

Annex 1

Recommendations^a for the production and quality control of smallpox vaccine, revised 2003

Recommendations published by WHO are intended to be scientific and advisory. Each of the following sections constitutes guidance for national regulatory authorities and for the manufacturers of biological products. If a national regulatory authority so desires, these Recommendations may be adopted as definitive national requirements, or modifications may be justified and made by a national regulatory authority. It is recommended that modifications to these Recommendations be made only on condition that the 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 national regulatory authorities, which may benefit from those details.

In these Recommendations, Part A describes the general provisions for the production and quality control of smallpox vaccine and is applicable to production of vaccine in all permissible substrates, including the use of a cell bank. Part B describes national control requirements. The terms “national regulatory authority” and “national control laboratory”, as used in these Recommendations, always refer to the country in which the vaccine is manufactured.

^a Previously called Requirements for Smallpox vaccine.

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Introduction

The Recommendations (formerly Requirements) for Production and Control of Smallpox Vaccines were last revised in 1965 (1). Since that time an intensified global eradication programme implemented from 1967 to 1980, and led by WHO, has resulted in the global eradication of smallpox (2). This was achieved by the globally coordinated use in national immunization programmes of effective vaccines that met the quality specifications in the 1965 Requirements. The last naturally occurring case of smallpox was reported in 1977. In addition to the availability of effective vaccines, an efficient infrastructure was established worldwide embracing the production, supply and administration of smallpox vaccine. Good surveillance, diagnosis of disease, training and public health information were additional important elements in successfully combating smallpox.

After human-to-human transmission of smallpox had been interrupted worldwide the Global Commission that certified eradication was of the opinion that the likelihood of reintroduction of smallpox from laboratories or natural or animal reservoirs was negligible. Nevertheless it recommended that it would be prudent for WHO and national health authorities to be prepared for unforeseen circum-

stances. One measure was to ensure that adequate reserves of potent vaccine remained available (3). Accordingly a global stockpile of vaccine was established, as were national stockpiles. In addition seed lots of vaccinia virus suitable for the preparation of smallpox vaccine were maintained in a designated WHO collaborating centre. However in March, 1986, the Committee on Orthopoxvirus Infections concluded that the maintenance of a global reserve by WHO was no longer indicated (4), and interest in maintaining stocks rapidly declined. A survey conducted by WHO in 2001 found that only small amounts of stockpiled smallpox vaccines still existed. These stocks are distributed quite unevenly around the world and are accessible to only a very selected part of the global population. Additional production would be needed to meet any major demand on vaccine supply such as might follow an intentional release of smallpox vaccine.

Global resumption of smallpox vaccine production would benefit from modern concepts of production and control, and modern regulatory expectations should be adhered to in the licensing process. These general principles should apply to new vaccine manufactured in embryonated eggs, or in primary or continuous cell lines, or animal skins. In addition, contemporary international reference materials to determine the potency of new vaccines and their immunogenicity in vaccinated individuals would be beneficial. The WHO Secretariat has followed these principles in producing this reformulated document and has included changes to bring the document into line with other WHO Recommendations published since the last revision.

General considerations

Since the cessation of routine smallpox vaccination after the successful eradication of the pathogen, population susceptibility has increased with each unvaccinated birth cohort. After the terrorist events of 11 September 2001 in the USA, and the subsequent anthrax-related incidents in the United States, heightened concern has been expressed by Member States about potential intentional release of microorganisms. Even though the risk of the deliberate use of smallpox against civilian populations is considered to be low, there is consensus regarding the need to:

- develop a coordinated response effort;
- include sufficient numbers of doses in a strategic reserve of vaccines;
- enhance the public health infrastructure; and
- improve disaster mitigation capacity.

Since the last revision of the WHO requirements for smallpox vaccines, the principles governing regulation of medicinal products, in particular biological medicinal products including vaccines, have evolved significantly in response to increased knowledge in this field and to advanced technologies. New vaccine production should take into account these developments, and all new vaccines, including those intended for strategic reserves, should be subject to the current national licensing processes for biological medicines. It is recognized however that the use of vaccines stockpiled from the eradication programmes or of new vaccines still undergoing evaluation may be justified, if supported by an appropriate risk–benefit analysis, in cases of national emergency.

Licensing of a new vaccine usually requires the demonstration of its efficacy against the natural infection in a clinical trial. This is not possible in the case of new smallpox vaccines because the natural infection has been eradicated. One approach that has been taken is to develop a new vaccine that is phenotypically similar to a vaccine known to be successful in the eradication initiative, but other approaches are also possible. Immunological correlates of protection are not defined for vaccinia virus. However pock formation in humans after smallpox vaccination is a marker of vaccine effectiveness. The formation of papules, vesicles and pustules with an appropriate appearance within a predefined time frame may be used as a marker to support the acceptability of a new smallpox vaccine. Other parameters such as levels of neutralizing antibodies (NA) or haemagglutination inhibiting (HI) anti-vaccinia virus antibodies can presently be considered only as supportive information. Further research is needed in this area. Challenge studies in a relevant animal model (e.g. mouse/vaccinia virus and monkey/monkeypox virus) may provide additional evidence on the protective efficacy of new smallpox vaccines.

Many general and specific aspects of the production and control of (live) viral vaccines, such as the origin, quality and certification of starting materials, cell substrate issues, specific pathogen free status of eggs, viral safety, method and process validation, testing procedures and principles of good manufacturing practice are not adequately covered by the 1965 WHO Guidelines. All of these issues have been addressed in this revision.

The 1965 requirements for smallpox vaccines focused on production on animal skin, in embryonated eggs and in chick embryo fibroblast cells (CEFs). Production in other cell lines was not covered. There is considerable interest in the production of new smallpox vaccines in

either Vero cells, human diploid cells or primary rabbit kidney cells, and other cells such as quail cells have been used in the past. A new section is therefore included that covers the production and control of cell substrate produced vaccine. Cell culture vaccines were not used in areas endemic for smallpox during the eradication campaigns and their efficacy against smallpox has not been demonstrated. Therefore an important parameter to establish is that the cell substrate does not have a negative effect on the safety and/or efficacy of the vaccine virus. Such effects are not predictable and considerable efforts are thus needed to show that a new cell culture-derived vaccine has similar preclinical and clinical properties to a comparator vaccine with a known safety and efficacy profile.

Adventitious agent testing for viruses in the vaccine virus seeds and product intermediates is complicated because complete neutralization of vaccinia virus is difficult to achieve. Testing for viral adventitious agents in eggs, animals and tissue culture might give ambiguous results. Although these tests remain the gold standard, supplementary testing to detect specific viral adventitious agents using validated polymerase chain reaction (PCR)-methodology or immunochemical methods is envisaged. The scope of the test programme depends on many parameters such as the nature and origin of the virus seed. For smallpox vaccines produced on animal skin, special attention should be given to the health status of the animals. Testing for viral adventitious agents in animal skin vaccine should depend on the animal species, the origin and on epizootiological considerations in the source country of the animals. Reference has been made to the guidelines on transmissible spongiform encephalopathy (TSE) and these should be adhered to. The bioburden of new vaccines produced in animal skin can be reduced by state-of-the art animal husbandry in dedicated facilities. A revised (stricter) specification for bioburden in the final product is introduced in these guidelines. However, since the production process on animal skin may be very difficult to validate, consistent sterility of the finished product may be difficult to achieve. The use of non-sterile final product may be justified because smallpox vaccine is administered in a very low volume by scarification rather than by intramuscular or intravenous inoculation, and specified pathogens are excluded from the vaccine. In addition the history of use of the vaccine produced on animal skin defined its safety profile and unambiguously demonstrated its efficacy.

