gene. The primary structure of the protein should be further characterized by suitable methods such as partial amino-acid sequence analysis and by peptide mapping. Mass spectrometry may be used to confirm the average molecular mass and the presence of the protein in the preparation.

Since it is known that conformational epitopes are essential for efficacy, it is essential that the morphological characteristics of the HBsAg particles and degree of aggregation should be determined. In addition, the protein, lipid, nucleic acid and carbohydrate content should be characterized and measured. Particle characterization may be done by atomic force and transmission electron microscopy, dynamic light scattering.

The gene products from lots produced during vaccine development should be shown to possess antigenic determinants characteristic of HBsAg by means of tests with monoclonal antibodies or polyclonal antibodies of defined specificity directed against epitopes of HBsAg known to be relevant to the protective efficacy of the vaccine.

B.3 Animal models

There is no adequate, relevant animal model for hepatitis B infection other than the chimpanzee. The efficacy of recombinant HBsAg vaccines has been demonstrated in challenge studies in this model by several manufacturers and therefore, such studies are no longer required for new vaccines based on the HBsAg protein.

The immunogenicity of new HBsAg vaccines and existing vaccines for which there has been a significant manufacturing change should be evaluated in non-clinical (e.g. in rabbits, guinea pigs, mice and possibly non-human primates). The non-clinical program should take into account the following:

- The titres of anti- HBsAg should be directly compared between the candidate vaccine and at least one licensed comparator, preferably one for which there has been extensive clinical use and generation of data supporting its effectiveness in routine use. If testing is performed due to a significant change in manufacturing then the candidate vaccine should be compared with the corresponding licensed vaccine.
- If it is proposed that a new candidate vaccine contains an adjuvant its inclusion should be supported by adequate immunogenicity data that, in addition to measuring humoral antibody, may include an assessment of the cellular immune response. Studies should compare the adjuvanted candidate vaccine with the HBsAg alone and/or with HBsAg administered in conjunction with a well established adjuvant (such as an aluminium salt).
- The potential need to evaluate other antibody responses and/or cellular immune responses, to characterize the immune response more in depth.

B.4 Nonclinical safety studies

As no effects other than on the immune system are expected with hepatitis B vaccines based on the absence of specific toxins, safety pharmacological studies are not required.

B.5 Toxicology studies

Toxicology studies should be undertaken in accordance with the *WHO guidelines for nonclinical evaluation of vaccines* (7). Such studies should reflect the intended clinical use of the vaccine in babies and young children.

If the vaccine is formulated with a novel adjuvant, nonclinical toxicology studies should be conducted as appropriate for the adjuvant concerned. Repeated dose toxicity study may be used to compare the safety profile of the novel adjuvant with the safety profile of an established vaccine formulation taking into account existing guidelines.

If a novel cell substrate (i.e. a substrate that had not been previously licensed or used in humans) is used for the production of a hepatitis B vaccine, safety aspects, such as potential immune responses elicited by residual host cell proteins, should be investigated in a suitable animal model.

Variations to the route of administration or to the vaccine formulation require evaluation of immunogenicity of the hepatitis B vaccine together with adequate animal safety/toxicological studies taking into account existing guidelines (7, 8).

Part C. Clinical evaluation

This section addresses the clinical evaluation of new hepatitis B vaccines and of existing vaccines for which a significant change to the manufacturing process is proposed. The content and extent of the clinical program will vary according to the each possible scenario and it is recommended that vaccine-specific requirements for clinical studies are discussed with the appropriate NRAs.

C.1 Consideration for clinical studies

In general, clinical trials should adhere to the principles described in the *WHO guidelines on good clinical practice* (19). General principles described in the *WHO guideline on regulatory expectations for clinical evaluation of vaccines* (8) apply to hepatitis B vaccines and should be followed. Some of the issues that are specific to the clinical development program for hepatitis B vaccines are discussed in the following sections and should be read in conjunction with the general guidance mentioned above. These recommendations should be viewed in the light of further data on the safety and immunogenicity of hepatitis B vaccines and any relevant data on similar type of vaccines that may become available in the near future.

With more than 20 years of clinical use of recombinant HBsAg vaccines in addition to the experience gained with use of the early plasma-derived vaccines there is sufficient experience to support the approval of new candidate vaccines (including those that may contain a novel adjuvant) and major changes to manufacturing of existing vaccines based on clinical studies that assess safety and immunogenicity in seronegative subjects.

Infant immunization is the most effective strategy to prevent hepatitis B infection and this approach has been incorporated in the immunization programs in over 177 countries (20). However, catch-up strategies, adult vaccination and vaccination of special populations are common and studies that address different types of usage are considered in the following sections.

C.2 Assessment of immune responses

The assessment of the immune response should be based on measurement of the anti-HBsAg antibody concentration in serum using a validated and standardized assay. An International Standard for hepatitis B immunoglobulin (anti-HBs) has been established and should be used in the assays to determine antibody responses in immunogenicity clinical studies.

