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Recommendations to assure the quality, safety and efficacy of enterovirus 71 vaccines (inactivated)

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Annex 3

Recommendations to assure the quality, safety and efficacy of enterovirus 71 vaccines (inactivated)

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

Abbreviations

BPL	beta-propiolactone
ELISA	enzyme-linked immunosorbent assay
EV71	enterovirus 71
HFMD	hand, foot and mouth disease
ICP	immune correlate of protection
Ig	immunoglobulin
IPV	inactivated poliomyelitis vaccine
MCB	master cell bank
NAT	nucleic acid amplification technique
NCL	national control laboratory
NIBSC	National Institute for Biological Standards and Control
NIFDC	National Institutes for Food and Drug Control
NRA	national regulatory authority
PCR	polymerase chain reaction
PDL	population doubling level
PSGL-1	P-selectin glycoprotein ligand-1
RD	rhabdomyosarcoma
SCARB2	scavenger receptor class B member 2
SCARB2	scavenger receptor class B member 2 (gene)
WCB	working cell bank

Introduction

Enterovirus 71 (EV71) was first isolated from the faeces of a female suffering from encephalitis in 1969 in California *(1)*. However, a retrospective study conducted in the Netherlands indicated that the virus could have emerged as early as 1963 *(2)*, a finding consistent with reports of possible worldwide EV71 epidemics in the late twentieth century *(3)*. EV71 has since been associated with a range of diseases, including hand, foot and mouth disease (HFMD), throughout the world and with epidemics in Asia, Europe and North America. The virus causes a range of effects from asymptomatic infection and mild HFMD to neurological disease with severe central nervous system complications and cardiopulmonary failure. In severe cases mortality rates can be high, especially in children aged 5 years and younger. EV71 is considered to be the most severe neurotoxic enterovirus and severe EV71 disease has become a major public health problem in China. In 2011, the WHO Regional Office for the Western Pacific issued A guide to clinical management and public health response for hand, foot and mouth disease (HFMD) *(4)* to support the treatment, prevention and control of HFMD.

Several vaccines against EV71 are currently under development and three inactivated EV71 vaccines have been licensed in China *(5–10)*. The WHO Expert Committee on Biological Standardization discussed the EV71 situation at its 67th meeting in 2016 and considered it to be of major regional significance *(11)*. The Committee noted that the joint efforts of the National Institutes for Food and Drug Control (NIFDC) and the National Institute for Biological Standards and Control (NIBSC) had resulted in the development of the First WHO International Standard for anti-EV71 serum (human), and recommended that consideration also be given to the development of a WHO written standard for EV71 vaccines. In addition, the First WHO International Standard for EV71 inactivated vaccine was established by the Committee in October 2019 following a collaborative study led by NIBSC and NIFDC *(12, 13)*. National standards for antigen content and neutralizing antibody responses for evaluating EV71 vaccines are also available in China *(14)* where they have supported the development and clinical assessment of such vaccines. In 2018, the Committee also endorsed a proposal to develop WHO international standards for enterovirus RNA for nucleic acid amplification technique (NAT)-based assays for EV71 *(15, 16)*.

Following requests from regulators and other stakeholders for WHO to develop recommendations to assure the quality, safety and efficacy of EV71 vaccines, a series of meetings was convened by WHO to review the current status of their development and licensure *(17)*. These meetings were attended by experts from around the world involved in the research, manufacture, regulatory assessment and approval, and control testing and release of EV71 vaccines. Participants were drawn from academia, national regulatory authorities (NRAs), national control laboratories (NCLs) and industry. The recommendations provided in the current document for the production, quality control and evaluation of inactivated EV71 vaccines have been based upon the experiences gained during the development and production of the first three licensed EV71 vaccines in China, the candidate EV71 vaccines now under development *(5–8, 18–22)* and other inactivated viral vaccines, such as inactivated poliomyelitis vaccines (IPVs) and hepatitis A vaccines *(23, 24)*.

Purpose and scope

These WHO Recommendations provide guidance to NRAs and manufacturers on the manufacturing processes, quality control and nonclinical and clinical evaluations needed to assure the quality, safety and efficacy of inactivated EV71 vaccines.

The guidance applies to EV71 vaccines prepared by the inactivation of whole EV71 virus for prophylactic use, grown in mammalian cells in culture, and using formaldehyde or other chemical inactivation procedures.

The document does not cover recombinant and other forms of subunit vaccines, vectored vaccines, virus-like particle vaccines or bivalent EV71-CA16 vaccines, which are at an early stage of development. However, some aspects outlined in this document may be relevant and may be taken into consideration during the development of such vaccines.

These WHO Recommendations should be read in conjunction with current WHO guidance documents on the nonclinical *(25)* and clinical *(26)* evaluation of vaccines, good manufacturing practices for biological products *(27)*, characterization of cell banks *(28)*, nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines *(29)* and lot release *(30)*.

Manufacturers and regulators should also take note of the decision of the Committee in 2018 to discontinue the inclusion of the innocuity test (also referred to as the abnormal toxicity test or general safety test) in routine lot release testing requirements for all vaccines in WHO Recommendations, Guidelines and other guidance documents for biological products *(31, 32)*. As this test is no longer recommended, it is not included in the routine testing requirements for EV71 vaccines provided in the current document.

Terminology

The definitions given below apply to the terms as used in these WHO Recommendations. These terms may have different meanings in other contexts.

Adjuvant: a vaccine adjuvant is a substance, or combination of substances, that is used in conjunction with a vaccine antigen to enhance (for example, increase, accelerate, prolong and/or possibly target) the specific immune response to the vaccine antigen and the clinical effectiveness of the vaccine.

Adventitious agents: contaminating microorganisms of the cell culture, or source materials used in its culture, that may include bacteria, fungi, mycoplasmas/spiroplasmas, mycobacteria, Rickettsia, protozoa, parasites, transmissible spongiform encephalopathy agents and endogenous/exogenous viruses that have been unintentionally introduced into the manufacturing process of a biological product.

Cell bank: a collection of vials of cells of uniform composition (though not necessarily clonal) derived from a single tissue or cell and used for the production of a vaccine, either directly or via a cell bank system.

Cell seed: a quantity of well-characterized cells that are frozen and stored under defined conditions, such as in the vapour or liquid phase of liquid nitrogen, in aliquots of uniform composition derived from a single tissue or cell, one or more of which would be used for the production of a **master cell bank (MCB)**. Cell seed is also referred to as a pre-MCB or seed stock. It may be made under conditions of good manufacturing practices or under the manufacturer's research and development conditions.

EV71 antigen: the virus-specific antigen produced in infected cell cultures and purified from such cultures. It can be assayed by methods such as enzyme-linked immunosorbent assay (ELISA) using EV71-specific antibodies. The antigen may consist of empty or full virus particles or both. The full and empty particles differ in their antigenic reactivity and both may be present in the final vaccine.

Final bulk: a formulated vaccine preparation from which the final containers are filled. The final bulk may be prepared from one or more purified antigen bulks formulated to contain all excipients and homogenous with respect to composition.

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

Immunogenicity: the capacity of a vaccine to induce antibody-mediated and/or cell-mediated immunity and/or immunological memory.

Master cell bank (MCB): a quantity of well-characterized cells of human or animal origin derived from a **cell seed** at a specific population doubling level (PDL) or passage level, dispensed into multiple containers, cryopreserved and stored frozen under defined conditions (such as the vapour or liquid phase of liquid nitrogen) in aliquots of uniform composition. The MCB is prepared from a single homogeneously mixed pool of cells and is used to derive all **working cell banks**. The testing performed on a replacement MCB (derived from the same clone or from an existing master or working cell bank) is the same as for the initial MCB, unless a justified exception is made.

Master seed lot: a quantity of virus suspension that has been processed at the same time to ensure a uniform composition, and passaged for a specific number of times that does not exceed the maximum approved by the NRA. It is characterized to the extent necessary to support development of the **working seed lot**.

Purified inactivated bulk: a purified pool of virus harvests in which the virus has been inactivated through the use of a validated method either before or after purification. It may be prepared from one **single harvest** or a number of single harvests and may yield one or more **final bulks**.

Seed lot: a preparation of live viruses constituting the starting material for the vaccine antigen. A seed lot is of uniform composition (though not necessarily clonal), is derived from a single culture process and is aliquoted into appropriate storage containers, from which all future vaccine production will be derived either directly or via a seed lot system.

Single harvest: a quantity or suspension derived from a batch of production cells inoculated with the same seed lot and processed together in a single production run.

Working cell bank (WCB): a quantity of well-characterized cells of animal or other origin, derived from the MCB at a specific PDL or passage level, dispensed into multiple containers, cryopreserved and stored frozen under defined conditions, such as in the vapour or liquid phase of liquid nitrogen, in aliquots of uniform composition. The WCB is prepared from a single homogeneously mixed pool of cells. One or more of the WCB containers is used for each production culture. All containers are treated identically and once removed from storage are not returned to stock.

Working seed lot: a quantity of virus of uniform composition derived from the **master seed lot** used at a passage level approved by the NRA for the manufacturing of vaccine.

General considerations

Clinical disease

HFMD was first reported in New Zealand in 1957 *(33)* and occurs mostly in young children, with a peak incidence at about 2 years of age. The common mild disease involves lesions on the mucosal surfaces of the mouth and spots on the palms of the hands and soles of the feet which resolve in a few days; this is not life threatening. However, a more severe and potentially fatal form of the disease was reported in 1969 *(1)* which is now recognized to encompass meningitis/encephalitis, autonomic nervous system dysregulation, cardiovascular collapse and pulmonary oedema. The overall mortality rate of HFMD is of the order of one per 1000 to 10 000 cases.