An animal model to predict the neurovirulence of vaccinia virus has been introduced. It had previously been shown that vaccinia viruses could be classified according to their high, medium and low neuropathogenic potential in humans (5). Although the histological pattern

that is seen in the mouse brain following intracerebral inoculation of different vaccinia strains is different to the histological changes found in the human brain following encephalitis after vaccination, the mouse model reflects the neuropathogenic potential of vaccinia strains in humans. The mouse model is introduced for phenotypic characterizations of both the seed material and of each lot of bulk suspension produced until sufficient validation data are available to reduce the frequency of testing.

Future research is anticipated to focus on highly attenuated vaccinia virus strains, or on other approaches such as inactivated vaccines, subunit vaccines or DNA vaccines, to facilitate the safe and effective immunization of vulnerable sectors of certain populations (such as the immunocompromised, the elderly, pregnant women and children with eczema). If such strains or approaches do not induce pock formation, then alternative markers of efficacy will be needed. Vaccinia strains that do not induce pocks are not covered in these guidelines.

The terms “national regulatory authority” and “national control laboratory”, as used in these recommendations, always refer to the country in which the vaccine is manufactured.

Part A. Manufacturing recommendations

A.1 Definitions

A.1.1 *International name and proper name*

The international name shall be “*Vaccinum variolae*”. The proper name shall be the equivalent name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the recommendations formulated below.

A.1.2 *Descriptive definition*

Vaccinum variolae is a preparation of live vaccinia virus grown in the membranes of the chick embryo or in in vitro cultures of suitable tissues or in the skin of living animals. The preparation should satisfy all the recommendations formulated below.

A.1.3 *International standard and reference preparations*

To allow for standardization of potency assays an International Reference Preparation of smallpox vaccine was established in 1962 (6, 7). This standard was derived from the Lister strain of virus and was

produced on the flanks of sheep before lyophilization and storage at -20°C . Initially held at the Statens Seruminstitut in Copenhagen, Denmark, the Reference Preparation was passed to the National Institute for Biological Standards and Control (NIBSC) in England in 1997. This Reference Preparation is available from NIBSC for calibration and establishment of in-house potency reference materials.

An international collaborative study of two new candidate International Reference Preparations is in progress. One of these candidate preparations will later be selected to replace the dwindling stocks of the current International Reference Preparation. Updated information may be found at www.who.int/biologicals.

An International Standard for Anti-Smallpox Serum was established in 1966 (8). Initially held at the Statens Seruminstitut in Copenhagen, Denmark, the Reference Preparation was passed to the National Institute for Biological Standards and Control (NIBSC) in England in 1997. Subsequently this material was found to be contaminated with hepatitis B virus and was destroyed.

A WHO informal consultation in 2002 recommended that a replacement preparation be obtained and studied for suitability in an international collaborative study. Such a standard would be used for the assay of variola and vaccinia antibodies and to calibrate vaccinia immunoglobulin preparations. National standards that are calibrated in International Units are known to exist and may serve as interim calibrants until a new international standard is prepared and characterized.

A.1.4 **Terminology**

Cell seed: A quantity of cells of human or animal origin stored frozen at -100°C or below in aliquots of uniform composition, one or more of which may be used for the production of a manufacturer's working cell bank.

Manufacturer's working cell bank (MWCB): A quantity of cells of uniform composition derived from one or more ampoules of the cell seed, which may be used for the production cell culture.

In normal practice, a cell bank is expanded by serial subculture up to a passage number (or population doubling, as appropriate) selected by the manufacturer, at which point the cells are combined to give a single pool and preserved cryogenically to form the MWCB. One or more of the ampoules from such a pool may be used for the production cell culture.

Production cell culture: A cell culture derived from one or more ampoules of the MWCB, or primary tissue, used for the production of a single harvest.

Adventitious agents: Contaminating microorganisms including bacteria, fungi, mycoplasmas, and endogenous and exogenous viruses.

Original seed: A batch of vaccine, or a seed preparation, with proven effectiveness and safety in the eradication initiative, from which the master seed is derived.

Virus master seed lot: A quantity of virus, physically homogeneous, derived from an original seed processed at one time and passaged for a number of times that does not exceed the maximum approved by the national regulatory authority.

Virus working seed lot: A quantity of virus, physically homogeneous, derived from the master seed by a limited number of passages by a method approved by the national regulatory authority. The virus working seed is used for production of vaccine without intervening passage.

Single harvest: A virus suspension harvested from a group of embryonated eggs or a group of cell cultures prepared from a single production run. For vaccines produced in animal skin, a single harvest is a quantity of material harvested from one animal.

Bulk suspension: A pool of a number of single harvests.

Final bulk: The finished biological preparation after completion of preparations for filling, homogeneous with respect to mixing of all components, and present in the container from which the final containers are filled. The final bulk may be prepared from one or more bulk suspensions.

Filling lot (final lot): A collection of sealed, final containers of freeze-dried vaccine that are homogeneous with respect to the risk of contamination during the filling process or the preparation of the finished vaccine. A filling lot must therefore have been filled or prepared in one working session.

Pock-forming unit: The smallest quantity of a virus suspension that will produce a single pock on the chick chorio-allantoic membrane.

Plaque-forming unit: The smallest quantity of a virus suspension that will produce a single plaque in monolayer cell cultures.

A.2 **General manufacturing recommendations**

The general manufacturing recommendations contained in Good manufacturing practices for biological products (9) should apply to establishments manufacturing smallpox vaccine, with the addition of the following:

Production areas should be decontaminated before they are used for the manufacture of smallpox vaccine.

The production of smallpox vaccine should be conducted by a separate staff which should consist of healthy persons, who should receive regular medical examinations. Steps should be taken to ensure that all such persons in the production areas and all relevant quality control staff are protected against vaccinia virus infection by immunization or other means. Steps should also be taken to minimize the risks of transmission of vaccinia virus from the production facility to the outside environment.

For new vaccine production in animal skins method strict adherence to good manufacturing practices will not be possible. It will also be very difficult to validate the manufacturing process. Therefore every effort should be made to minimize contaminating microbial agents in the vaccine by meticulous controls of facilities, personnel, animals used for production and by specific tests on the product.

A.3 Production control

A.3.1 *Control of source materials*

A.3.1.1 *Virus strains*

Strains of vaccinia used in the production of smallpox vaccine should be identified by historical records, which should include information on their origin. Only vaccinia strains that are approved by the national regulatory authority should be used. They should be shown to yield immunogenic vaccines that produce typical vaccinal lesions in the skin of humans. For new vaccines, neutralizing antibodies or haemagglutination inhibition antibodies, or an inhibition to response to revaccination, may be used to assess immunogenicity.

Vaccine strains known to protect humans against variola include, but are not restricted to, the Lister-Elstree, and the New York City Board of Health (NYCBOH) strains. The Tiantan strain, and other derivatives of the Lister-Elstree strain, were also used in some countries.

The WHO seed virus, based on the Lister-Elstree strain, is held by WHO Collaborating Centres. Although WHO has taken every possible precaution to ensure that these seeds meet the recommendations for smallpox vaccine it should be emphasized that, in each country, the national regulatory authority must accept responsibility for the quality of vaccines produced from the seeds and used in that country. Requests for the seed virus should be made through WHO.

A.3.1.1.1 *Virus seed lot system*

Vaccine production should be based on the virus seed lot system. The number of passages required to produce vaccine single harvests from

the original seed should be limited and approved by the national regulatory authority.

The virus master seed lot may be produced by cloning from an original seed.

The passages between master and working virus seeds should be in the same general type of substrate as used for vaccine production. For example if the vaccine is produced in embryonated eggs, the working seed lot should be produced from the master seed by passage in embryonated eggs and not by passage in cell cultures or in animal skin.

If different substrates have been utilized for master and working virus seeds, adequate validation of this change must be conducted to rule out changes in the quality of the vaccine.

Vaccine should be produced from the virus working seed with no intervening passage.