The use of validated quantitative assays is critical for the evaluation of immune responses, testing should be conducted by laboratories that implement quality assurance of testing procedures. Assay validation data should be reviewed and approved by the NRA. Assay validation involves demonstration that the performance characteristics of the method meet the requirements for its intended use (21). The protocols for assay validation studies should identify and justify the choice of the parameters to be studied along with the pre-defined acceptance criteria. The validation report should include a detailed description of the processing and storage of samples, reference standards and reagents and generation of the calibration curve.

See section C3.3.1 regarding analysis of the immunogenicity data.

C.3 Clinical studies

New hepatitis B vaccines should be compared directly with at least one licensed vaccine for which there is considerable clinical experience in routine use. The selection of the comparator should be discussed with NRAs and should be selected taking into account the total antigen content of the candidate vaccine and the study population. Where possible it is preferred that a candidate vaccine (whether or not monovalent) should be compared with a monovalent licensed vaccine. However, this may not be feasible in studies in infants due to the need to deliver numerous antigens concomitantly using multivalent HBsAg-containing vaccines. More information regarding the clinical evaluation of hepatitis B containing combination vaccines is discussed in a separate document.

In studies performed to support major changes to manufacture of a licensed vaccine the candidate vaccine should be compared with the existing vaccine (i.e. manufactured according to the licensed process).

New HBsAg vaccines should usually be tested initially in healthy adult volunteers. In this regard it is important to take into account that the immune response to HBsAg is age dependent and decreases in magnitude with increasing age of adults. After the age of 30-40 years antibody levels indicative of protection are achieved after a primary vaccinations series in less than 90% of subjects and in only 65–75% of vaccinees aged more than 60 years (20). Therefore studies my restrict enrolment of adults by age or may employ age stratification.

Once immunogenicity is demonstrated in adults further studies may be conducted in younger target populations according to the intended use (e.g. newborns, infants etc). As one of the most important uses of the hepatitis B vaccine is to prevent infection in infants born to carrier mothers, unless a true efficacy trial is performed in this group, clinical studies may potentially include a measure of the kinetics of antibody acquisition in comparison to vaccines that have an established efficacy in this situation rather than a simple comparison of seropositive rates or GMTs following the last dose of the primary series. As the birth dose is never in combination with other vaccines the comparison could be made following the first dose with is particularly important in prevention of maternal transmission of hepatitis B virus.

The amount of recombinant HBsAg administered per dose requires justification based on non-clinical studies and, if necessary, formal dose-ranging studies in adults. However, these may not always be necessary if the non-clinical data and mode of manufacturing are considered to support the dose appropriate at least for the initial clinical studies. In this regard, it should be noted that it is usual that lower doses of HBsAg are administered to subjects aged less than approximately 12-15 years compared to adults (e.g. half the adult dose is commonly used in infants and children up to a selected age). Therefore any new recombinant HBsAg-containing vaccine should be evaluated for dose-related immunogenicity according to age.

The primary series schedule(s) that are examined will likely follow those already approved for other recombinant HBsAg-containing vaccines according to specific target populations. However, if a candidate vaccine is proposed to contain an antigen dose and/or an adjuvant that is considerable different to licensed vaccines then a formal evaluation of schedule may be necessary according to specific populations (e.g. by age and/or other host factors).

Enrolment should usually be limited to subjects who have no history of hepatitis B vaccination or disease. It is preferred that studies should screen participants prior to enrolment for the presence of HBsAg or anti-HBc antibody. If results of these tests are available only after the first vaccination is given, any subjects with a positive result should be eliminated from the primary analysis of immunogenicity.

Studies in neonates may include those born to HBsAg positive and/or negative mothers depending on the study objectives and may stratify neonates accordingly. Studies in infants may be limited to those born to HBsAg negative mothers (with no birth dose of hepatitis B-specific immunoglobulin) with or without a prior birth dose of vaccine according to the objectives and may employ stratification accordingly.

One other scenario that requires a specific clinical development program concerns vaccines that contain high doses of antigen and/or an adjuvant that are intended for populations known to respond poorly or not at all to standard primary courses. In such cases the studies need to be tailored according to the properties of the vaccine (e.g. to justify the dose of antigen and the adjuvant content in specific target populations). It may be appropriate to enroll subjects who have shown no detectable antibody response to a complete primary series in order to evaluate the benefit of a higher dose and/or adjuvanted vaccine. See also section C.3.3.

C.3.1 Immunogenicity endpoints

C.3.1.1 Primary analysis

The protective efficacy of hepatitis B vaccines has been shown to be directly related to the induction of anti-HBs antibody. An anti-HBs antibody concentration of ≥10 mIU is generally considered to be a marker of protection against hepatitis B (20) and studies should determine the percentage of seronegative individuals who achieve this antibody level at approximately 4 weeks after completion of a primary series.