The frequency of reported HFMD cases is geographically highly variable with most cases occurring in East Asian countries, particularly China but including Viet Nam, Thailand, Singapore, Malaysia and the Republic of Korea. Normally, only few cases of severe disease are reported in Europe or the USA with reports of mild HFMD also being less common – though underreporting of the latter is very likely. Typically, the total combined number of cases in Europe and the USA is of the order of several hundred per year, whereas in 2008 there were 488 955 cases and 128 deaths in China alone *(4, 34, 35)*. HFMD is now a reportable disease in China (in contrast to Europe and the USA) and during the period 2013–2018 between 300 000 and 400 000 cases occurred in May–June each year, with a small number of deaths *(4, 34, 35)*. The reason for the differences in disease burden in different geographical areas is not yet clear.

Enteroviruses and their epidemiology

The causative agents of HFMD are picornaviruses, most often of human enterovirus species A. The picornaviruses belong to the Picornaviridae, a family of small non-enveloped viruses with a single-stranded positive-sense RNA genome of about 7500 nucleotides. The enteroviruses are a recognized taxon by the International Committee on Taxonomy of Viruses and are sub-classified into numerous species including the four human enteroviruses A, B, C and D *(36)*. The archetypal human picornavirus is poliovirus (belonging to species C) but the most common cause of HFMD is species A enteroviruses, chiefly the Coxsackie A viruses and EV71. The relative frequency of different enterovirus species found varies from year to year when clinical isolates or environmental samples such as sewage are examined.

In recent years, Coxsackie viruses such as A16, A10 and A6 have caused most of the HFMD cases in Asia. However, other human enteroviruses of the Picornaviridae family have also being implicated and as the different serotypes are antigenically distinct, the development of a vaccine based on a single serotype to protect against all mild HFMD cases is unlikely using existing technologies. There is therefore interest in the development of combination vaccines containing several serotypes. However, while mild HFMD is caused by many strains of enterovirus, the great majority of severe disease in recent years has been caused by EV71 which accounts for 70% of severe HFMD cases and 90% of HFMD-related deaths in China *(35)*. As a result, EV71 has been the main focus of vaccine development.

EV71 isolates can be clustered according to their genomic sequence into at least eight genogroups (A–H) *(36, 37)* but belong to one serotype. Genogroups B and C have been of greatest interest because of their frequency of isolation and implication in disease in Eastern Asia, and can each be sub-classified into five subgenogroups (C1–C5 and B1–B5) *(36, 37)*. C4 is by far the major genogroup circulating in China, while B4, B5 and C5 are found in other Asian countries. In contrast, strains of genogroups C1 and C2 are predominantly found in Europe where severe disease is uncommon. It is possible that this has some effect on the disease burden, with C4 being particularly virulent. However, an outbreak of HFMD with severe disease caused by a C1 genogroup strain occurred in Spain in 2016 *(38, 39)*. At the time of development of the current document, little information was available on the situation in South America, possibly due to inadequate surveillance or because EV71 infection is less common in this region *(40–42)*. A recent study showing that the EV71 genotype C exists in Peru was the first report of this lineage circulating in South America *(42)*. Previously, low levels of EV71 genotype B had been identified in the State of Para, Brazil *(40)*.

Animal models

A valid animal model would be useful in vaccine development to measure protective efficacy and potency, as well as to resolve issues related to virulence. As yet, the available models are imperfect. Neonatal mice are susceptible to EV71 by intracerebral inoculation and neonatal (but not adult) rhesus monkeys develop symptoms of HFMD on infection. Adult or infant mice are not susceptible to infection. Infant rhesus monkeys have been demonstrated to develop HFMD symptoms upon inoculation with the virus and could therefore be used as a model of protection *(43, 44)*. The neurovirulence of EV71 was demonstrated in cynomolgus monkeys and this model would be useful for challenge-protection studies for candidate EV71 vaccines *(45, 46)*. Picornaviruses are believed to use specific receptors to infect human cells. Human P-selectin glycoprotein ligand-1 (PSGL-1) is expressed in leukocytes and involved in their binding to endothelial cells in the early stages of inflammation, and has been identified as a receptor for EV71. However, the disease produced by clinical EV71 strains in transgenic mice carrying PSGL-1 was not enhanced compared to non-transgenic strains. Human scavenger receptor class B member 2 (SCARB2) has also been identified as a receptor for EV71. Transgenic mice carrying the *SCARB2* gene are more susceptible to infection and disease than non-transgenic controls but the effect is not dramatic – two-week old transgenic mice develop mild symptoms and then recover *(47–50)*.

Vaccines against EV71

Three vaccines against EV71 have been licensed in China, all using C4 genogroup strains. Candidate vaccines containing B4 and B5 genogroups are in development elsewhere but have not yet reached the licensing stage. In addition, the development of vaccines against Coxsackie A16, A6 and A10 is being considered with a view to developing combination/multivalent vaccines. The efficacy of the three licensed EV71 vaccines after two doses ranges from 90.0% to 97.4% after one year of surveillance *(5–7)* to 95.1% after two-year follow-up *(8)*.

Licensed EV71 vaccines are produced from virus grown on mammalian cells and inactivated by validated methods – similar to the approach used for IPVs and hepatitis A vaccines. Tissue culture grown virus harvests comprise two types of particle forms; one containing the RNA genome (full) and one that does not (empty). In the case of both polioviruses and EV71 viruses, the two particle forms have different antigenic and immunogenic properties; poliomyelitis vaccines are purified so that they contain little if any empty virus particles but EV71 vaccines contain both types; potentially complicating potency assays. The atomic structures of both full and empty particles of EV71 viruses and polioviruses have been resolved by X-ray crystallography and cryogenic electron microscopy *(51, 52)*.

Specific issues in the development of inactivated EV71 vaccines include:

- The degree to which a vaccine based on one genogroup will protect against the others is not established. Although there is good cross-neutralization between genogroups, including by sera induced by vaccination *(53, 54)*, it has not been established that this translates into good cross-protection in humans. One recent collaborative study indicated that assays of antigen content work acceptably on all genogroups tested. However clinical cross-protection has not been demonstrated. Thus, the C4 genogroup vaccines may or may not protect against other genogroups.
- There is a lack of a convenient and convincing animal model, with the model most accurately reflecting human disease at present being infant rhesus monkeys. This makes the study of protective efficacy and immunogenic potency difficult other

than by clinical trial. Neonatal mice are susceptible to disease and transgenic mice carrying the *SCARB2* gene which encodes EV71 receptors have been developed and can prove useful without fully imitating human pathogenesis.

- Virological issues include the existence of full and empty particle forms in the licensed products. These two particle forms differ in their antigenic and immunogenic properties, thus complicating potency assays. It is not clear whether current national and international vaccine reference standards and antigen potency assays are suitable for specifically detecting one or other of the particle forms *(55)*.

International reference materials

Subsequent sections of this document refer to WHO reference materials that may be used in laboratory or clinical evaluations. The following key standards are currently used in the control of EV71 vaccines.

- A WHO international standard for anti-EV71 serum is available for the standardization of diagnostic tests for use in seroprevalence studies and for assessing immunity. This standard was established by the WHO Expert Committee on Biological Standardization in 2015 as the First WHO International Standard for anti-EV71 serum (human) (NIBSC code 14/140) with an assigned value of 1000 IU/ampoule *(56, 57)*. The preparation is held and distributed by NIBSC and NIFDC.
- A WHO international reference reagent for EV71 neutralization assays (low-titre) is also available for use in the standardization of virus neutralization assays. This reference reagent was established by the Committee in 2015 as the First WHO International Reference Reagent for EV71 neutralization assays (NIBSC code 13/238) with an assigned value of 300 IU/ampoule *(56, 57)*. The preparation is held and distributed by NIBSC and NIFDC.
- In 2019, the First WHO International Standard for EV71 inactivated vaccine (NIBSC code 18/116) was established by the Committee with an assigned unitage of 3625 IU/ampoule *(12, 13)*. In addition, WHO international reference reagents for EV71 genogroups C4 and B4 inactivated vaccine (NIBSC codes 18/120 and 18/156 respectively) were established with assigned unitages of 300 and 250 IU/ampoule respectively *(12, 13)*. These preparations are held and distributed by NIBSC. The First WHO International Standard for EV71 inactivated vaccine is intended for use in in vitro assays to measure the antigen content of vaccine products through the calibration of secondary reference preparations. However, it is known that full and empty virus particles (known to be present in EV71 vaccine preparations from all manufacturers) differ in their antigenicity and immunogenicity. The proportion of empty/full virus particles in the WHO international standard is not known. It is also not known whether this matters for the overall assessment of vaccines or if the current international standard is suitable for accurately measuring antigen content across manufacturers. International standards and reference reagents for the control of in vivo potency assays are under investigation *(13)*.
- Product-specific national standards for EV71 neutralizing antibody and EV71 antigen were established by NIFDC following collaborative studies conducted by NIFDC and the three main vaccine manufacturers in China *(14)*. This has helped

to ensure the accuracy, comparability and repeatability of anti-EV71 neutralizing antibody and EV71 antigen detection assays, and hence improve EV71 vaccine standardization. In addition, NIFDC has also developed a new national reference for in vivo vaccine potency. These preparations are held and distributed by NIFDC.