Phenotypic differences between clonal derivatives of vaccinia have been shown. It is theoretically possible that multiple passages may select variants present in the original seed. Restricting the number of passages from original seed to vaccine single harvests should minimize this risk.

A large working seed lot should be set aside as the basic material to be used by the manufacturer for the preparation of batches of vaccine.

All virus seed lots in liquid form should be stored at a temperature of -60°C or below. Seed lots that are freeze-dried need not be stored at -60°C or below. The available data show that potency is retained when seed lots are stored at temperatures of -20°C or below.

A.3.1.1.2 Tests on virus seed lots

The virus master and working seed lots should be identified as vaccinia by suitable tests. A sensitive test should be conducted to exclude the presence of other orthopoxviruses.

Molecular tests such as restriction fragment length polymorphism or partial sequencing, especially of terminal DNA sequences which show the greatest variation between vaccinia strains, may be useful as identity tests.

The potency of the virus master and working seed lots should be determined as described in section A.3.3.4.

The virus master and working seed lot used for the production of vaccine batches in embryonated eggs or cell cultures should be free from detectable adventitious agents. Seed lots produced in embryonated eggs should comply with the recommendations in sections A.3.1.2 and A.3.2.1 and seed lots produced in cell cultures should comply with the recommendations in section A.3.1.3 and A.3.2.2 or A.3.2.3 as appropriate.

Whereas testing for adventitious bacteria, mycoplasma and fungi should use standardized methods, testing of vaccine virus seeds for viral adventitious agent's might be more complex because complete neutralization of vaccinia virus may be difficult to achieve. Should this be the case, the seed lot may be diluted to the dilution used as inoculum for production of vaccine prior to testing for viral adventitious agents. Supplementary specific testing for viral adventitious agents using validated PCR-methodology or the use of immunochemical methods could also be appropriate.

Seed lots to be used for production in embryonated eggs or cell culture should also be tested for carry-over of potential adventitious agents from the original seed. Given that the complete passage history of the original seed is unlikely to be known, and that more than one species may have been used in the passage history, this additional testing should at least cover important adventitious agents of concern.

The passage history of the original seed is likely to have included sheep, calves and humans and may have included rabbits, goats or water buffaloes.

The burden of contaminating microbial agents in virus master and working seeds prepared in animal skins should be limited by meticulous controls of facilities, personnel, animals used for production and by specific tests on the seeds. However it may be difficult to ensure that seed lots produced in animal skins are totally free from adventitious agents. Such lots should also comply with the recommendations in sections A.3.1.4 and A.3.2.4. The absence of specific human pathogens should be confirmed by additional testing procedures (bacterial and fungal cultures, virus culture and PCR testing for viral agents).

All master and working seed lots should comply with the current guidelines to minimize the risks of transmission of animal transmissible spongiform encephalopathies (10).

The neurovirulence of master and working seed viruses should meet the criteria for acceptability given in section A.3.3.5.1 (test for neurovirulence in mice). The original seed should be used, where possible, as comparator in these tests. Where original seeds are not available for this purpose equivalent materials may be used.

As an alternative to mice, a neurovirulence test may be conducted in rabbits.

A.3.1.2 Eggs

If the vaccine is to be produced in embryonated eggs or primary chick embryo fibroblasts, the eggs to be used should be from a closed, specific-pathogen-free, healthy flock. This flock should be monitored at regular intervals for agents pathogenic to birds. These include

Mycobacterium avium, fowlpox virus, avian leucosis virus (ALV) and other avian retroviruses; Newcastle disease virus and other avian parainfluenza viruses; avian encephalomyelitis virus; infectious laryngotracheitis virus; avian reticulo-endotheliosis virus; Marek's disease virus; infectious bursal disease virus; *Haemophilus paragallinarum*; *Salmonella gallinarum*; *Salmonella pullorum*; *Mycoplasma gallisepticum*, and *Mycoplasma synoviae*.

In some countries, all birds are bled when a colony is established, and thereafter 5% of the birds are bled each month. The resulting serum samples are screened for antibodies to the relevant pathogens. Any bird that dies should be investigated to determine the cause of death.

The flock must not have been vaccinated with live Newcastle disease virus vaccine.

It is recommended that eggs should be obtained from young birds.

A.3.1.3 *Cell cultures*

Smallpox vaccines may be produced in human diploid cells, in continuous cell lines, in primary rabbit kidney cells or in primary chick embryo fibroblast cells. For vaccines produced in human diploid cells and continuous cell lines sections A.3.1.3.1 and A.3.1.3.2 should apply; for production in primary rabbit kidney cells section A.3.1.3.3 should apply; and for production in primary chick embryo fibroblasts section A.3.1.2 should apply to the source materials. Section A.3.1.3.4 applies to all types of cell culture.

A.3.1.3.1 **Cell seed and manufacturer's working cell bank**

The use of a cell line such as MRC-5 or Vero cells for the manufacture of smallpox vaccines should be based on the cell seed system. The cell seed should be approved by the national control authority. The maximum number of passages (or population doublings) by which the MWCB is derived from the cell seed should be established by the national regulatory authority.

WHO has established a cell bank of Vero cells characterized in accordance with the requirements in the report of the WHO Expert Committee on Biological Standardization (11), which is available as a well characterized starting material to manufacturers for preparation of their own master and working cell seeds on application to the Coordinator, Quality Assurance and Safety of Biologicals, World Health Organization, Geneva, Switzerland.

A.3.1.3.2 **Identity test**

Cell seed should be characterized according to the requirements for animal cells lines used as substrates for biologicals production (14), as appropriate to continuous cell lines or human diploid cells.

The MWCB should be identified by means, inter alia, of biochemical (e.g. isoenzyme analysis), immunological and cytogenetic marker tests, approved by the national regulatory authority.

A.3.1.3.3 Primary rabbit kidney cells for production of smallpox vaccines

Rabbits, 2–4 weeks old, may be used as the source of kidneys for cell culture. Only rabbit stock approved by the national regulatory authority should be used as the source of tissue and they should be derived from a closed, healthy colony. A closed colony is a group of animals sharing a common environment and having their own caretakers who have no contact with other animal colonies. The animals are tested according to a defined programme to ensure freedom from specified pathogens or their antibodies.

No generally agreed testing programme is available. Agents that may be considered include the following viruses: myxoma virus, rabbit poxvirus, parainfluenza viruses, Sendai virus, reovirus type 3, rotavirus, and rabbit parvovirus; mycoplasma species; the following bacteria: *Bordetella bronchiseptica*, *Clostridium perfringens*, *Clostridium pilliforme*, *Chlamydia psittaci*, *Citrobacter rodentium*, *Clostridium spiroforme*, *Francisella tularensis*, *Listeria*, *Mycobacterium tuberculosis*, *Pasteurellaceae*, *Pseudomonas aeruginosa*, *Salmonella* species, *Staphylococcus aureus*, *Yersinia enterocolitica*; *Toxoplasma gondii*; ticks and endoparasites.

When new animals are introduced into the colony, they should be maintained in quarantine in vermin-proof quarters for a minimum of 2 months and shown to be free from specified pathogens. Animals to be used to provide kidneys should not previously have been used for experimental purposes, especially those involving infectious agents. The colony should be monitored for zoonotic viruses and markers of contamination at regular intervals.

At the time the colony is established, all animals should be tested to determine freedom from antibodies to possible viral contaminants for which there is evidence of capacity for infecting humans or evidence of capacity to replicate in vitro in cells of human origin. A test for retroviruses using a sensitive polymerase chain reaction (PCR)-based reverse transcriptase (Rtase) assay should also be included. The results of such assays may need to be interpreted with caution because Rtase activity is not unique to retroviruses and may derive from other sources, such as retrovirus-like elements that do not encode a complete genome (12). Nucleic acid amplification tests for retrovirus may also be used.