For the comparison between the candidate and reference vaccine the protocol and analysis plan should pre-define a well-justified non-inferiority margin (22) for the comparison of the percentage of subjects with •10mIU/ml anti-HBs.

C.3.1.2 Secondary analysis

- In all studies it is appropriate that protocols should plan at least for a secondary analysis of percentages that achieve •100 mIU/ml anti-HBs and to present reverse cumulative distributions (23). In addition, it is recommended that secondary analyses should compare the Geometric mean titres (GMTs) between vaccines and studies may plan for a formal comparison of GMT ratios.

It is expected that at least some of the clinical studies, including those in the primary target population(s), should be conducted with different lots manufactured using the same process as for the vaccine intended for the market. However, as indicated in the *WHO Guidelines on clinical evaluation of vaccines: regulatory expectations* (8) a formal clinical trial to demonstrate lot-to-lot consistency is not normally required unless there is a particular concern with respect to the manufacturing consistency of the product and the potential impact that this may have on the efficacy and safety of the vaccine. If performed, lot-to-lot consistency should be designed in accordance with the principles outlined in section B.3.3.3 of the *WHO Guidelines on clinical evaluation of vaccines: regulatory expectations* (8).

C.3.2 Persistence of anti-HBs antibody

Long-term observations of efficacy in various age groups have indicated that the loss of detectable anti-HBs antibody in subjects who had responded satisfactorily to a primary series does not necessarily indicate lack of protection due to the effect of persistence of immune memory. That is, a number of long-term follow-up studies from various epidemiological settings have confirmed that HBsAg-carrier status or clinical HBV-disease rarely occurs in subjects who responded to a primary series even when the anti-HBsAg concentrations decline to ≤10 mIU/ml over time (20). However, the persistence of ≥10mIU/ml anti-HBsAg should be evaluated for any new hepatitis B vaccine. The total duration of serological follow-up should be discussed and planned in advance in conjunction with NRAs.

Since it is current opinion that additional doses of HBsAg-containing vaccine after completion of the initial vaccination series may not be needed (the occasional exception being routine use of an additional dose in toddlers who received a course with certain vaccines during infancy) it may be difficult to justify administration of a further dose solely to assess immune memory. However, it is of interest and potential benefit to administer an additional dose of a HBsAg-containing vaccine to subjects who have failed to maintain ≥10mIU/ml anti-HBsAg. In these instances the titres obtained after the additional dose should be compared with the titres observed shortly after the last dose of the initial vaccination series. Careful attention should be paid to the documentation of safety associated with additional doses.

C.3.3 Studies in special populations

There are several host factors that have been described in association with lack of response or poor responses to hepatitis B vaccines (e.g. male gender, age over 40 years, smoking, obesity and several underlying diseases that include advanced HIV infection, chronic renal failure, chronic hepatic disease and diabetes). Clinical studies may be conducted to specifically assess the safety and immunogenicity of new recombinant HBsAg-containing vaccines in populations at risk of not responding adequately to vaccination. The design of such studies should take into consideration the potential need for a higher antigen dose and/or adjuvant.

C.3.4 Concomitant administration with other vaccines

The potential for immune interference between hepatitis B vaccines and other routine vaccines that might need to be given at the same time for convenience should be investigated in order to make recommendations regarding concomitant use.

C.4 Post-marketing studies

The manufacturer has a responsibility to assess safety and effectiveness following initial approval of a new hepatitis B vaccine, in particular when formulated with other components as part of a combination vaccine. NRAs should ensure that adequate pharmacovigilance plans are in place regarding these activities at the time of first licensure. There should be specific commitments made by manufacturers to provide data to NRAs on a regular basis and in accordance with national regulations. The data that are collected and submitted to the responsible NRAs should be assessed rapidly so that action can be taken if there are implications for the marketing authorization.

The collection of reliable and comprehensive data on effectiveness involves close co-operation between manufacturers and public health authorities. Therefore, pre- and post-approval discussions

between vaccine manufacturers responsible for placing the product on the market and national and international public health bodies are essential for ensuring that reliable effectiveness data are collected in the post-marketing period in selected countries/regions.

Part D. Recommendations for national regulatory authorities

D.1 General

The general recommendations for control laboratories given in Guidelines for national authorities on quality assurance for biological products (24) should apply. These guidelines specify that no new biological substance should be released until consistency of manufacturing and quality as demonstrated by a consistent release of batches has been established. The detailed production and control procedures and any significant changes in them should be discussed with and approved by the NRA. For control purposes, the NRA should obtain the working reference from manufacturers

D.2 Release and certification

A vaccine lot should be released only if it fulfils the national requirements and/or Part A of the present Recommendations. A protocol based on the model given in Appendix 2, signed by the responsible official of the manufacturing establishment, should be prepared and submitted to the NRA in support of a request for release of vaccine for use.