Part A. Manufacturing recommendations

A.1 Definitions

A.1.1 International name and proper name

The international name of the vaccine should be “enterovirus 71 vaccine (inactivated)”. The proper name should be the equivalent of the international name in the language of the country in which the vaccine is licensed.

The use of the international name should be limited to vaccines that meet the specifications given below.

A.1.2 Descriptive definition

An EV71 vaccine (inactivated) consists of a sterile suspension of EV71 grown in cell cultures, concentrated, purified and inactivated. The antigen may be formulated for delivery with a suitable adjuvant. The preparation should meet all of the specifications given below.

A.2 General manufacturing recommendations

The general guidance provided in WHO good manufacturing practices for pharmaceutical products: main principles *(58)* and WHO good manufacturing practices for biological products *(27)* should be applied to the design, establishment, operation, control and maintenance of manufacturing facilities for EV71 vaccine.

Staff involved in the production and quality control of inactivated EV71 vaccine should be shown to be immune to EV71.

A.3 Control of source materials

A.3.1 Virus strains and seed lot system

A.3.1.1 Virus strains

Strains of EV71 used in the production of EV71 vaccine should be identified by historical records, which should include information on their origin and subsequent manipulation or passage (for example, the genogroup and subgenogroup of EV71). Strain identity should be determined by infectivity tests and immunological methods, and by full or partial genomic sequencing.

Only virus strains that are approved by the NRA and that yield a vaccine complying with the recommendations set out in these WHO Recommendations should be used.

A.3.1.2 Virus seed lot system

Vaccine production should be based on the virus seed lot system. Unless otherwise justified and authorized, the virus in the final vaccine should not have undergone more passages from

the virus master seed lot than were used to prepare a vaccine shown to be satisfactory with respect to safety and efficacy.

Virus master and working seed lots should be stored in a dedicated temperature-monitored system that ensures stability during storage (for example, at or below −60 °C).

A.3.1.3 Tests on virus master and working seed lots

Each virus master and working seed lot used for the production of vaccine lots should be subjected to the tests listed in this section.

Each virus master and working seed lot should have been derived from materials that comply with the recommendations made in sections A.3.2 and A.3.3 below and should be approved by the NRA.

A.3.1.3.1 Tests for adventitious agents

The virus master and working seed lots used for the production of vaccine lots should be free from adventitious agents.

A sample of at least 20 mL of each virus master seed lot should be tested for the presence of adventitious agents. The sample should be neutralized by a high-titred antiserum against EV71. If the virus cannot be completely neutralized, alternative testing methods should be explored. Any alternative method used should be validated and approved by the NRA.

> If polyclonal antisera are used, the immunizing antigen used for the preparation of the antiserum should not be the same as the production seed.
>
> The immunizing antigen should be shown to be free from adventitious agents and should be grown in cell cultures free from adventitious microbial agents that might elicit antibodies that could inhibit the growth of any adventitious agents present.

The sample should be tested in susceptible cells such as Vero, human rhabdomyosarcoma (RD) or human diploid cells. The tissue cultures should be incubated at 37 °C and observed for 2 weeks. Within this observation period, at least one subculture of supernatant fluid should be made in the same tissue culture system. The sample should be inoculated in such a way that the dilution of the supernatant fluid in the nutrient medium does not exceed 1 in 4. The area of the cell sheet should be at least 3 cm^2 per mL of supernatant fluid. At least one culture vessel of the cell cultures should remain uninoculated and should serve as a control. The cells inoculated with the supernatant fluid and the uninoculated control cultures should be incubated at 37 °C and observed at appropriate intervals for an additional 2 weeks.

The virus master seed lot passes the test if there is no evidence of the presence of adventitious agents. For the test to be valid, not more than 20% of the culture vessels should have been discarded for any reason by the end of the observation period.

> New molecular methods with broad detection capabilities are being developed for the detection of adventitious agents. These methods include: (a) degenerate NAT for whole virus families, with analysis of the amplicons by hybridization, sequencing or mass spectrometry; (b) NAT with random primers followed by analysis of the amplicons on large oligonucleotide micro-arrays of conserved viral sequencing, or digital subtraction of expressed sequences; and (c) high-throughput sequencing. These methods might be used in the future to supplement existing methods or as alternative methods to both in vivo and in vitro tests after appropriate validation and with the approval of the NRA *(28)*.
>
> The theoretical risk of the presence of potential human, simian, bovine or porcine adventitious agents in the seed lots, which may be derived from the use of bovine serum or porcine trypsin, should be

assessed. If necessary, viruses such as bovine polyomavirus, porcine parvovirus or porcine circovirus may be screened for using specific assays, such as molecular NAT-based assays *(28)*.

The need for adventitious virus testing on working seed lot should be based on risk assessment. However, sterility testing for bacteria, fungi and mycoplasmas should be conducted.

A.3.1.3.2 Identity test

The strain identity of the seed lot should be determined by infectivity tests. The test for antigen content described in section A.4.4.2.2 below can be used to identify the seed lot.

A.3.1.3.3 Virus titration

The virus concentration of the seed lot should be determined by titration of infectious virus using validated tissue culture methods. This titration should be carried out in not more than 10-fold dilution steps using 10 cultures per dilution, or by any other arrangement yielding equal precision.

The use of human RD, human diploid or Vero cells in microtitre plates is suitable for this purpose *(28)*.

A.3.2 Cell lines

The general production precautions, as formulated in WHO good manufacturing practices for biological products *(27)*, should apply to the manufacture of EV71 vaccine, with the additional requirement that, during production, only one type of cell should be introduced or handled in the production area at any one time. Vaccines may be produced in a human diploid cell line or in a continuous cell line.

A.3.2.1 Master cell bank (MCB) and working cell bank (WCB)

The use of a cell line for the manufacture of EV71 vaccine should be based on the cell bank system. The cell seed and cell banks should conform to 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 *(28)*. The MCB should be approved by the NRA. The maximum number of passages (or population doublings) by which the WCB is derived from the MCB and the maximum number of passages of the production cultures should be established and confirmed through process validation and characterization of end-of-production cell culture by the manufacturer and approved by the NRA.

The WHO Vero reference cell bank 10-87 is considered suitable for use as a cell seed for generating an MCB *(59)* and is available to manufacturers on application to the Group Lead, Norms and Standards for Biologicals, Technical Specifications and Standards, Department of Health Product Policy and Standards, Access to Medicines and Health Products Division, World Health Organization, Geneva, Switzerland.

A.3.2.2 Identity test

Identity tests on the MCB and WCB should be performed in accordance with 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 *(28)* and should be approved by the NRA.

The cell banks should be identified by means of tests such as biochemical tests (for example, isoenzyme analysis), immunological tests, cytogenetic marker tests and DNA fingerprinting or sequencing. The tests used should be approved by the NRA.

A.3.3 Cell culture medium

Where serum is used for the propagation of cells it should be tested to demonstrate freedom from bacterial, fungal and mycoplasmal contamination – as specified in Part A, sections 5.2 *(60)* and 5.3 *(61)* of the WHO General requirements for the sterility of biological substances – as well as freedom from infectious viruses. Suitable tests for detecting viruses in bovine serum are given in Appendix 1 of 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 *(28)*.

Validated molecular tests for bovine viruses may replace the cell culture tests of bovine sera if approved by the NRA. As an additional monitor of quality, sera may be examined for freedom from bacteriophage and endotoxin. Gamma irradiation may be used to inactivate potential contaminant viruses, while recognizing that some viruses are relatively resistant to gamma irradiation.

The source(s) of animal components used in the culture medium should be approved by the NRA. The components should comply with the current *WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (62)*. The serum protein concentration should be reduced by rinsing the cell cultures with serum-free medium and/or by purification of the virus harvests.

> In some countries, control tests are carried out to detect the residual animal serum content in the final vaccine.

Human serum should not be used. If human serum albumin is used at any stage of product manufacture, the NRA should be consulted regarding the requirements, as these may differ from country to country. At a minimum, it should meet the WHO Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives *(63)*. In addition, human albumin, as with all materials of animal origin, should comply with the current *WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (62)*.

> Manufacturers are encouraged to explore the possibilities of using serum-free media for the production of EV71 vaccine.

Penicillin and other beta-lactams should not be used at any stage of manufacture because they are highly sensitizing substances in humans. Other antibiotics may be used during early stages of production. In this case, the use of antibiotics should be well justified, and they should be cleared from the manufacturing process at the stage specified in the marketing authorization. Clearance should be demonstrated through a residual removal study (or studies) and acceptable levels should be approved by the NRA.

Bovine or porcine trypsin used for preparing cell cultures should be tested and found to be free of cultivatable bacteria, fungi, mycoplasmas and infectious viruses, as appropriate *(28)*. The methods used to ensure this should be approved by the NRA.

> In some countries, irradiation is used to inactivate potential contaminant viruses. If irradiation is used, it is important to ensure that a reproducible dose is delivered to all lots and to the component units of each lot. The irradiation dose must be low enough for the biological properties of the reagents to be

retained but also high enough to reduce virological risk. Therefore, irradiation cannot be considered a sterilizing process *(28)*.

Recombinant trypsin is available and should be considered; however, it should not be assumed to be free from risk of contamination and should be subject to the usual considerations for any reagent of biological origin *(28)*.

The source(s) of trypsin of bovine origin, if used, should be approved by the NRA and should comply with the current *WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (62)*.

A.4 Control of vaccine production

A.4.1 Control cell cultures

A fraction of the production cell culture equivalent to at least 5% of the total or 500 mL of cell suspension, or 100 million cells, 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 NRA should determine the size and treatment of the cell sample to be examined.