After the colony is established, it should be monitored by testing a representative group of animals consisting of at least 5% of the animals that are bled at intervals acceptable to the national regulatory authority.

As an example of how often to monitor the rabbit colony, it is suggested that they are bled at monthly intervals in line with the sampling frequency of birds used in the production of chick embryo fibroblast cells (section A.3.1.2).

In addition, the colony should be screened for pathogenic bacteria, including mycobacteria, fungi and mycoplasma, as agreed with the national regulatory authority. The screening programme should test all of the animals over a defined period of time, as agreed with the national regulatory authority.

Any animal that dies should be investigated to determine the cause of death. If the presence of an infectious agent is demonstrated in the colony, the national control authority should be informed and the manufacture of smallpox vaccine may be discontinued. In this case, manufacture should not be resumed until a thorough investigation has been completed and precautions have been taken against the infectious agent being present in the product, and only then with the approval of the national control authority.

At the time of kidney harvest, the animals should be examined for the presence of any abnormalities and if kidney abnormalities or other evidence of pathology is found, those animals are not be used for smallpox vaccine production.

Each group of control cultures derived from a single group of animals used to produce a single virus harvest should remain identifiable as such until all testing, especially for adventitious agents, is completed.

A.3.1.3.4 Cell culture medium

Serum used for the propagation of cells should be tested to demonstrate freedom from bacteria, fungi and mycoplasmas, according to the requirements given in Part A, sections 5.2 and 5.3 of the revised Requirements for Biological Substances No. 6 (13), and from infectious viruses. Suitable tests for detecting viruses in bovine serum are given in Appendix 1 of the Recommendations for Production and Control of Poliomyelitis Vaccine (Oral) (14).

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.

Irradiation may be used to inactivate potential contaminant viruses.

The acceptability of the sources(s) of any components of bovine, sheep or goat origin used in culture media should be approved by the national regulatory authority. These components should comply with current guidelines in relation to animal transmissible spongiform encephalopathies (10).

Human serum should not be used. If human albumin is used it should meet the revised Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives (Requirements for Biological Substances No. 27) (15), as well as current guidelines in relation to human transmissible encephalopathies (10).

The use of human albumin as a component of a cell culture medium requires careful consideration due to potential difficulties arising from the shorter expiry period of albumin in relation to the intended long-term storage of smallpox vaccines.

Penicillin and other β -lactams should not be used at any stage of the manufacture.

Other antibiotics may be used at any stage in the manufacture provided that the quantity present in the final product is acceptable to the national control authority. Nontoxic pH indicators may be added, e.g. phenol red in a concentration of 0.002%. Only substances that have been approved by the national regulatory authority may be added.

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

The source(s) of trypsin of bovine origin, if used, should be approved by the national regulatory authority. Bovine trypsin, if used, should comply with current guidelines in relation to animal transmissible spongiform encephalopathies (10).

A.3.1.4 *Animals used for production of animal skin vaccine*

If vaccine is prepared in animal skins, animals of a species approved by the national control authority, in good health, and not previously employed for experimental purposes should be used.

Manufacturers are encouraged to use animals from closed or intensively monitored colonies where these are available.

The animals should be kept in well-constructed and adequately ventilated animal rooms in cages spaced as far apart as possible. Adequate precautions should be taken to prevent cross-infection between stalls or cages. For large animals, not more than one animal should be housed per stall. For small animals, not more than two animals should be housed per cage and cage-mates should not be interchanged. The animals should be kept in the country of manufacture of the vaccine in quarantine groups for a period of not less than six weeks before use.

A quarantine group is a colony of selected, healthy animals kept in one room, with separate feeding and cleaning facilities, and having no contact with other animals during the quarantine period.

If at any time during the quarantine period the overall death rate of a group of animals reaches 5%, animals from that entire group should not be used for vaccine production. The groups should be kept continuously in isolation, as in quarantine, even after completion of the quarantine period, until the animals are used. After the last animal of a group has been taken, the room that housed the group should be thoroughly cleaned and decontaminated before being used for a fresh group.

Animals to be inoculated should be anaesthetized and thoroughly examined. If an animal shows any pathological lesion relevant to its use in the preparation of a seed lot or vaccine, it should not be used, nor should any of the remaining animals from that quarantine group be used unless it is evident that their use will not impair the safety of the product.

If ruminants are to be used for vaccine production, special attention is required to ensure that the animals comply with the current guidelines on animal transmissible spongiform encephalopathies given in *the Report of a WHO Consultation on Medical and Other Products in Relation to Human and Animal Transmissible Spongiform Encephalopathies* (10). This means the animals used should be less than two years of age and sourced from herds that have had no cases of BSE, have been actively monitored, and have never been fed mammalian-derived protein (other than milk).

Where possible ruminants used for vaccine production should also be from a BSE-free country, have a fully documented breeding history, and have come from a herd in which any new genetic material introduced is from herds with the same BSE-free status.

A.3.2 Control of vaccine production

A.3.2.1 Production in specific pathogen free embryonated eggs

A.3.2.1.1 Tests on uninoculated eggs

A sample of 2% of, but in any case not less than 20 and not more than 50, uninoculated embryonated eggs from the batch used for vaccine production should be incubated under the same conditions as the inoculated eggs. At the time of virus harvest, the uninoculated eggs should be processed in the same manner as the inoculated eggs, and the extract from the control embryos should be shown to be free from haemagglutinating agents and from adenoviruses, avian retroviruses such as avian leukosis virus, and other extraneous agents by tests approved by the national control authority.

A test for retroviruses using a sensitive PCR-based reverse transcriptase (Rtase) assay may be used. The results of such assays need to be interpreted with caution because Rtase activity is not unique to infectious retroviruses and may derive from other sources, such as mammalian polymerase or incomplete retrovirus-like elements that do not encode a complete genome (12). Nucleic acid amplification tests for retrovirus may also be used.

A.3.2.1.2 Single harvests

After inoculation and incubation at a controlled temperature, only living, typical chick embryos should be harvested. The age of embryos at the time of harvest should be reckoned from the initial introduction of the eggs into the incubator and should be no more than 12 days. After homogenization and centrifugation, the embryonic extract should be kept at -60°C or below until further processing.

A.3.2.1.3 Bacterial and fungal sterility and mycoplasma tests

A volume of at least 10 ml of each single harvest should be tested for bacterial and fungal sterility, and mycoplasma, according to the revised Requirements for Biological Substances No. 6 (16)

A.3.2.2 Production in primary chick embryo fibroblasts, human diploid cells or continuous cell cultures

A.3.2.2.1 Control of cell cultures

At least 5% or 1000 ml of the cell suspension at the concentration and cell passage level employed for seeding vaccine production cultures should be used to prepare control cultures.

If bioreactor technology is used, the national regulatory authority should determine the size and treatment of the cell sample to be examined.

A.3.2.2.1.1 Tests of control cell cultures

The treatment of the cells set aside as control material should be similar to that of the production cell cultures, but they should remain uninoculated for use as control cultures for the detection of extraneous viruses.

These control cell cultures should be incubated under similar conditions to the inoculated cultures for at least two weeks, and should be examined during this period for evidence of cytopathic changes. For the test to be valid, not more than 20% of the control cell cultures should have been discarded for nonspecific, accidental reasons.

At the end of the observation period, the control cell cultures should be examined for degeneration caused by an extraneous agent. If this examination, or any of the tests specified in this section, shows

evidence of the presence in a control culture of any adventitious agent, the vaccinia grown in the corresponding inoculated cultures should not be used for vaccine production.

A.3.2.2.1.2 Tests for haemadsorbing viruses

At the end of the observation period, 25% of the control cells should be tested for the presence of haemadsorbing viruses using guinea-pig red blood cells. If the latter have been stored, the duration of storage should not have exceeded seven days and the storage temperature should have been in the range 2–8 °C .