A statement signed by the appropriate official of the NRA should be provided if requested by a manufacturing establishment and should certify whether or not the lot of vaccine in question meets all national requirements, as well as Part A of these Recommendations. The certificate should also state the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, the date of the last satisfactory HBsAg potency test as well as assigned expiry date on the basis of shelf life should be stated. A copy of the official national release document should be attached. The certificate should be based on the model given in Appendix 3. The purpose of the certificate is to facilitate the exchange of recombinant hepatitis B vaccines between countries.

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Appendix 1

Methodological considerations. Potency tests for recombinant hepatitis B vaccines.

Background

Recombinant hepatitis B vaccines were licensed in the mid-1980s. WHO published requirements for hepatitis B vaccines produced by recombinant DNA techniques in yeast and mammalian cells in 1989 (1) and these were revised to include the use of *in vitro* potency tests in 1997 (2).

Because of the diversity in the reactivity of vaccines containing HBsAg produced by different manufacturing processes and to which different adjuvants or immunostimulants have been added, recombinant hepatitis B vaccines produced by different manufacturers must be considered as different products. In view of such differences, the establishment of an International Standard that would be suitable for the standardization of assays of vaccines from all manufacturers is unlikely to succeed. Furthermore, the stability of such a vaccine is unlikely to be adequate for long term use and in the calibration of secondary standards. Manufacturers therefore establish a product specific reference preparation which is traceable to a lot of vaccine shown to be efficacious in clinical trials. This vaccine will serve as a working standard and be included in all potency tests. The NRA approves the reference preparation used and the potency limits applied. The performance of this reference vaccine should be monitored by trend analysis using control charts of relevant test parameters (e.g. ED_{50} for in-vivo assays,) and it should be replaced when necessary.

Potency tests

Potency tests should reflect the activity of the vaccine and should be able to distinguish vaccines of low potency which may be of reduced immunogenicity in humans. At the time when the WHO requirements for recombinant hepatitis B vaccines were published it was considered that assays that determine the HBsAg content of adjuvanted vaccines would be difficult to standardize. Therefore, it was proposed that immunogenicity in mice should form the basis for determining vaccine potency by comparing the antibody response induced by the test and the vaccine reference preparation and that the specification for potency should be approved by the NRA.

Several manufacturers have since developed and validated *in vitro* potency tests which are suitable to monitor product consistency of their individual vaccines. As a result of the implementation of the *in vitro* test, the mouse potency test is no longer being performed by these manufacturers on every final lot. Since the vaccines in question were well established and had been used in millions of individuals, an amendment to the Requirements was published in 1997 (2) to permit the use of a validated *in vitro* test to determine vaccine potency as one parameter to monitor consistency of production with specifications approved by the NRA.

The *in vivo* assay should be used to establish consistency of production of a new hepatitis B vaccine and in vaccine stability studies. In addition, the *in vivo* potency test should be used to characterize the vaccine after significant changes in the manufacturing process.

In vivo potency tests

A suitable quantitative potency test in mice has been developed (3, 4). Briefly, groups of 10-20 mice, five weeks of age or weight range (17-22 g),, are immunized intraperitoneally with a series of at least three dilutions of the reference and test vaccine using a suitable diluent. Some manufacturers

use a diluent which contains the same concentration of alum as the vaccine. The strain of mice used for this test must give a suitable dose-response curve with the reference and test vaccine. The concentrations of vaccine tested should be selected to permit the calculation of 50% seroconversion to antibodies against HBsAg. The ED_{50} for both test and reference vaccine should lie within the doses administered. Terminal bleeds are taken after 28 or 42 days or when an adequate antibody response has developed. Individual sera are assayed for antibodies to HBsAg

Points which should be considered in establishing such an assay are:

- Strain and sex of mice used must give a suitable dose response to the reference and test vaccine.
- Number of mice per dilution required to meet the validity criteria of the test.
- Nature and composition of the diluent used to prepare the dilutions of the test vaccine (e.g. containing the adjuvant at the same concentration as used in the vaccine).
- Number of dilutions and appropriate selection of doses to be tested.
- The concentrations of vaccine tested should be selected to permit the calculation of the dilution giving 50% seroconversion (i.e. ED_{50}).
- Assay used to determine the concentration of antibodies to HBsAg in sera (e.g. specificity of monoclonal antibody used).
- Calculation of the cut-off value (threshold). The value is either calculated from the responses of control group of mice immunized with diluent (e.g. mean OD of negative control + 2 standard deviations) or using a threshold expressed in mIU/ml as an arbitrary value eg 10 mIU/ml which is the level indicative of seroprotection in humans.,. It is important that the choice of cut-off generates an optimal dose response with the specific dilution range.
- Statistical approach used to analyse the results (e.g. probit).
- Interpretation of results - ED_{50}/relative potency
- The establishment of an in-house mouse anti-HBsAg reference serum or panel of high, medium and low titre sera could be used to monitor kit performance and assist in the evaluation of new kits or comparison of results from different laboratories (e.g. manufacturer and NCL)
- The 95% confidence limits of the potency estimates for each test vaccine should fall in the range 33 - 300%.