A.4.1.1 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 any adventitious agents.

These control cell cultures should be incubated under conditions as similar as possible to the inoculated cultures for at least 2 weeks, and should be tested for the presence of adventitious agents as described below. For the test to be valid, not more than 20% of the control cell cultures should have been discarded for any reason by the end of the test period.

At the end of the observation period, the control cell cultures should be examined for evidence of degeneration caused by an adventitious agent. If this examination or any of the tests specified in this section shows evidence of the presence of any adventitious agent in the control culture, the EV71 grown in the corresponding inoculated cultures should not be used for vaccine production.

If not tested immediately, samples should be stored at −60 °C or below.

A.4.1.2 Tests for haemadsorbing viruses

At the end of the observation period, at least 25% of the control cells should be tested for the presence of haemadsorbing viruses using guinea-pig red blood cells. If the latter cells have been stored, the duration of storage should not have exceeded 7 days and the storage temperature should have been in the range of 2–8 °C. In tests for haemadsorbing viruses, calcium and magnesium ions should be absent from the medium.

Some NRAs require, as a test for haemadsorbing viruses, that other types of red blood cells, including cells from humans, monkeys and chickens (or other avian species), are also used instead of guinea-pig cells alone.

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

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

In some countries the sensitivity of each new lot of red blood cells is demonstrated by titration against a haemagglutinin antigen before use in the test for haemadsorbing viruses.

A.4.1.3 Tests for other adventitious agents in cell supernatant fluid

At the end of the observation period, a sample of the pooled supernatant fluid from each group of control cultures should be tested for adventitious agents. For this purpose, 10 mL of each pool should be tested in the same cells, but not the same lot of cells, as those used for the production of vaccine.

A second indicator cell line should be used to test an additional 10 mL sample of each pool. When a human diploid cell line is used for production, a simian kidney cell line should be used as the second indicator cell line. When a simian kidney cell line is used for production, a human diploid cell line should be used as the second indicator cell line *(28)*.

The pooled fluid should be inoculated into culture vessels of these cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1 part in 4. The area of the cell sheet should be at least 3 cm^2 per mL of pooled fluid. At least one culture vessel of each kind of cell culture should remain uninoculated and should serve as a control.

The inoculated cultures should be incubated at the same temperature as that of the production of virus antigen and observed at appropriate intervals for a period of at least 14 days.

Some NRAs require that, at the end of this observation period, a subculture is made in the same culture system and observed for at least an additional 14 days. Furthermore, some NRAs require that these cells should be tested for the presence of haemadsorbing viruses.

For the tests to be valid, not more than 20% of the culture vessels should have been discarded for any reason 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 lot of cells from which the control cells were taken should be discarded.

Some selected viruses may be screened for using specific validated assays which are approved by the NRA, such as molecular NAT-based assays *(28)*.

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

A.4.1.4 Identity tests

At the production level, the control cells should be identified using tests approved by the NRA.

Suitable methods include, but are not limited to, biochemical tests (for example, isoenzyme analyses), immunological tests, cytogenetic tests (for example, for chromosomal markers), morphological identification and tests for genetic markers (for example, DNA fingerprinting or sequencing).

A.4.2 Cell cultures for vaccine production

A.4.2.1 Observation of cultures 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 visually for degeneration caused by infective agents.

If this examination shows evidence of the presence of any adventitious agent in a cell culture then the culture should not be used for vaccine production.

A.4.3 Control of single harvests

After inoculation of the production cells with virus, the culture conditions of inoculated and control cell cultures should be standardized and kept within limits agreed with the NRA.

Samples required for the testing of single harvests should be taken immediately on harvesting.

Samples may be taken after storage and filtration with the agreement of the NRA.

A.4.3.1 Identity test

The single harvests or pool of single harvests should be identified as EV71 by serum neutralization in cell culture infectivity assays using specific antibodies, or by molecular methods such as NAT-based assays. The test for antigen content described in section A.4.4.2.2 below can be used to identify the single harvest.

A.4.3.2 Sterility tests for bacteria, fungi and mycoplasmas

A volume of at least 10 mL of each single harvest should be tested for bacterial, fungal and mycoplasmal contamination using appropriate tests, as specified in Part A, sections 5.2 *(60)* and 5.3 *(61)* of the WHO General requirements for the sterility of biological substances, or by methods approved by the NRA.

NAT alone or in combination with cell culture, with an appropriate detection method, may be used as an alternative to one or both of the compendial mycoplasma detection methods following suitable validation and the agreement of the NRA *(28)*.

In some countries this test is performed on the purified virus harvest instead of on the single harvest.

A.4.3.3 Virus titration

The virus concentration of each single harvest should be determined by titration of infectious virus using validated tissue culture methods to monitor production consistency and as a starting point for monitoring the inactivation curve. This titration should be carried out in not more than 10-fold dilution steps using 10 cultures per dilution, or by any other arrangement yielding equal precision.

The use of human RD, human diploid or Vero cells in microtitre plates is suitable for this purpose *(28)*. The same cells should be used for virus titrations throughout the production process.

Information on virus titre will help in selecting single harvests that can be expected to meet potency requirements after inactivation.

The virus titration may be carried out on the pooled harvest after demonstration of consistency of production at the stage of the single harvest.

A.4.4 Control of virus pools

Several single harvests may be mixed to prepare a pool of virus before inactivation. The order in which the purification, filtration and inactivation of virus pools is conducted should be carefully established by the manufacturer to ensure consistent full virus inactivation and absence of residual infectivity. Based on experience of the production of IPVs, the WHO

Recommendations to assure the quality, safety and efficacy of poliomyelitis vaccines (inactivated) *(23)* recommends purification, filtration and inactivation steps in that order.

> The requirement for filtration before and during inactivation was introduced into the IPV production process following the Cutter incident during which a number of paralytic polio cases occurred in children following vaccination with a defective IPV *(23)*. The vaccine used was later found to contain aggregates which led to incomplete virus inactivation likely due to formaldehyde not accessing some virus particles inside the aggregates.

Any deviation from the production sequence shown to be acceptable for IPV for a vaccine against a virus similar to poliovirus (such as EV71) should be fully validated and justified in terms of yielding a product of equivalent safety.

Inactivation of virus may be performed before or after purification according to current approved procedures for the production of licensed vaccines. The method of purification and inactivation as well as the agent used for inactivation should be appropriately validated and should be approved by the NRA.

If inactivation of the virus pool is conducted after purification, please see section A.4.4.1 below. If inactivation of the virus pool is conducted before purification please see section A.4.4.2 below.

A.4.4.1 Purification of virus pools

Each pool of virus should be purified. Removal of host cell protein should be assessed during process validation *(28)*.

> An acceptable method is to clarify the virus suspension by filtration, to concentrate the virus by ultrafiltration and, thereafter, to collect the virus peak after passing it through a gel-filtration column. Further purification is achieved by passing the virus through an ion-exchange column. Other purification procedures resulting in acceptable release criteria may be used.

A.4.4.2 Tests on virus pools (purified or not) before inactivation

A.4.4.2.1 Virus titration

The virus concentration of each virus pool should be determined by titration of infectious virus using validated tissue culture methods. This titration should be carried out in not more than 10-fold dilution steps using 10 cultures per dilution, or by any other arrangement yielding equal precision.

> The use of human RD, human diploid or Vero cells in microtitre plates is suitable for this purpose *(28)*. The same cells should be used for virus titrations throughout the production process.
>
> Information on virus titre will help in selecting pools that can be expected to meet potency requirements following inactivation.

A.4.4.2.2 Virus antigen content

The antigen content of each virus pool should be determined using a validated immunochemical method and should be calculated using a reference vaccine calibrated against the First WHO International Standard for EV71 inactivated vaccine (see **International reference materials** above) and expressed in IU.

A.4.4.2.3 Specific activity

The ratio of virus concentration or antigen content to the total protein content (specific activity) of the virus pool before inactivation should be within the limits of material shown to be safe and effective in clinical trials and approved by the NRA. This would ensure a consistent ratio of chemical agent to the viral protein, and thus a consistent inactivation process.

A. 4.4.3 Filtration before inactivation (purified or not)

In order to avoid interference with the inactivation process, virus aggregation should be prevented or aggregates should be removed immediately before and during the inactivation process. For this reason, each virus pool should be filtered before inactivation.

> Satisfactory results have been reported with several filter types but a final filtration using a 0.22 µm filter should be used.

Inactivation should be initiated as soon as possible and, in any case, not later than 72 hours after filtration.

> It is preferable to start inactivation within 24 hours of filtration. Since the purpose of the filtration step is to remove particulate matter and other interfering substances that may diminish the effectiveness of the inactivation process and since aggregates tend to increase on standing after filtration, efforts should be made to keep within this time limit.

A sample of the filtered virus pool should be retained and its virus titre determined as described in section A.4.4.2.1 above.

> The main purpose of determining the titre of filtered virus pools destined for inactivation is to provide the starting titre to monitor the kinetics of inactivation.

A.4.5 Control of inactivated bulk

A.4.5.1 Inactivation procedure

The virus in the filtered pools should be inactivated using a validated method approved by the NRA. Prior to inactivation, the concentration of the filtered pool (based on viral titre, virus antigen or protein content) should be adjusted to the acceptable range established during the process validation.

> Most manufacturers currently use formaldehyde as the method for inactivation but at least one manufacturer is using other inactivating agents such as beta-propiolactone (BPL).