In tests for haemadsorbing viruses, calcium and magnesium ions should be absent from the medium.

As an additional test for haemadsorbing viruses the national regulatory authority may require that other types of red blood cells, including cells from humans (blood group O), monkeys and chickens (or other avian species), should be used in addition to guinea-pig cells.

A reading should be taken after incubation for 30 minutes at 2–8 °C and again after a further incubation for 30 minutes at 20–25 °C.

If a test with monkey red cells is performed, readings should also be taken after a final incubation for 30 minutes at 34–37 °C.

A.3.2.2.1.3 Tests for other adventitious agents

At the end of the observation period, a sample of the pooled fluid from each group of control cultures, and a sample of pooled cell homogenate from each group of control cultures, should be tested for adventitious agents. For this purpose, 10ml of each pool should be tested in the same cells, but not the same batch of cells, as those used for the production of virus, and additional 10-ml samples of each pool should be tested in human cells sensitive to measles and at least one other sensitive cell system.

The test of cell homogenates is added as an additional test for adventitious agents because of the potential difficulties in neutralizing vaccinia virus in single harvests.

The pooled fluid should be inoculated into bottles of these cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1 in 4. At least one bottle of each kind of cell culture should remain uninoculated and should serve as a control.

The inoculated cultures should be incubated at a temperature of 35–37 °C and should be observed for a period of at least 14 days.

For the tests to be valid, not more than 20% of the culture vessels should have been discarded for nonspecific accidental reasons by the end of the test period.

If any cytopathic changes due to adventitious agents occur in any of the cultures, the virus harvests produced from the batch of cells from which the control cells were taken should be discarded.

If these tests are not performed immediately, the samples should be kept at a temperature of -60°C or below.

A.3.2.2.1.4 Additional tests if chick cell cultures are used for production

If chick cell cultures are used, a sample of fluids pooled from the control cultures should be tested for adenoviruses and for avian retroviruses such as avian leukosis virus, by a method approved by the national regulatory authority.

A test for retroviruses using a sensitive PCR-based Rtase assay may be used. The results of such assays need to be interpreted with caution because Rtase activity is not unique to retroviruses and may derive from other sources, such as retrovirus-like elements that do not encode a complete genome (12). Nucleic acid amplification tests for retrovirus may also be used.

A.3.2.2.1.5 Identity test

At the production level, and for vaccines produced in human diploid cells or continuous cells only, the cells should be identified by means of tests approved by the national regulatory authority.

Suitable tests are isoenzyme analysis, immunological tests and cytogenetic marker tests.

A.3.2.2.2 Cell cultures for vaccine production

A.3.2.2.2.1 Tests for adventitious agents

On the day of inoculation with the virus working seed lot, each cell culture or a sample from each culture vessel should be examined for degeneration caused by infective agents. If such examination shows evidence of the presence in a cell culture of any adventitious agent, the culture should not be used for vaccine production.

If animal serum is used for cell cultures before the inoculation of virus, the medium should be removed and replaced with serum-free maintenance medium, after the cells have been washed with serum-free medium, if appropriate. If suspension cultures are used, testing should be conducted to ensure that serum levels are reduced to acceptable levels. This testing may be performed at a later stage of production.

A.3.2.2.2.2 Tests for bacteria, fungi and mycoplasmas

A volume of at least 20 ml of the pooled supernatant fluids from the production cell culture should be tested for bacterial and fungal sterility and for mycoplasmas. The tests for bacterial, fungal and

mycoplasmal sterility should be performed as described in the revised General Requirements for the Sterility of Biological Substances (Requirements for Biological Substances No. 6) (13).

A.3.2.2.3 Control of single harvests

A.3.2.2.3.1 Single harvest

After inoculation of the production cells with the virus working seed lot, neither inoculated nor control cell cultures should at any time be at a temperature outside the range approved by the national regulatory authority for the relevant incubation periods. The optimal range for pH, multiplicity of infection, cell density and time of incubation should be established for each manufacturer, and be approved by the national regulatory authority.

The virus suspension should be harvested not later than that number of days after virus inoculation agreed by the national regulatory authority.

It is advisable that the inoculated cell cultures should be processed in such a manner that each virus suspension harvested remains identifiable as a single harvest and is kept separate from other harvests until the results of all the tests described in Part A sections A.3.2.2.1, A.3.2.2.2, A.3.2.2.3.3, A.3.2.2.3.4 and A.3.2.2.3.5 have been obtained.

A.3.2.2.3.2 Sampling

Samples required for the testing of single harvests should be taken immediately on harvesting. If the tests for adventitious agents as described in Part A, section A.3.2.2.3.3 are not performed immediately, the samples taken for these tests should be kept at a temperature of -60°C or lower, and subjected to no more than one freeze-thaw cycle.

A.3.2.2.3.3 Tests of neutralized single harvests for adventitious agents

For the purposes of the recommendations specified in this section of Part A, the volume of each single harvest taken for neutralization and testing should be at least 10ml and should be such that a total of at least 50ml or the equivalent of 500 doses of final vaccine, whichever is the greater, has been withheld from the corresponding bulk suspension.

The antisera used for neutralization should be of nonhuman origin and should have been prepared using virus cultured in cells from a species different from that used in the production of the vaccine. Samples of each virus harvest should be tested in human cells sensitive to measles and at least one other sensitive cell system.

Complete neutralization of vaccinia virus may be difficult to achieve at high virus concentrations. If this is the case, specific tests can supplement non-

specific testing with standard tissue culture tests or eggs. Specific tests could include PCR, immunochemical tests or antibody production tests in animals. The extent of testing for specific adventitious agents may vary and depends on the agents that could be present based on the nature and origin of the substrate used for vaccine production and the origin of the virus seed. The national regulatory authority should approve the test programme for viral adventitious agents. Use of scarce biological reagents, such as high-titred vaccinia neutralizing sera, may be decreased by not testing for viral adventitious agents at the level of the single harvest but testing instead at later stages of the manufacturing process, for example at the level of the final bulk. This option should first be approved by the national regulatory authority.

The neutralized suspensions should be inoculated into bottles of these cell cultures in such a way that the dilution of the suspension in the nutrient medium does not exceed 1 in 4. The area of the cell sheet should be at least 3 cm² per ml of neutralized suspension. At least one bottle of each kind of cell culture should remain uninoculated; it should serve as a control; it should be maintained by nutrient medium containing the same concentration of the specific antiserum used for neutralization.

Animal serum may be used in the propagation of the cells, but the maintenance medium used after inoculation of the test material should contain no added serum other than the smallpox neutralizing antiserum.

The inoculated cultures should be incubated at a temperature of 35–37°C and should be observed for a period of at least 14 days.

If adequately justified lower temperatures may be used.

For the tests to be valid, not more than 20% of the culture vessels should have been discarded for nonspecific accidental reasons by the end of the test period.

If any cytopathic changes due to adventitious agents occur in any of the cultures, the virus harvest should be discarded.

A.3.2.2.3.4 Additional tests if chick cell cultures are used for production

A volume of each neutralized virus pool equivalent to 100 human doses of vaccine or 10 ml, whichever volume is greatest, should be tested in a group of fertilized chicken's eggs by the allantoic route of inoculation, and a similar sample should be tested in a separate group of eggs by the yolk-sac route of inoculation. In both cases 0.5 ml of inoculum should be used per egg.

The virus pool passes the test if, at the end of a 3–7-day observation period, there is no evidence of the presence of any adventitious agents. If an adventitious agent is detected in the uninoculated controls, the test should be repeated.

A.3.2.2.3.5 Sterility tests

A volume of at least 10 ml of each single harvest should be tested for bacterial, fungal and mycoplasmal sterility according to the requirements given in the revised Requirements for Biological Substances No. 6 (16).