***In vitro* potency tests**

Several manufacturers have validated *in vitro* potency tests based on the determination of HBsAg in dilutions of the vaccine using a commercial detection kit or another method which quantifies the HBsAg antigen content present in the vaccine. Two manufacturers have described a method based on an inhibition approach in which dilutions of the vaccine are incubated with a fixed amount of polyclonal anti-HBsAg antibodies where detection of the unbound antibody is directly related to the amount of HBsAg in the vaccine (5, 6, 7). Whichever type of assay is used, the validation studies should show that the assay is suitable to verify consistency of production.

In vitro potency tests should be able to distinguish vaccine of low potency, which may include vaccines of low immunogenicity in humans (e.g. lots tested during dose finding clinical studies) and vaccine samples with artificially reduced potency obtained following incubation for 7 days or 15 days at 60°C or by incubation overnight at 37°C with 100 ppm of hydrogen peroxide (6, 7). Although vaccines which have been frozen are known to be of low immunogenicity in man due to an effect on the adjuvant, such vaccines may not necessarily give low potencies in *in vitro* assays.

When a manufacturer introduces a change in the test method, this must be fully validated according to the ICH guidelines and, preferably, the test run in parallel with the previous assay. If this is not possible, vaccine lots assessed using the old method may be used so that any potential variation are detected and managed appropriately.

Important parameters to determine during validation include the assessment of commercial lots and reference vaccine concurrently at optimal dilutions to produce dose-response curves suitable for quantitative analysis by an appropriate statistical method. The statistical validity of the assay should be assessed, the potency of the test relative to the reference vaccine should be estimated and the assay's precision (i.e. confidence interval) should be calculated. The 95% confidence limits of the potency estimates for each test vaccine should fall in the range 80 - 125%. Acceptance criteria for the *in vitro* assay should be established based on the assay of a suitable number of consecutive final lots.

The vaccine formulation may influence the parameters used in the assay, therefore, it is recommended that optimization of the assay is carried out when a new formulation is tested (e.g. when the level of preservative is modified). Each manufacturer should set a specification for the *in vitro* test which ensures that vaccines that pass this test would also pass the mouse immunogenicity test.

Several factors must be considered when validating an assay, namely

- The specific reagents and/or the type of commercial kit used, where the type of HBsAg used may differ from the vaccine HBsAg (e.g. manufacturing process, expression system etc).
- The adjuvant used in the vaccine and the possible need for a pre-treatment step (e.g. with detergent).
- The reference preparation used (e.g. monovalent HBsAg, combination vaccine etc).
- Nature of the diluent used to prepare the dilutions of the test and reference vaccine.
- Statistical approach use to analyse the results.
- Establishment of test specification based on data from assays on a series of typical production batches of vaccines which pass the mouse immunogenicity test.

The validation of an *in vitro* potency test should be based on ICH principles (ICH Topic Q 2 CPMP/ICH/381/95) and include:

- Specificity
- Precision (including repeatability, intermediate precision and reproducibility)
- Linearity
- Range (limits of quantification)
- Robustness should be documented during assay development

***In vitro* assay for antigen quantification of aqueous bulk (non-adjuvanted antigen).**

The same *in vitro* assay used to determine vaccine potency is generally used to determine HBsAg content of the aqueous bulk. Therefore, it is important to minimize the impact of changes in commercial kits and to use appropriate reference preparations. This reference material could be a representative bulk of known HBsAg protein content or a highly purified preparation of HBsAg of known protein content stored in single use aliquots. It is important to note that the reference materials based on adjuvanted product is not suitable for use in assays of non-adjuvanted

intermediate bulks of HBsAg. Although formulation of final vaccine bulk is generally based on protein content, some manufacturers have chosen to use HBsAg content for this in-process step and the standardization and monitoring of this assay is therefore critical.

Establishment of product-specific reference

The vaccine potency (*in vivo* and *in vitro*) should be assessed against a product-specific reference preparation. The first (primary) reference preparation should be established using a vaccine lot found to be effective and safe in clinical trials or alternatively a vaccine lot that is traceable to vaccine lot of proven effectiveness and safety. Points to consider when establishing a product-specific reference preparation include:

- Source
- Quantity (availability)
- Full characterisation
- Evaluation in the mouse potency test
- Evaluation in an *in vitro* potency test
- Stability studies (accelerated degradation and real time stability)
- Establishment of control charts to monitor reference performance.