The method of inactivation should have been shown to consistently inactivate EV71 virus without destroying the antigenic and immunogenic activity. Inactivation of the virus pool may take place before or after purification depending on the approved production process. The progress of inactivation should be monitored by suitably spaced determinations of virus titres. The inactivation period should usually exceed the time taken to reduce the titre of live virus to undetectable amounts by a factor of at least 2 and be agreed to by the NRA.

A second filtration should be carried out during the process of inactivation. This step is taken after the virus titre has fallen below detectable levels but before the first sample for the safety test is taken. Following this filtration step, the inactivation process should continue.

The kinetics of viral inactivation should be established by each manufacturer and approved by the NRA. During the validation studies, an inactivation curve should be established with at least four time points showing the decrease in live virus concentration

with time. The consistency of the inactivation process should be monitored; the virus titre and antigen content of each pool before, during and at the end of inactivation should be determined.

A record of consistency in the effectiveness and kinetics of inactivation should be established by the production of at least five consecutive lots and, if broken, a root-cause analysis should be performed and a further five consecutive filtered purified virus pools should be prepared and shown to be satisfactory for establishing this record.

A.4.5.2 Purification of inactivated virus pool

If inactivation is conducted using a non-purified virus pool, the inactivated pool should be purified as described in section A.4.4.1 above.

A.4.5.3 Tests on purified inactivated bulk

A.4.5.3.1 Test for effective inactivation

After removal or neutralization (as appropriate) of the inactivating agent, the absence of residual live EV71 virus should be verified by inoculating a quantity of the inactivated virus bulk equivalent to 5% of the lot (or not less than 1000 doses of vaccine) into sensitive cell cultures of the same type as those used for vaccine production.

The virus sample should be incubated for no less than 21 days making no fewer than two cell passages during that period. The dilution of the sample in the nutrient fluid should not exceed 1 in 4 and the area of the cell sheet should be at least 3 cm^2 per mL of sample. One or more culture vessels of each lot of cultures should be set aside to serve as uninoculated control culture vessels with the same medium. The sensitivity of the assay should be demonstrated.

> If formaldehyde has been used as the inactivating agent, samples of vaccine for tissue culture tests are generally neutralized at the time of sampling by the addition of bisulfite. Usually, the samples are subsequently dialysed or another validated method used.
>
> It is possible to conduct tissue culture tests on non-dialysed material. However, this is often found to be toxic to cells, even with a dilution of 1 in 4. If in such tests, nonspecific degeneration of cells occurs, or if the sensitivity of the tissue culture system is reduced, the test should be repeated on dialysed material. The virus antigen content after dialysis should be determined to ascertain whether the viral antigen was lost during the dialysis process.

If infectious virus is detected, the bulk should not be used for further processing. The isolation of live virus from an inactivated bulk should be regarded as a break in the manufacturing consistency record and a production process review and revalidation should be undertaken.

It is important to demonstrate that each test retains sensitivity to detect partially inactivated EV71 virus. At the end of the observation period, the cell culture used for the detection of residual live virus should be challenged with a validated amount of live EV71 virus of the same strain as that of the inactivated virus bulk. The details of the challenge procedure should be approved by the NRA. It is recommended that the ability to detect infectious virus is checked concurrently for each test by including a positive control at the beginning of each test. Positive control flasks should be inoculated with a low quantity of virus close to the detection limit of the method.

The problem of detecting residual active virus in an inactivated vaccine is not the same as that of measuring infective virus in untreated suspensions. Other similar viruses that have been exposed to the action of formaldehyde without becoming inactivated have been

shown to require a much longer period to produce cytopathic changes than untreated virus. For this reason, it is desirable that tissue cultures in tests for the presence of residual active virus are observed for as long a time as is technically possible. A satisfactory tissue culture system for this purpose depends, therefore, not only on the sensitivity of the cells used for the preparation of the cultures but also on the nutrient fluid.

Serum added to the nutrient fluid should be tested for inhibitors to EV71 at serum concentrations up to 50%. Only serum free from inhibitors should be used.

Maintenance of the cultures in good condition may require frequent changes of culture medium. However, it should be borne in mind that early changes of fluid may result in unadsorbed virus being removed and the validity of the test would thus be impaired. Therefore, the fluid should be changed no earlier than 5–7 days after inoculation.

A.4.5.3.2 Sterility tests for bacteria and fungi

Each purified inactivated bulk should be tested for bacterial and fungal sterility as specified in Part A, section 5.2 of the WHO General requirements for the sterility of biological substances *(60)*, or by methods approved by the NRA.

A.4.5.3.3 Antigen content

The EV71 antigen content of each purified inactivated bulk should be determined by the use of a validated immunochemical method and should be calculated using a reference vaccine calibrated against the First WHO International Standard for EV71 inactivated vaccine (see **International reference materials** above). The results obtained should be expressed in IU and be within the required limits established by the NRA.

A.4.5.3.4 Residual inactivating agent

The concentration of free residual formaldehyde or any other chemical used for inactivating the virus should be determined by a method approved by the NRA. The maximum acceptable limit should be approved by the NRA.

If BPL is used then its hydrolysation kinetics should be determined during the validation of the inactivation process to ensure that no residual BPL is present in the inactivated bulk at the end of the inactivation step.

A.4.5.3.5 Residual cellular DNA

If continuous cell lines are used for production, the purification procedure should have been shown to consistently reduce the level of residual host cell DNA *(28)*. The amount and size of residual host cell DNA should not exceed the maximum levels agreed with the NRA, taking into consideration issues such as those discussed in 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 *(28)*. Human diploid cell lines have been used successfully for many years for the production of viral vaccines and the residual cellular DNA deriving from these cells is not considered to pose any significant risk *(28)*.

This test can be performed on the purified virus bulk and may be omitted from routine testing, with the agreement of the NRA, if the manufacturing process is validated to achieve this specification *(28)*.

If assessed, the size distribution of the DNA may be considered as a characterization test, taking into account the amount of DNA detectable using appropriate methods, as approved by the NRA *(28)*.

A.4.5.3.6 Residual chemicals

If chemical substances are used during the purification process, tests for these substances should be carried out. Their concentration should not exceed the limits approved for the particular product.

A.4.5.3.7 Test for residual animal serum protein

If animal serum has been used in the cell culture system, a sample of purified bulk should be tested. The residual amount of serum albumin should be less than 50 ng per single human dose.

A.4.6 Control of final bulk

Preservatives, excipients or other substances that might be added to form the final bulk should have been shown, to the satisfaction of the NRA, to have no deleterious effect on the immunizing potency and safety profile of the EV71 antigens. Preservative efficacy should be demonstrated during product development using a method approved by the NRA.

The operations necessary for preparing the final bulk from the purified inactivated bulk should be conducted in such a manner as to avoid contamination of the product. In preparing the final vaccine bulk, any substances that are added to the product (such as diluents, stabilizers or adjuvants) should have been shown, to the satisfaction of the NRA, not to impair the safety and efficacy of the vaccine in the concentrations used. Until the final 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.4.6.1 Sterility tests for bacteria and fungi

The final bulk should be tested for bacterial and fungal sterility as specified in Part A, section 5.2 of the WHO General requirements for the sterility of biological substances *(60),* or by methods approved by the NRA.

A.4.6.2 Potency tests

Each final bulk should be tested using an in vivo assay for immunogenicity and in vitro antigen content assay approved by the NRA, unless this is to be performed on final product. Product-specific reference preparations may be used in these tests.

The EV71 antigen content of each final bulk should be determined using a validated immunochemical method and calculated using a reference vaccine calibrated against the First WHO International Standard for EV71 inactivated vaccine (see **International reference materials** above). The in vitro assay found to be the most suitable for measuring the antigen content is the EV71 antigen ELISA. The results obtained should be within the required limits established by the NRA.

Once consistency of production has been established for a suitable number of consecutive final bulks, the in vivo assay may be omitted for the purpose of routine lot release, with the agreement of the NRA. This can occur once it has been demonstrated that the acceptance criteria for the EV71 antigen determination are such that the in vitro test yields a comparable result to the in vivo assay in terms of acceptance or rejection of a lot. This demonstration should include testing of sub-potent lots, produced experimentally if necessary by heat treatment or other means of diminishing the immunogenic activity.

If an adjuvant is used in the final bulk, a desorption or treatment step may be necessary before performing the EV71 antigen ELISA.

If the final bulk is formulated with other antigens into a combination vaccine, the suitability of performing the EV71 antigen ELISA on the final bulk will have to be determined. If the EV71 antigen ELISA is not suitable for a particular combination, an in vivo assay should be used.

The potency of the final bulk should be approved by the NRA.

A.4.6.3 Preservative content (if applicable)

If preservative is added, its concentration in the final bulk should be determined by a method approved by the NRA. The preservative used and concentration permitted should be approved by the NRA. The preservative should not adversely affect the quality of the antigens.

A.4.6.4 Adjuvant (if applicable)

Each final vaccine bulk should be assayed for adjuvant content. This test may be omitted if it is to be performed on the final lot. Where aluminium compounds are used, the aluminium content should not exceed 1.25 mg per single human dose.

A.5 Filling and containers

The requirements concerning filling and containers given in WHO good manufacturing practices for pharmaceutical products: main principles *(58)* and WHO good manufacturing practices for biological products *(27)* should apply to vaccine filled in the final form. Single- and multi-dose containers may be used.

Care should be taken to ensure that the materials of which the container and (if applicable) the transference devices and closure are made do not adversely affect the quality of the vaccine.