A.3.2.3 *Production in primary rabbit kidney cells*

A.3.2.3.1 *Tests of cell cultures used for vaccine production*

On the day of inoculation with virus working seed lot, each cell culture should be examined for degeneration caused by an infective agent. If this examination reveals evidence of the presence in a cell culture of any adventitious agent, none of the entire group of cultures concerned should be used for vaccine production.

On the day of inoculation with the virus working seed lot, a sample of at least 30 ml of the pooled fluid is removed from the cell cultures of the kidneys of each group of animals used to prepare the primary cell suspension. The pooled fluid should be tested in primary kidney-cell cultures prepared from the same species, but not the same group of animals, as that used for vaccine production. The pooled fluid should be inoculated into bottles of these cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1 in 4. The area of the cell sheet should be at least 3 cm² per ml of pooled fluid. At least one bottle of each primary cell culture should remain uninoculated and should serve as a control.

The cultures should be incubated at a temperature of 34–36°C and should be observed for a total period of at least 4 weeks. During this observation period and after not less than 2 weeks of incubation, from each of these cultures at least one subculture of fluid should be made in the same tissue culture system. The subculture should also be observed for at least 2 weeks.

Serum may be added to the original culture at the time of subculturing, provided that the serum does not contain antibody or other inhibitors to adventitious agents of the cell culture donor species.

For the tests to be valid, not more than 20% of the culture vessels should have been discarded for nonspecific accidental reasons by the end of the respective test periods.

If, in these tests, evidence is found of the presence of an adventitious agent, the single harvest from the whole group of cell cultures concerned should not be used for vaccine production.

If these tests are not done immediately, the samples of pooled cell-culture fluid should be kept at a temperature of –60°C or below.

A.3.2.3.2 Tests of control cell cultures

Cultures prepared on the day of inoculation with the virus working seed lot from 25% of the cell suspension obtained from the kidneys of each group of animals used to prepare the primary cell suspension should remain uninoculated, and should serve as controls. These control cell cultures should be incubated under the same conditions as the inoculated cultures for at least two weeks, and should be examined during this period for evidence of cytopathic changes. For the tests to be valid, not more than 20% of the control cell cultures should have been discarded for nonspecific, accidental reasons.

At the end of the observation period, the control cell cultures should be examined for degeneration caused by an infectious agent. If this examination or any of the tests specified in this section shows evidence of the presence in a control culture of any adventitious agent, the vaccinia grown in the corresponding inoculated cultures from the same group should not be used for vaccine production.

A.3.2.3.2.1 Tests for haemadsorbing viruses

At the time of harvest or not more than four days after the day of inoculation of the production cultures with the virus working seed lot, a sample of 4% of the control cell cultures should be taken and should be tested for haemadsorbing viruses. At the end of the observation period, the remaining control cell cultures should be similarly tested. The tests should be made as described in Part A, section A.3.2.2.1.2.

A.3.2.3.2.2 Tests for other adventitious agents

At the time of harvest, or not more than seven days after the day of inoculation of the production cultures with the working seed lot, a sample of at least 20 ml of the pooled fluid from each group of control cultures should be taken and tested in primary kidney-cell cultures, as described in Part A, section A.3.2.3.1.

At the end of the observation period for the original control cell cultures, similar samples of the pooled fluid should be taken and the tests in primary kidney-cell cultures should be repeated, as described in Part A, section A.3.2.3.1.

In some countries, fluids are collected from the control cell cultures at the time of virus harvest and at the end of the observation period. Such fluids may then be pooled before testing for adventitious agents.

A.3.2.3.3 Tests for neutralized single harvests in primary kidney-cell cultures

The specifications given in part A section A.3.2.2.3.3 should apply with the addition that each neutralized single harvest should be

additionally tested in primary kidney-cell cultures prepared from the same species, but not the same group of animals, as that used for vaccine production.

A.3.2.3.4 *Sterility tests*

A volume of at least 10 ml of each single harvest should be tested for bacterial, fungal and mycoplasmal sterility according to the requirements given in the revised Requirements for Biological Substances No. 6 (13).

A.3.2.4 *Production in animal skins*

A.3.2.4.1 *Vaccines produced in the skin of living animals*

The animals should be free of ectoparasites, and each animal should be kept under veterinary supervision for at least two weeks prior to the inoculation of the seed virus. Before inoculation the animals should be cleaned, and thereafter kept in scrupulously clean stalls until the vaccinal material is harvested.

The use of bedding, unless sterilized and changed frequently, should be avoided. The stalls, including feed boxes, should be designed so as to make cleaning easy, and dust-producing food should be avoided.

During a period of five days before inoculation, and during incubation, the animals should remain under veterinary supervision; they should remain free from any sign of disease, and rectal temperatures should be recorded daily. If any abnormal rise in temperature occurs, or if any clinical sign of disease is observed, the production of vaccine from the group of animals concerned should be suspended until the cause of these irregularities has been resolved. The prophylactic and diagnostic procedures adopted to exclude the presence of infectious disease should be submitted for approval to the national regulatory authority.

According to the species of animal used and the diseases to which that animal is liable in the country where the vaccine is being produced, the prophylactic and diagnostic procedures to be used will vary. They must exclude the possibility of transmitting diseases within the country where the vaccine is prepared, but consideration should also be given to the danger of spreading diseases to other countries or continents to which the vaccine may be shipped.

Special attention should always be given to foot-and-mouth disease, transmissible spongiform encephalopathies, brucellosis, Q fever, tuberculosis and dermatomycosis, but in some areas it will also be necessary to consider diseases such as contagious pustular dermatitis (orf), pulpy kidney disease, sheep pox, anthrax, rinderpest, haemorrhagic septicaemia, Rift Valley fever and many others.

The inoculation of seed virus should be made on parts of the animal that are not liable to be soiled by urine and faeces. The surface used

for inoculation shall be so shaved and cleaned as to procure the nearest possible approach to surgical asepsis. If any antiseptic substance deleterious to the virus is used in the cleaning process it shall be removed by thorough rinsing with sterile water prior to inoculation. During inoculation, the exposed surface of the animal not used for inoculation shall be covered with sterile covering.

Many workers prefer to inoculate the ventral surface of female animals. If male animals are used this area is more liable to soiling by urine and faeces than the flank, which may be equally susceptible to vaccinia virus and easier to keep clean, especially since the animal tends to rest on the uninoculated side.

It is recommended that the animal be anaesthetized during the process of shaving, cleaning and inoculation.

After inoculation the area may be covered with suitable antibiotics.

Before the collection of the vaccinal material, any antibiotic should be removed and the inoculated area should be subjected to a repetition of the cleaning process. The uninoculated surfaces should be covered with sterile covering.

Before harvesting, the animal should be killed painlessly. The animals should be exsanguinated before harvesting to avoid heavy admixture of the vaccinal material with blood.

The vaccinal material from each animal should be collected separately taking aseptic precautions.

All animals used in the production of vaccine should be examined by autopsy. Special attention should be paid to examining the central nervous system for evidence of transmissible spongiform encephalopathy. If evidence of any generalized or systemic disease other than vaccinia is found, or evidence of encephalopathy, the vaccinal material from the entire group of animals exposed should be discarded.

A.3.3 Control of bulk suspension

A.3.3.1 *Preparation of bulk suspension*

The bulk suspension should be treated to remove cell debris.

The national regulatory authority may require the further purification of harvests derived from continuous cell lines to remove cellular DNA, and/or the use of DNAase treatment to reduce the size of DNA fragments. If the harvests are derived from human diploid or primary cell cultures, further purification is not required.

A.3.3.2 *Sampling*

Samples of the bulk suspension prepared as described in section A.3.3.1 should be taken immediately and, if not tested immediately, should be kept at a temperature of -60°C or below until the tests described in the following sections are performed.

A.3.3.3 *Identity test*

The vaccinia virus in the bulk suspension should be identified by serological or molecular tests.