Replacement of product-specific reference

Standards should be routinely monitored and they should be replaced before they begin to show loss of activity. The shelf-life of standards may be longer than the shelf life of vaccine for routine use if data to demonstrate stability of an individual vaccine for this period are available. The shelf-life should be established under the defined storage conditions and maintenance of sterility. A replacement working standard should be a typical batch of vaccine preferably of similar potency to the previous standard.

Points to consider in the establishment and replacement of a reference vaccine:

- Documentation of the procedure for replacing standards.
- Information of product/reference stability and establishment of shelf-life.
- Procedure to monitor loss of potency (e.g. trending of relevant values such as changes in dose response curves, changes of values compared to an internal reference preparation such as a non-adjuvanted stable HBsAg etc).
- Definition of acceptable limits of trended values (e.g. mean initial potency minus 3 standard deviations).
- Review of batch record of new reference vaccine to ensure it complies with the specifications in the marketing authorisation.
- Calibration of new reference vaccine against current reference using both *in vivo* and *in vitro* tests.

Issues relating to potency tests on the hepatitis B component of combination vaccines

Optimization of *in vitro* assays should be undertaken when used with combination vaccines containing HBsAg as there is evidence that some vaccine components may interfere in such tests (7). If an *in vitro* assay is not suitable for a particular combination, an *in vivo* assay should be used. This should be performed at the level of the final bulk.

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If a monovalent hepatitis B vaccine is used as a reference in *in vivo* potency assays of combination vaccines, consideration must be given to the adjuvant effect of whole cell pertussis and whether the release specification applied to monovalent vaccines is applicable.

References

1. WHO Requirements for hepatitis B vaccines made by recombinant DNA techniques. WHO Technical Report Series No. 786, Annex 2.
2. WHO Requirements for hepatitis B vaccines made by recombinant DNA techniques (amendment). WHO Technical Report Series No. 889, Annex 4.
3. Ferguson M, Seagroatt V, Schild GC. A collaborative study to establish the International Reference Reagent for hepatitis B vaccine containing plasma-derived hepatitis B surface antigen. *Journal of Biological Standardization,* 1989, 17:151-160.
4. Ferguson M, Heath A, Minor PD. Report of a collaborative study for assessing the potency of hepatitis B vaccines. *Biologicals*. 1990, 18:345-350.
5. Chovel, ML; Fernandez de Castro, A. Comparison between in vitro potency tests for Cuban Hepatitis B vaccine: contribution to the standardization process. *Biologicals,* 2004, 32:171-176.
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Appendix 2

Summary protocol for manufacturing and control of hepatitis B vaccines.

The following protocol is intended for guidance, and indicates the information that should be provided as a minimum by the manufacturer to the NRA.
Information and tests may be added or deleted if necessary , to be in line with the marketing authorization approved by the NRA.

It is thus 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 license and with the relevant WHO recommendations of 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 accompanies 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 of the country in which the vaccine was produced/released stating that the product meets national requirements as well as Part A recommendations of this document published by WHO.

1. Summary information on the final vaccine lot

International name: _____

Trade name: _____

Batch number(s): _____

Finished product (final vaccine lot) _____

Final vaccine bulk: _____

Type of container: _____

Total number of containers in this batch: _____

Number of doses per container: _____

Composition (antigen concentration) / volume of single human dose: _____

_____

Date of last potency test by the manufacturer _____

Date of expiry: _____

Storage temperature: _____

Product license (marketing authorization) number: _____

Marketing authorization issued by _____

Name and address of manufacturer: _____

Name and address of product license holder if different: _____

2. Production information

Batch number(s) of aqueous bulk(s): _____

Site(s) of manufacture of aqueous bulk(s): _____

Date of manufacture of aqueous bulk: _____

Site of manufacture of finished product (final vaccine lot) _____

Date of manufacture of finished product (final vaccine lot):: _____

A genealogy of the lot numbers of all vaccine components used in the formulation of the final product will be informative.

The following sections are intended for the reporting of the results of the tests performed during the production of the vaccine

3. Starting materials

The information requested below is to be presented on each submission. Full details on Master and working seed-lots and cell banks upon first submission only and whenever a change has been introduced.

Cell banks

Source of HBsAg (expression system) _____

Master cell bank (MCB) lot number & preparation date: _____

Population doubling level (PDL) of MCB _____

Date of approval of protocols indicating compliance with the requirements of the relevant monographs and with the marketing authorisation: _____

Manufacturer's working cell bank (MWCB) lot number & preparation date: _____

Population doubling level (PDL) of MWCB _____

Date of approval of protocols indicating compliance with the requirements of the relevant monographs and with the marketing authorisation: _____

Production cell lot number: _____

Storage condition: _____

<u>Identification of cell substrate</u>

Method: _____

Specification: _____

Date of test : _____

Result: _____

_____

Nature and concentration of antibiotics or selecting agent (s) used in production cell culture maintenance medium:

_____

Identification and source of starting materials used in preparing production cells including excipients and preservatives (particularly any materials of human or animal origin e.g. albumin; serum): _____

4. Fermentation

Mammalian cells

Provide information on cells corresponding to each single harvest.