Manufacturers should provide the NRA with adequate data to prove the stability of the product under appropriate conditions of storage and transport.

A.6 Control tests on the final lot

Samples should be taken from each final lot for the tests described in the following sections. The following tests should be performed on each final lot of vaccine (that is, in the final containers). Unless otherwise justified and authorized, the tests should be performed on labelled containers from each final lot by means of validated methods approved by the NRA. All tests and specifications, including methods used and permitted concentrations, should be approved by the NRA.

A.6.1 Inspection of final containers

Every container in each final lot should be inspected visually or mechanically, and those showing abnormalities should be discarded and recorded for each relevant abnormality. A limit should be established for the percentage of rejections permitted before triggering an investigation of the cause, potentially resulting in lot failure.

A.6.1.1 Appearance

The appearance of the vaccine should be described with respect to its form and colour.

A.6.2 Identity test

An identity test should be performed on at least one labelled container from each final lot using an appropriate method. The potency test described in section A.6.4 below may serve as the identity test.

A.6.3 Sterility tests for bacteria and fungi

Each final lot should be tested for bacterial and fungal sterility as specified in Part A, section 5.2 of the WHO General requirements for the sterility of biological substances *(60)*, or by methods approved by the NRA.

A.6.4 Potency test

The potency of each final lot should be determined using an in vivo assay and a validated in vitro antigen content assay (see section A.4.6.2 above) if such a test has not been performed on the final bulk. Potency should be calculated using a reference vaccine calibrated against the First WHO International Standard for EV71 inactivated vaccine (see **International reference materials** above) or other reference preparation.

If the use of an adjuvant in the final bulk interferes with the assay, a desorption or treatment step may be necessary. If treatment/desorption is not possible, the interference of the adjuvant should be documented and an in vivo assay should be performed (see section A.4.6.2 above).

The potency of the vaccine should be approved by the NRA.

A.6.5 Preservative content

Where appropriate, the preservative content of each final lot should be determined. The method used and content permitted should be approved by the NRA. This test may be omitted if conducted on the final bulk.

A.6.6 Endotoxin content

The endotoxin content of each final lot should be determined using a method approved by the NRA. Endotoxin levels should be consistent with levels found to be acceptable in vaccine lots used in pre-licensure clinical trials and should be approved by the NRA.

A.6.7 Residual formaldehyde

The concentration of free residual formaldehyde in each final lot should be determined using a method approved by the NRA. The acceptable maximum limit should be approved by the NRA. This test may be omitted if performed on the final bulk.

A.6.8 pH

The pH of each final lot should be determined and should be within limits approved by the NRA.

A.6.9 Adjuvant and degree of adsorption (if applicable)

If an adjuvant is used in the formulation, each final lot should be assayed for adjuvant content. Where aluminium compounds are used, the aluminium content should not exceed 1.25 mg per single human dose.

The degree of adsorption of the antigen to the aluminium compounds (aluminium hydroxide or hydrated aluminium phosphate) in each final lot should be assessed.

Both tests may be omitted for routine lot release upon demonstration of consistency of production, subject to the agreement of the NRA.

A.6.10 Residual antibiotics (if applicable)

If any antibiotics are added during vaccine production, the residual antibiotic content should be determined and should be within limits approved by the NRA. This test may be omitted for routine lot release once consistency of production has been established to the satisfaction of the NRA.

> If aluminium adsorption has an impact on the test, then testing for antibiotic content may be done at the purified inactivated bulk stage.

A.6.11 Extractable volume

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

For vaccines filled into multi-dose containers, the extractable content should be checked and should be shown to be sufficient for the intended number of doses.

A.7 Records

The requirements given in WHO good manufacturing practices for pharmaceutical products: main principles *(58)* and WHO good manufacturing practices for biological products *(27)* should apply.

A.8 Retained samples

The requirements given in WHO good manufacturing practices for pharmaceutical products: main principles *(58)* and WHO good manufacturing practices for biological products *(27)* should apply.

A.9 Labelling

The requirements given in WHO good manufacturing practices for pharmaceutical products: main principles *(58)* and WHO good manufacturing practices for biological products *(27)* should apply, and additionally the label on the container or package, or the leaflet accompanying each container, should include the following information:

- the designation(s) of the strain(s) of EV71 contained in the vaccine
- the cell substrate used for the preparation of vaccine
- the antigen content
- the method and inactivating agent used to inactivate the virus
- the nature and amount of any stabilizer and preservative present in the vaccine
- the nature and amount of adjuvant, if applicable.

> 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 antibiotics present in the vaccine, if any, may also be included.

A.10 Distribution and transport

The requirements given in WHO good manufacturing practices for pharmaceutical products: main principles *(58)* and WHO good manufacturing practices for biological products *(27)* should apply. Further guidance is provided in the WHO Model guidance for the storage and transport of time- and temperature-sensitive pharmaceutical products *(64)*.

A.11 Stability testing, storage and expiry date

A.11.1 Stability testing

Adequate stability studies form an essential part of vaccine development. Current guidance on the evaluation of vaccine stability is provided in the WHO Guidelines on stability evaluation of vaccines *(65)*. Stability testing should be performed at different stages of production when intermediate product is stored, namely on single harvests, purified inactivated bulk, final bulk and final lot. Stability-indicating parameters should be defined or selected appropriately according to the stage of production. During vaccine production a shelf-life should be assigned to all in-process materials – particularly intermediates such as single harvests, purified inactivated bulk and final bulk.

The stability of the vaccine in its final containers, maintained at the recommended storage temperature up to the expiry date, should be demonstrated to the satisfaction of the NRA. As a guide, containers from at least three consecutive final lots derived from different bulks may be tested.

Accelerated stability tests may be undertaken to provide additional information on the overall characteristics of the vaccine and may also aid in assessing comparability should the manufacturer decide to change any aspect of manufacturing.

The formulation of the vaccine should be stable throughout its shelf-life. Acceptable limits for stability should be agreed with the NRA. Following licensure, ongoing monitoring of vaccine stability is recommended to support shelf-life specifications and to refine the stability profile *(65)*. Data should be provided to the NRA in accordance with local regulatory requirements.

The final stability testing programme should be approved by the NRA and should include an agreed set of stability-indicating parameters, procedures for the ongoing collection and sharing of stability data, and criteria for rejecting vaccine(s).

A.11.2 Storage conditions

EV71 vaccine (inactivated) should be stored at all times at a temperature of 2–8 °C.

If a vaccine has been shown to be stable at temperature ranges higher than the approved 2–8 °C range, it may be stored under extended controlled temperature conditions for a defined period, subject to the agreement of the NRA *(66)*.

A.11.3 Expiry date

The expiry date should be based on the shelf-life as supported by stability studies and approved by the NRA. The start of the dating period should be based on the date of blending of the final bulk, the date of filling or the date of the first valid potency test on the final lot, and should be agreed with the NRA.

> Where an in vivo potency test is used, the date of the potency test is the date on which the test animals were inoculated with the final bulk.

Part B. Nonclinical evaluation of enterovirus 71 vaccines (inactivated)

Nonclinical evaluation of a new EV71 vaccine should follow the principles outlined in the WHO guidelines on nonclinical evaluation of vaccines *(25)* which provide details on the design, conducting, analysis and evaluation of nonclinical studies. Further guidance on the general principles for the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines can be found in separate WHO Guidelines *(29)*. The following sections B.1–B.3 provide specific guidance on addressing important issues related to the nonclinical development of a new inactivated whole EV71 virus vaccine.

B.1 Product characterization and process development

The vaccine lots used in nonclinical studies should be adequately characterized as described in Part A of these WHO Recommendations, taking into consideration the stage of product development. Both the antigen(s) of the vaccine and the end product need to be clearly defined, and the manufacturing process carefully monitored for all crucial steps so as to confirm the consistency of production. It is essential that sufficient data are generated to confirm the full inactivation of vaccine virus and the absence of virulent virus in the end product. Furthermore, sufficient vaccine stability data are necessary to support its suitability for use in the nonclinical studies.

It is crucially important that vaccine manufacturing processes are appropriately standardized and controlled to ensure consistency of production. The extent of product characterization may vary according to the stage of development. To support their validity, nonclinical studies should be carried out on vaccine lots that are adequately representative of the concurrent clinical lots in terms of their physicochemical data, stability, qualitative and quantitative impurity profiles, and formulation.

B.2 Nonclinical immunogenicity and protection studies

B.2.1 Evaluation of immunogenicity in animal models

Unless otherwise justified, the immunogenicity of any new EV71 vaccine needs to be characterized in relevant animal models (for example, mice, rats or rabbits) before proceeding to human trials. These proof-of-concept nonclinical studies should reflect the clinically proposed use of the vaccine, including the administration route, and should include an evaluation of serum neutralizing antibody response against isolated virus strains or pseudoviruses *(5–7, 67, 68),* and dose-range testing of the antigen. The immune response to the candidate vaccine should ideally be assessed after each dose of vaccine, and whenever possible against a licensed EV71 vaccine used as an active control. Data on cross-neutralizing antibodies should be obtained from nonclinical immunogenicity studies using a range of isolated heterologous viruses or pseudoviruses of different subgenogroups. These data may guide selection of the doses, dosing regimen and administration route to be evaluated in clinical trials.

When a candidate EV71 vaccine is formulated with a new adjuvant, a rationale for the selection of the adjuvant should be provided and the benefit of its inclusion in the vaccine formulation should be demonstrated by the immunogenicity data.