Care should be taken to ensure that the sera used are monospecific by titrating them against homotypic and heterotypic viruses of known virus titre. Monoclonal antibodies may be useful in this test. Molecular tests such as restriction fragment length polymorphism or partial sequencing, especially of terminal DNA sequences which show the greatest variation between vaccinia strains, may be useful as identity tests.

A.3.3.4 *Virus concentration*

The amount of infective vaccinia virus per ml of filtered bulk suspension should be determined in the chick egg chorioallantoic membrane, or in cell cultures, in comparison with a reference preparation assayed in the same system.

The virus concentration as determined by this test should be the basis for the quantity of virus used in the neurovirulence tests in mice (Part A, section A.3.3.5.1) and for preparing the final bulk (Part A, section A.3.4). The detailed procedures for carrying out this test and for interpreting the results should be those approved by the national regulatory authority.

A.3.3.5 *Test for consistency of virus characteristics*

The vaccinia virus in the bulk suspension prepared as described in section A.3.3.1 should be tested in comparison with the working seed virus with regard to certain characteristics, as described below, to ensure that the vaccine virus has not undergone changes during its multiplication in the production culture system. From the results of these tests for successive batches of vaccine a critical assessment may be made of the consistency of vaccine quality (see Part B, section B.2).

A.3.3.5.1 *Test for neurovirulence*

The neurovirulence of the bulk suspension should be compared to an original seed (or equivalent) by intracerebral inoculation of suckling mice.

Direct inoculation of vaccinia virus into the central nervous system of suckling mice has been shown to discriminate between clonal derivatives of

vaccinia. It is not a model of post-infectious, demyelinating disease since the pathology is quite distinct. The purpose of the test is to show consistency of production and that the each new filtered bulk suspension does not significantly differ in neurovirulence phenotype from the comparator.

Suckling 3–5-day-old CD-1 outbred mice are inoculated intracerebrally with 20 µl of the filtered bulk suspension or the comparator vaccine. The target titre of the inoculum is 5.0 log₁₀ pfu/ml. The titre of virus in the inoculum should be confirmed by titration of the residual inocula, and should be within 0.5 log₁₀ pfu of the target. The mice are observed for up to 21 days and the mortality ratio and survival times are compared between groups. The mortality ratio of the filtered bulk suspension should not exceed that of the control comparator using by Fisher's exact test, and the filtered bulk suspension should not show more rapid time to death than the comparator control, based on a log rank test.

Other test systems in mice may be used to discriminate between acceptable and unacceptable batches, and should be approved by the national regulatory authority.

The national regulatory authority may approve neurovirulence tests in other species such as rabbit.

A.3.3.6 Tests for cellular DNA

For viruses grown in continuous cell culture, the bulk suspension should be tested for residual cellular DNA. The removal process should be shown by calculation to consistently reduce the level of cellular DNA to less than 10 ng per human dose. This test may be omitted, with the agreement of the national regulatory authority, if the manufacturing process is validated to achieve this specification.

The cytoplasmic replication cycle and specific structures needed for genome replication and encapsidation of vaccinia virus make it very unlikely that host cell DNA is also encapsidated during smallpox vaccine production. Integration of foreign DNA into the vaccinia virus genome is only possible by specific encapsidation or homologous recombination, the latter process requiring a minimum length of homologous DNA sequences. From these observations it seems extremely unlikely that cellular DNA sequences would be integrated. However, the evolution of viral genes with cellular homologues suggests that under selective pressure this may happen, but only rarely.

A.3.4 Final bulk

The operations necessary for preparing the final bulk should be conducted in such a manner as to avoid contamination of the product.

The dilution and mixing procedures involved in preparing the final bulk should be those approved by the national control authority.

A.3.4.1 Preservatives, stabilizers and additives

Any stabilizers, preservatives or additives that may be added to the bulk suspension should have been shown to the satisfaction of the

national control authority not to impair the safety or efficacy of the vaccine and to improve the stability of the vaccine in the concentrations used. If phenol is present its concentration should not exceed 0.5% and it should comply with pharmacopoeial specifications.

Human albumin may present difficulties if used as a stabilizer. The expiry period of albumin may be less than that of the vaccine, especially where long-term stockpiling of smallpox vaccines is intended. There may also be difficulties if a batch of human albumin is subject to a recall from the market.

A.3.4.2 Tests for bacteria and fungi

For vaccines other than those prepared on animal skins, the final bulk should be tested for bacterial and fungal sterility in accordance with the requirements given in Part A, section A.5, of the revised Requirements for Biological Substances No. 6 (Requirements for the Sterility of Biological Substances) (16). If phenol or other preservatives are used, this test should be performed on samples taken before any preservatives are added.

A.3.4.3 Bioburden tests

For vaccines produced on animal skins only, a volume of final bulk agreed by the national regulatory authority should be tested after the addition of preservatives for bacterial bioburden using the tests given in the revised Requirements for Biological Substances No. 6 (13). From the number of colonies that appear on the plates the number of living bacteria in 1 ml of final bulk should be calculated. If this number exceeds 50, the final bulk should be discarded. In addition the tests described in part A sections A.3.4.3.1, A.3.4.3.2, A.3.4.3.3 and A.3.4.3.4 shall also be performed.

A.3.4.3.1 Test for the presence of *Escherichia coli*

At least three 1-ml samples of a 1:100 dilution of the final bulk after addition of preservatives should be cultured on plates of a medium suitable for differentiating *E. coli* from other bacteria. The plates shall be incubated for 48 hours at 35°C–37°C. If *E. coli* is detected, the final bulk should be subjected to further processing or discarded.

The need for further treatment should be an exceptional occurrence since the presence of *E. coli* in this test might indicate a heavy faecal contamination. It should be justified by a report into the manufacture of the batch. In some countries further treatment is not permitted.

A.3.4.3.2 Test for the presence of haemolytic streptococci, coagulase-positive staphylococci, or any other pathogenic microorganisms known to be harmful if introduced into the human body by the process of vaccination

At least three 1-ml samples of a 1: 100 dilution of the final bulk after addition of preservatives should be cultured on plates of blood agar. The plates should be incubated for 48 hours at 35°C–37°C and the colonies appearing should be examined. If any of the organisms mentioned are detected, the final bulk should be discarded.

In some countries culture of the final bulk after addition of preservatives in salt meat broth is made for the purpose of detecting staphylococci.

A.3.4.3.3 Test for the presence of *Bacillus anthracis*

Any colony seen on any of the plates used in the tests described in Part A, sections A.3.4.3., A.3.4.3.7., A.3.4.3.2 and A.3.4.3.3 that morphologically resembles *B. anthracis* should be examined. If the organisms contained in the colony are non-motile, further tests for the cultural character of *B. anthracis* should be made, including pathogenicity tests in suitable animals. If *B. anthracis* is found to be present, the final bulk, and any other associated bulks, should be discarded.

In countries where anthrax presents a serious risk, this test should be based on tests of each single harvest. The application of molecular tests for *B. anthracis* is encouraged.

A.3.4.3.4 Test for the presence of *Clostridium tetani* and other pathogenic spore-forming anaerobes

A total volume of not less than 10 ml of the final bulk after addition of preservatives, preferably taken from the depth of the bulk and not from the upper surface, should be distributed in equal amounts into ten tubes, each containing not less than 10 ml of a medium suitable for the growth of anaerobic microorganisms. The tubes should be held at 65°C for 1 hour to reduce the content of non-spore-forming organisms, after which they should be incubated for at least one week between 35°C and 37°C. From every tube showing growth, subcultures should be made on to plates of a suitable medium which should be incubated anaerobically at the same temperature. All anaerobic colonies should be examined and identified and if *Cl. tetani* or other pathogenic spore-forming anaerobes are present the final bulk should be discarded.