Ratio or proportion of control to production cell cultures: _____

Volume of control cells: _____

Period of observation of cultures: _____

Percentage rejected for non-specific reasons: _____

Result: _____

Haemadsorbing viruses

Type(s) of RBC: _____

Storage time and temperature of RBC: _____

Incubation time and temperature of RBC: _____

% cultures tested: _____

Date of start of test : _____

Date of end of test : _____

Result: _____

Tests on supernatant fluids for other adventitious agents (if relevant)

Date of sampling from production cell cultures: _____

Type of simian cells: _____

Quantity of sample inoculated: _____

Incubation temperature: _____

Date of start of test : _____
Date of end of test : _____
% of viable culture at the end _____
Result: _____

Type of human cells: _____
Quantity of sample inoculated: _____
Incubation temperature: _____
Date of start test: _____
Date of end of test: _____
% of viable culture at the end: _____
Result: _____

Type(s) of other diploid cells: _____
Quantity of sample inoculated: _____
Incubation temperature: _____
Date of start of test: _____
Date of end of test : _____
% of viable culture at the end: _____
Result: _____

Mammalian and yeast cells

Bacteria and fungi _____
Method _____
Media and temperature of incubation: _____
Volume inoculated: _____
Date of inoculation : _____
Date of end of observation : _____
Result: _____

Mycoplasmas (for mammalian cells) _____
Method: _____
Media: _____
Volume inoculated: _____
Date of start of test : _____
Date of end of test : _____
Result: _____

5. Single harvests (or pools)

Batch number(s): _____
Date of inoculation: _____

Date of harvesting: _____

Volume(s) of fermentation paste, storage temperature, storage time and approved storage period: _____

Culture purity or sterility for bacteria and fungi

- Method: _____
- Media and temperature of incubation: _____
- Volume inoculated: _____
- Date of start of test : _____
- Date of end of test : _____
- Result: _____

Plasmid retention

- Method: _____
- Specification: _____
- Date of test: _____
- Result: _____

In addition, the following tests if mammalian cells are used

Adventitious agents

- Method: _____
- Specification: _____
- Date of test: _____
- Result: _____

Mycoplasmas

- Method: _____
- Media: _____
- Volume inoculated: _____
- Date of start of test : _____
- Date of end of test : _____
- Result: _____

Mycobacterium spp. (if applicable)

- Method: _____
- Media and temperature of incubation: _____
- Volume inoculated: _____
- Date of start of test : _____
- Date of end of test : _____

Result: _____

Reverse transcriptase assay

Method: _____
Specification: _____
Date of test: _____
Result: _____

6. Control of aqueous bulk (purified antigen)

Batch number(s) of purified bulk: _____
Date(s) of purification(s): _____
Volume(s), storage temperature, storage time and approved storage period: _____

Purity (add PAGE photographs if applicable)

Method: _____
Specification: _____
Date of test: _____
Result: _____

Protein content

Method: _____
Specification: _____
Date of test: _____
Result: _____

HBsAg Antigen content / Identity

Method: _____
Specification: _____
Date of test: _____
Result: _____

Ratio of HBsAg antigen : protein content

Specification: _____
Result: _____

Bacteria and fungi

Method: _____
Media and temperature of incubation: _____
Volume inoculated: _____
Date of start of test : _____

Date of end of test :
Result:

Lipid
Method:
Specification:
Date of test:
Result:

Carbohydrate
Method:
Specification:
Date of test:
Result:

Potential hazards e.g. residual chemical(s) (if relevant)
Method:
Specification:
Date of test:
Result:

Residual DNA (if applicable)
Method:
Specification:
Date of test:
Result:

Bacterial endotoxins
Method:
Specification:
Date of test:
Result:

Albumin content
(if mammalian cells and animal serum are used for production)
Method:
Specification:
Date of test:
Result:

Tests on adjuvant bulk

Adjuvant or mineral vehicle concentration (if applicable)

Method:
Specification:
Date of test:
Result:

Degree of adsorption (if applicable)

Method:
Specification:
Date of test:
Result:

Bacteria and fungi

Method:
Media and temperature of incubation:
Volume inoculated:
Date of start of test :
Date of end of test :
Result:

pH

Method:
Specification:
Date of test:
Result:

7. Final vaccine bulk

Batch number(s) of aqueous bulk: _____

Formulation date: _____

Batch number(s) of all components used during adjuvant formulation: _____

Volume, storage temperature, storage time and approved storage period: _____

Bacteria and fungi

Method: _____
Media and temperature of incubation: _____
Volume inoculated: _____
Date of start of test : _____
Date of end of test : _____