The immunogenicity studies in animals may additionally be considered, when appropriate, as part of a comparability exercise to demonstrate the reproducibility of the manufacturing process whenever major changes are introduced during the different stages of

process development or during the validation phase of a new candidate EV71 vaccine manufacturing process.

B.2.2 Challenge-protection studies

Current evidence suggests that serological immune responses play an essential role in mediating protection by formalin-inactivated whole EV71 virus vaccines. Animal studies conducted in newborn mice, transgenic mice and nonhuman primates have demonstrated that vaccination with inactivated EV71 vaccines induces protective immunity against EV71 and that protection in challenged animals is primarily mediated by neutralizing antibodies *(44, 50, 53, 69)*. Importantly, human efficacy trials conducted with several formalin-inactivated EV71 vaccines have shown a strong correlation between vaccine-induced serum neutralizing antibodies and protection against EV71-associated diseases *(5–7)*. Based on these observations, it is considered that, for a similarly manufactured candidate EV71 vaccine, no further challenge-protection studies in animal models need to be performed.

However, challenge-protection studies may be useful for any candidate EV71 vaccine based on a novel production process or intended to have novel mechanisms of action.

Since evidence from epidemiological studies and clinical trials for cross-protection against EV71 disease has thus far been limited, any claims of cross-protection should in general be supported by appropriate animal data. Specifically, challenge-protection studies should be conducted in appropriate animal models to evaluate the potential for protection against heterologous viruses of different genogroups, as this could indicate the breadth of protection.

B.3 Nonclinical safety studies

For a new EV71 vaccine based on inactivated whole EV71 virus, a repeat-dose toxicity study in a relevant animal species is generally needed to assess potential local and systemic toxicity and any other undesirable effects. Omission of standalone local tolerance and single-dose toxicity studies is possible if assessment of acute toxic effects and local tolerance has been incorporated into the repeat-dose toxicity study.

If the candidate vaccine contains a novel adjuvant, the principles set out in the WHO Guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines *(29)* should be followed. For example, consideration should be given to assessing the toxicity of the adjuvant alone.

For candidate EV71 vaccines manufactured using novel cell substrates, efforts should be made during the nonclinical safety study to explore biomarkers indicative of potential allergic reactions – for example, by measuring type 2 CD4+ T-helper cell responses.

Part C. Clinical evaluation of enterovirus 71 vaccines (inactivated)

C.1 Introduction

Clinical trials should adhere to the principles described in the WHO Guidelines for good clinical practice (GCP) for trials on pharmaceutical products *(70)* and the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations *(26)*. This section focuses only on issues relevant or specific to the clinical development of inactivated EV71 vaccines.

At present, no efficacy data on cross-protection are available from completed clinical trials with inactivated EV71 vaccines due to the distinct regional circulation of specific subgenogroups. In addition, no internationally recognized immune correlate of protection

(ICP) or surrogate marker of protection has been established. Although immunogenicity results from clinical trials suggest that a neutralizing antibody titre of 1:16 to 1:32 might be related to protection, further analysis based upon the use of a scaled logit model has indicated that significantly higher levels of neutralizing antibodies might be needed to achieve protection *(5–7, 54, 67)*.

If cross-protection against heterologous EV71 viruses is to be claimed then appropriate nonclinical and/or clinical studies should be conducted to evaluate the potential for such cross-protection (see section B.2.1 above). In addition, data demonstrating the ability of antibodies obtained from vaccinated individuals to neutralize various subgenogroups of EV71 viruses in vitro, including recently circulating isolates, are expected to be provided. Continuous evaluation of protective vaccine efficacy post licensure is encouraged due to the evolution of new EV71 strains or a rapid change of subgenogroups in different countries and regions, which may result in outbreaks. Should new subgenogroups emerge in a country in which the vaccine is licensed and used, further clinical investigation of potential cross-protection may be needed.

C.2 Assays

General guidance on the use and validation of assays for the evaluation of immune responses is provided in section 5.3.3 of the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations *(26)*.

Sections C.2.1 and C.2.2 provide specific guidance on the following assays relevant to the investigation of immune responses to inactivated human EV71 vaccines in clinical trials and to the confirmation of vaccine efficacy in pivotal studies respectively:

- serological assays for establishing the baseline serostatus of trial subjects and evaluating the humoral immune response to vaccination (see also section C.3); and
- detection assays for laboratory confirmation of HFMD and herpangina caused by EV71 infection, in vaccine efficacy trials (see also section C.4).

C.2.1 Serological assays

C.2.1.1 Functional antibody

The direct measurement of anti-EV71 neutralizing antibody is well established. Neutralizing antibody has been estimated using methods such as plaque reduction neutralization assays employing either isolated virus strains or pseudoviruses *(5–7, 67, 68)*. Sponsors are encouraged to develop high-throughput assays for anti-EV17 neutralizing antibody. These assays should be standardized using the First WHO International Standard for anti-EV71 serum (human) and the First WHO International Reference Reagent for EV71 neutralization assays (see **International reference materials** above). In addition, a reference neutralizing antibody panel for the evaluation of neutralizing antibody responses has been established in China *(14)*.

C.2.2 Virus detection assays

Since HFMD and herpangina can be caused by different human enteroviruses, including EV71, Coxsackie and echoviruses, appropriate RNA or virus detection assays are required to confirm the presence of EV71 in throat and vesicle swabs and/or stool samples (see section C.4.2 below). International guidance on enterovirus diagnostics and characterization should be taken into consideration *(4)*.

For other enterovirus infections, laboratory confirmation of diagnosis based on cell culture, virus isolation and virus identification remains a standard approach. The use of established cell lines such as human RD or Vero cells is recommended for virus isolation.

Various quantitative polymerase chain reaction (PCR) assays are commercially available. Although several EV71-specific PCR systems have been described, the ability of an assay to reliably detect EV71 RNA from specific subgenogroups should be taken into account when selecting the method to be used in clinical trials. In general, it is recommended that the subgenogroup be determined based upon VP1 gene sequences.

Sponsors should provide full details of the methodology applied, and appropriate controls should be used.

In addition, EV71 infection can be confirmed by the use of anti-EV71 immunoglobulin M (IgM) assays.

C.3 Immunogenicity

C.3.1 Formulation, dose and regimen

C.3.1.1 Primary series

EV71 vaccines will be used mainly or exclusively in regions with relatively high rates of clinically apparent infections. In naturally primed individuals the first dose of EV71 vaccine may elicit large increments in antibody due to an anamnestic response. In contrast, multiple doses of the same vaccine may be required to achieve similar antibody levels in EV71-naive subjects. Since pre-vaccination testing for EV71 serostatus will not be practical in routine use, it is important that the primary series should be selected on the basis of the immune responses observed in subjects who were seronegative prior to vaccination.

In the absence of an internationally established ICP for EV71 – that is, one meeting the WHO definition of an ICP *(26)* – the selection of the vaccine dose and regimen may be based on the reaching of a plateau antibody response unless this is precluded by concerns over reactogenicity. It is desirable that immunogenicity studies should explore the minimum number of doses and the shortest dose interval(s) required to achieve a plateau immune response.

C.3.1.2 Cross-protection

The ability of a candidate EV71 vaccine to protect against a range of wild-type strains covering the main EV71 genogroups may vary according to the vaccine strain used. For example, lower cross-neutralization against an atypical C2-like strain was observed in naturally infected EV71 patients *(71)* and in clinical trials using B4-based vaccine strains *(22)*.

In clinical trials in which vaccine-elicited antibody is determined against the antigen in the vaccine, it is recommended that neutralizing activity is also measured using antigens derived from a range of circulating wild-type EV71 strains from different (sub)genogroups. If marked differences are observed in measured antibody levels using vaccine versus non-vaccine strains, and/or by EV71 subgenogroup, it would be of particular interest to assess whether a similar effect is observed for functional antibody levels in naturally infected individuals.

C.4 Efficacy

C.4.1 Requirement for a demonstration of vaccine efficacy

It is currently recommended that licensure of a candidate EV71 vaccine should be based on evidence of its protective efficacy against clinically apparent HFMD and herpangina. The following considerations apply:

- At the time of preparing these WHO Recommendations, three vaccines against human EV71 had been licensed in one country (see **General considerations** above) *(4, 9, 17)*.
- These licensed vaccines are not yet widely used internationally. As a result, the use of a control group that does not receive vaccination against EV71 is possible.
- In jurisdictions in which a licensed vaccine is available, it is possible that individual NRAs may consider that licensure can be based on a trial that evaluates the efficacy of the candidate vaccine relative to that of the licensed vaccine in a population similar to that in which the efficacy of the licensed vaccine was established.
- The lack of an established ICP against EV71 does not rule out immunobridging a candidate vaccine to a licensed vaccine that has been shown to be efficacious. However, this approach is only possible if both vaccines contain the same antigen(s) so that anti-EV71 neutralizing antibody immune responses can be compared directly. In addition, the demonstration of efficacy of all three licensed vaccines was confined to EV71 subgenogroup C4 and it is not known whether protective efficacy may vary between genogroups circulating in different regions.

Taking these considerations into account, the focus of the following sections is on clinical development programmes that include vaccine efficacy trials in which the control group does not receive vaccination against EV71. However, most of the recommendations are also applicable to trials in which the control group receives a licensed vaccine against EV71. Clinical programmes leading to licensure based on immunobridging are not addressed in the following guidance. The general principles to be considered are discussed in sections 5.6.2 and 6.3.3 of the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations *(26)*.