Organisms resembling pathogenic *Clostridia* found in the tube culture from which the subculture was made may be tested for pathogenicity by inoculation into animals as follows: groups of not less than two guinea-pigs and five mice are used for each tube culture to be tested: 0.5 ml of the cultures is mixed with 0.1 ml of a freshly prepared 4% solution of calcium chloride and injected intramuscularly into each of the guinea-pigs; 0.2 ml of

the cultures mixed with 0.1 ml of this calcium chloride solution are injected intramuscularly into each of the mice. The animals are observed for one week. If any animal develops symptoms of tetanus, or if any animal dies as a result of infection with spore-forming anaerobes, the final bulk should be discarded.

If other methods are used for this test, they should have been demonstrated, to the satisfaction of the national regulatory authorities, to be at least equally effective to the test above for detecting the presence of *Cl. tetani* and other pathogenic spore-forming anaerobes.

A.4. **Filling and containers**

The requirements concerning filling and containers given in Good Manufacturing Practices for Biological Products (9) should apply to vaccine filled in the final form.

Care should be taken to ensure that the material of which the container is made does not adversely affect the virus content of the vaccine under the recommended storage conditions.

A.5. **Control tests on final product**

Samples should be taken from each filling lot for the tests described in the following sections.

A.5.1 **Identity test**

The vaccinia virus should be identified by an appropriate method.

Appropriate methods include serology, growth characteristics and molecular methods.

A.5.2 **Tests for bacteria and fungi**

Vaccines other than those produced in animal skins should be tested for bacterial and fungal sterility according to the requirements given in Part A, section 5, of the revised Requirements for Biological Substances No.6 (Requirements for the Sterility of Biological Substances) (13).

A.5.3 **Virus titration**

The vaccinia titre should be determined using assays that include a reference preparation. Dried vaccine should be reconstituted to the form in which it is to be used for human inoculation before the test is made. The minimum virus titre is $8.0 \log_{10}$ pock forming units/ml, or the validated equivalent in plaque forming units or TCID₅₀ units, unless a lower titre is justified by clinical study, and this should be maintained to the end of the shelf-life of the batch.

The 95% confidence intervals of the assays should not differ by a factor of more than 0.5 log 10 from the estimated number of infectious units in the vaccine.

For the test of virus concentration in cell cultures, an international collaborative study is in progress that will provide valuable information on the most appropriate method to recommend. Results of this study are expected in 2003.¹

A.5.4 Accelerated degradation test

Representative final containers of the vaccine should be incubated at an elevated temperature for a defined period of time. For freeze-dried vaccines this should be 37°C for 4 weeks. For non-lyophilized vaccines, other temperatures and time periods may be determined on a case-by-case basis by the national regulatory authority.

The purpose of this test is to show that each new batch of vaccine is consistent, when exposed to heat stress, with the batches that were tested in real-time stability studies and used to determine the shelf-life of the vaccine.

The total virus content in both exposed and unexposed vials should be determined concurrently with that of a reference preparation. The vaccine passes the test when the loss on exposure is not greater than a factor of 1.0 log 10 infectious units per human dose, and the residual titre after heating is greater than that specified in section A.5.3.

A.5.5 Preservative content

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

A.5.6 Endotoxin content

Each filling lot should be tested for endotoxin if this has not been done on the final bulk. The method used and content permitted should be approved by the national regulatory authority.

A.5.7 Test for pH

The pH of each filling lot should be determined and be within limits approved by the national regulatory authority.

¹ Results of the study are available in document WHO/BS/03.1977

A.5.8 Protein content

The protein content of each filling lot, if not done on the final bulk, should be determined and be within limits approved by the national regulatory authority.

A.5.9 Ovalbumin content

For vaccines produced in embryonated eggs only, the ovalbumin content of each filling lot, if not done on the final bulk, should be determined and be within limits approved by the national regulatory authority.

A.5.10 Residual moisture

The residual moisture content of each filling lot of freeze-dried vaccine shall be determined and be within limits approved by the national regulatory authority.

A.5.11 General safety (innocuity) test

Each filling lot should be tested for unexpected toxicity (sometimes called abnormal toxicity) using a general safety (innocuity) test approved by the national regulatory authority.

This test may be omitted for routine lot release once consistency of production has been well established to the satisfaction of the national regulatory authority and when good manufacturing practices are in place. Each lot, if tested, should pass a test for abnormal toxicity. However it should be noted that preliminary experiments may be needed to determine the sample volume to use for this product in this test.

A.6. Records

The recommendations given in Good Manufacturing Practices for biological products (9) should apply.

A.7. Samples

The requirements given in Good Manufacturing Practices for Biological Products (9) should apply.

A.8. Labelling

The requirements given in Good Manufacturing Practices for Biological Products (9) should apply, with the addition of the following.

The label on the container or package should include the following information:

- the designation of the strain of vaccinia virus contained in the vaccine;
- the minimum amount of virus contained in one ml;
- the substrate used for the preparation of the vaccine;
- the nature and amount of any stabilizer, preservative or additives present in the vaccine; and
- the nature and amount of any stabilizer, preservative or additives present in the diluent.

No vaccine should be released for distribution without an adequate indication of the expiry date of the vaccine. This may be displayed on the primary or secondary packaging.

It is desirable for the label to carry the names both of the producer and of the source of the bulk material, if the producer of the final vaccine did not prepare it. The nature and amount of the antibiotics present in the vaccine, if any, may be included.

A.9. Distribution and shipping

The requirements given in Good Manufacturing Practices for Biological Products (9) should apply.

A.10. Storage and expiry date

The statements concerning storage temperature and expiry date appearing on the primary or secondary packaging should be based on experimental evidence and should be submitted for approval to the national regulatory authority.

A.10.1 Storage conditions

Before being released by the manufacturing establishment, all vaccines in final containers should be kept continuously in the frozen state at a temperature below -20°C .

The maximum duration of storage should be fixed with the approval of the national regulatory authority and should be such as to ensure that the minimum titre specified on the label of the container (or package) will still be maintained after release by the manufacturing establishment until the end of the shelf-life, if the conditions under which the vaccine is stored are in accordance with what is stated on the label. The maximum duration of storage at $2-8^{\circ}\text{C}$ or below -20°C may be specified.

Since vaccinia virus batches may be stockpiled for special contingencies, very long-term storage may be envisaged. Under these exceptional circumstances it is permissible for batches to be retested at defined intervals for extension of the storage period. The retesting should involve the accelerated degradation test given in Part A, section A.5.4. If the batch complies with the specifications given in Part A, section A.5, the storage period can be extended by the same amount as the original period.

A.102 Expiry date

The expiry date should be fixed with the approval of the national regulatory authority and should relate to the date of the last satisfactory determination, performed in accordance with Part A, section A.5.3, of virus concentration, i.e. the date on which the test system was inoculated.

Part B. Recommendations for national regulatory authorities

B.1 General

The general recommendations for national regulatory authorities given in Guidelines for National Authorities on Quality Assurance for Biological Products (16), which specify that no new biological substance should be licensed until consistency of production has been established, should apply.

The detailed production and control procedures and any significant changes in them should be discussed with and approved by the national regulatory authority. The national regulatory authority should obtain the International Standard for virus titre and, where necessary, establish national working reference preparations by comparison with this preparation.

B.2 Release and certification

A vaccine lot should be released only if it satisfies Part A of the present Recommendations. Before any vaccine lot is released from a manufacturing establishment, the requirements for consistency of production given in Guidelines for National Authorities on Quality Assurance for Biological Products (16) should be met.

A statement signed by the appropriate official of the national regulatory laboratory 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 the present Recommendations. The certificate should further state the date of the last satisfactory determination of virus concentration, the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, a copy of the official national release document should be attached.

The purpose of the certificate is to facilitate the exchange of smallpox vaccine between countries. National Regulatory Authorities should consider re-certification of vaccine lots at the time of distribution.

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