Result: _____

Identity

Method: _____
Specification: _____
Date of test: _____
Result: _____

Adjuvant or mineral vehicle concentration (if applicable)

Method: _____
Specification: _____
Date of test: _____
Result: _____

Degree of adsorption (if applicable)

Method: _____
Specification: _____
Date of test: _____
Result: _____

pH

Method: _____
Specification: _____
Date of test: _____
Result: _____

Preservative

Method: _____
Specification: _____
Date of test: _____
Result: _____

Potency test: *in vivo* assay (if applicable)

Species, strain, sex and weight specifications: _____
Dates of vaccination, bleeding: _____
Date of assay: _____
Batch number of reference vaccine and assigned potency: _____
Vaccine doses (dilutions) and number of animals responding at each dose: _____
ED_{50} of reference and test vaccine: _____

Potency of test vaccine vs. reference vaccine with 95% fiducial limits of mean: _____

Validity criteria: _____

8. Final vaccine lot

Batch number: _____
Date of filling: _____
Type of container: _____
Filling volume: _____
Number of containers after inspection: _____

Appearance
- Method: _____
- Specification: _____
- Date of test: _____
- Result: _____

Identity
- Method: _____
- Specification: _____
- Date of test: _____
- Result: _____

Bacteria and fungi
- Method _____
- Media and temperature of incubation: _____
- Volume inoculated: _____
- Date of start of test : _____
- Date of end of test : _____
- Result: _____

pH
- Method: _____
- Specification: _____
- Date of test: _____
- Result: _____

Osmolality
- Method: _____
- Specification: _____

Date of test: _____

Result: _____

Preservatives (if applicable)

Method: _____

Specification: _____

Date of test: _____

Result: _____

Pyrogenic substances

Method: _____

Specification: _____

Date of test: _____

Result: _____

Adjuvant content

Method: _____

Specification: _____

Date of test: _____

Result: _____

Protein content (or calculated value)

Method: _____

Specification: _____

Date of test: _____

Result: _____

Degree of adsorption (if applicable)

Method: _____

Specification: _____

Date of test: _____

Result: _____

Potency:

In vitro assay

Method: _____

Batch number of reference vaccine and assigned potency: _____

Specification: _____

Date of assay: _____

Result: _____

If an *in vivo* assay is used (may be performed at final bulk stage)

Species, strain, sex and weight specifications: _____

No. of mice tested _____

Dates of vaccination, bleeding: _____

Date of assay: _____

Batch number of reference vaccine and assigned potency: _____

Vaccine doses (dilutions) and number of animals responding at each dose: _____

ED50 of reference and test vaccine _____

Potency of test vaccine vs. reference vaccine with 95% fiducial limits of mean: _____

Validity criteria: _____

Date of start of period of validity: _____

General safety (unless deletion authorised)

Test in mice

No. of mice tested : _____

Volume and route of injection: _____

Date of injection: _____

Date of end of observation: _____

Specification: _____

Result: _____

Test in guinea-pigs

No. of guinea-pigs tested:

Volume and route of injection;

Date of injection:

Date of end of observation:

Specification :

Result:

Freezing point (if applicable)

Method: _____

Specification: _____

Date of test: _____

Result: _____

Appendix 3

Model certificate for the release of recombinant hepatitis B vaccine by a national regulatory authority

This certificate is to be provided by the national regulatory authority of the country where the vaccines have been manufactured, upon request by the manufacturer

Certificate No_____

LOT RELEASE CERTIFICATE

The following lot(s) of recombinant hepatitis B vaccine produced by _____[1] in _____[2], whose numbers appear on the labels of the final containers, meet all national requirements[3] and Part A[4] of the *WHO recommendations to assure the quality, safety and efficacy of recombinant hepatitis B vaccines* (_____)[5], and comply with *Good Manufacturing Practices for Pharmaceutical Products: Main Principles*[6] and *Good Manufacturing Practices for Biological Products*[7].

As a minimum, this certificate is based on examination of the summary protocol of manufacturing and control.

Final Lot No.	No. of released human doses in this final vaccine lot	Expiry date
_____	_____	_____

The Director of the National Regulatory Authority (or Authority as appropriate):

Name (Typed) _____

Signature _____

Date _____

[1] Name of manufacturer
[2] Country of origin
[3] If any national requirements are not met, specify which one(s) and indicate why release of the lot(s) has nevertheless been authorized by the national regulatory authority
[4] With the exception of provisions on distribution and shipping, which the national regulatory authority may not be in a position to assess.
[5] WHO Technical Report Series, No. ___, YYYY, Annex __.
[6] WHO Technical Report Series, No. 908, 2003, Annex 4.
[7] WHO Technical Report Series, No. 822, 1992, Annex 1.

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