C.4.2 Considerations for efficacy trial design

C.4.2.1 Primary objective

The primary objective will be to demonstrate that the candidate vaccine protects against clinically apparent (that is, symptomatic) HFMD and herpangina caused by EV71 infection regardless of the genogroup (see section C.4.2.4).

- It is not required for efficacy to be shown against asymptomatic EV71 infection as such infections are of no clinical significance.
- It is not required for vaccine efficacy trials to be powered to demonstrate genogroup-specific efficacy (see section C.4.2.2).

C.4.2.2 Trial sites

Efficacy trials will be conducted in endemic areas in which the estimated attack rate for HFMD and herpangina due to EV71 infection is sufficient to complete enrolment into an adequately powered vaccine efficacy trial within a reasonable time frame. Sites may be chosen on the basis of available public health disease-surveillance data and/or pre-trial evaluations of epidemiology conducted by the sponsor. In three prior efficacy trials *(6–9)* the EV71 viruses that caused clinically apparent HFMD and herpangina were limited to strains of

the C4 subgenogroup circulating at the trial sites in the years in which they were conducted. Sponsors are encouraged to consider selecting sites in a range of geographical areas in which strains of different genogroups are circulating and/or to conduct separate vaccine efficacy trials in regions with different genogroup distributions.

C.4.2.3 Subject selection criteria

Because of the age-dependent incidence and severity of EV71 infections it is likely that vaccine efficacy trials will target infants and children. An upper age limit may be set depending on the age-specific attack rates.

C.4.2.4 Primary end-point

In accordance with the recommended primary objective above, the primary end-point should be clinically apparent HFMD or herpangina that is confirmed to be due to EV71 infection. Sponsors could consider appointing an independent data-monitoring committee to review the data and determine which subjects meet the case definition to be counted in the primary analysis.

C.4.2.4.1 Clinical features for the case definition

The clinical features that cause subjects to present to study site staff or to a local designated health care facility for laboratory investigations for acute HFMD or herpangina should be identified with the aim of capturing as many cases as possible while limiting unnecessary investigations. On this basis, it is reasonable to define a possible case of HFMD or herpangina requiring laboratory investigation as an illness presenting with febrile illness accompanied by a papular or vesicular rash in the characteristic distribution on the oral mucosa, hands, feet or buttocks. A severe case of HFMD should be defined as associated with neurological, respiratory or circulatory complications as described by WHO *(4)*.

C.4.2.4.2 Laboratory confirmation of HFMD or herpangina caused by EV71 infection

It is recommended that laboratory confirmation of HFMD and herpangina cases should be conducted in a designated qualified central laboratory. If more than one central laboratory is necessary for practical reasons, it is essential that all of the laboratories use identical methodologies, and consideration should be given to testing a randomly selected subset of samples at each laboratory to assess concordance. The laboratory methods used should be validated.

The confirmation of EV71 as causative of the clinical picture should be based on any of the following:

- detection of EV71 RNA in vesicle/throat swabs or in stool;
- virus isolation and analysis of VP1 sequence;
- detection of IgM against EV71 – which is often detectable at the time of onset of clinical symptoms but which may peak after 1–2 weeks.

To avoid cases being missed, protocols should plan for appropriately timed repeat specimens to be collected from individuals with a first negative test for EV71 RNA (for example, 3–7 days after the first sample).

Samples obtained at first presentation and repeat specimens should also be tested to detect infection with other enteroviruses, such as Coxsackie and echoviruses, that can also cause HFMD and which regularly co-circulate with EV71 in affected countries.

C.4.2.5 Primary, secondary and other analyses

In a vaccine efficacy trial, it may be permissible for the primary analysis to include only confirmed cases of HFMD and herpangina caused by EV71 as follows:

- in subjects who completed the vaccination series within predetermined visit windows, if more than one dose is required; and
- with symptom onset occurring more than a defined period after the only or final dose of the series, taking into account what is known about the timing of the post-dose anti-EV71 IgG peak.

This approach gives the most optimistic estimation of vaccine efficacy.

If the primary analysis is confined to cases counted as described above, it is essential that predefined secondary analyses are carried out to estimate vaccine efficacy based on confirmed cases of clinically apparent HFMD and herpangina caused by EV71 infection, defined and counted as follows:

- all cases in subjects who received at least one assigned dose as randomized, and regardless of adherence to study visit windows;
- cases that occurred at any time after the last dose received (that is, counted from the day of dosing) in those who completed the assigned number of doses;
- cases that occurred after each sequential dose, depending on the number of doses in the series and counted from the day of dosing.

Vaccine efficacy should be explored according to EV71 genogroup if this is feasible, depending on the numbers of cases that occur due to individual genogroups.

It is recommended that an additional analysis should explore any differences in clinical or laboratory features (including severity) between cases that occur in the candidate vaccine group and the control group (whether the control group receives placebo or a licensed vaccine against EV71). The analysis should take into account whether the severity observed in individual subjects could reflect coinfection with other enteroviruses.

C.4.2.6 Case ascertainment

It is recommended that an active case-ascertainment strategy is used throughout the time frame of a vaccine efficacy trial. This is essential at least up to the time of the primary analysis, which may be conducted after a specific number of total cases has been accumulated or after a predefined period in which a sufficient number of cases are expected to occur to estimate vaccine efficacy.

C.4.2.7 Duration of protection

While the primary analysis may lead to licensure, it is recommended that trials continue to use active case ascertainment to follow up subjects for several years to provide data on waning vaccine protection, without un-blinding of treatment assignment at the level of the individual. These data can then be reported at some point after licensure of the vaccine and may point to a need for further doses to be administered at intervals to maintain protection, or a need to change the vaccine strains used.

C.4.2.8 Vaccine effectiveness

The need for vaccine effectiveness studies should be established at the time of licensure.

If longer-term follow-up within a pre-licensure trial is not considered to be feasible, the duration of vaccine protection should be investigated within a vaccine effectiveness study and/or as part of routine disease surveillance conducted by public health authorities. Furthermore, the efficacy of the vaccine against individual subgenogroups should be explored as part of a vaccine effectiveness study and/or during routine disease surveillance.

C.5 Safety

Evaluation of the safety of candidate EV71 vaccines should be undertaken in accordance with the recommendations made in section 7 of the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations *(26)*. If the primary series consists of several vaccine doses it is important to document whether reactogenicity increases with sequential doses. Additionally, the safety of post-primary doses should be evaluated. There may be special considerations for vaccine safety depending on the vaccine construct and the intended target population.

If a candidate vaccine is evaluated in a large pre-licensure trial, and if the safety profile documented during immunogenicity trials did not give rise to any major concerns, it may be acceptable for a full assessment of safety (that is, including detailed documentation of local and systemic reactogenicity, as well as all unsolicited adverse events) to be confined to a randomized subset of the total subjects. Any serious adverse event occurring in any subject enrolled at any of the trial sites should be documented.

Part D. Recommendations for NRAs

D.1 General recommendations

The guidance for NRAs and NCLs given in the WHO Guidelines for national authorities on quality assurance for biological products *(72)* and WHO Guidelines for independent lot release of vaccines by regulatory authorities *(30)* should be followed. These guidelines specify that no new biological product should be released until consistency of lot manufacturing and quality has been established and demonstrated by the manufacturer.

The detailed production and control procedures, as well as any significant changes in them that may affect the quality, safety or efficacy of inactivated EV71 vaccines *(73)*, should be discussed with and approved by the NRA. For control purposes, the relevant international reference preparations currently in force should be obtained for the purpose of calibrating national, regional and working standards as appropriate *(74)*. The NRA may obtain from the manufacturer the product-specific or working reference to be used for lot release.

Consistency of production has been recognized as an essential component in the quality assurance of inactivated EV71 vaccines. The NRA should carefully monitor production records and quality control test results for clinical lots, as well as for a series of consecutive lots of the final product.

D.2 Official release and certification

A vaccine lot should be released only if it fulfils all national requirements and/or satisfies Part A of these WHO Recommendations *(30)*.

A summary protocol for the manufacturing and control of enterovirus 71 vaccines (inactivated), based on the model summary protocol provided below in Appendix 1 and signed by the responsible official of the manufacturing establishment, should be prepared and submitted to the NRA/NCL in support of a request for the release of a vaccine for use.

A lot release certificate signed by the appropriate NRA/NCL official should then be provided if requested by the manufacturing establishment, and should certify that the lot of vaccine meets all national requirements and/or Part A of these WHO Recommendations. The certificate should provide sufficient information on the vaccine lot, including the basis of the release decision (by summary protocol review or independent laboratory testing). The purpose of this official national lot release certificate is to facilitate the exchange of vaccines between countries and should be provided to importers of the vaccines. A model NRA/NCL Lot Release Certificate for enterovirus 71 vaccines (inactivated) is provided below in Appendix 2.

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Appendix 1

Model summary protocol for the manufacturing and control of enterovirus 71 vaccines (inactivated)

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

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

The section concerning the final product should be accompanied by a sample of the label and a copy of the leaflet (package insert) 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 (see Appendix 2) from the NRA or NCL of the country in which the vaccine was produced and/or released stating that the product meets all national requirements as well as Part A of these WHO Recommendations.

1. Summary information on final lot

International name of product: _____
Commercial/trade name: _____
Product licence (marketing authorization) number: _____
Country: _____
Name and address of manufacturer: _____
Name and address of licence holder, if different: _____
Final packaging lot number: _____
Type of container: _____
Number of containers in this final lot: _____
Final container lot number: _____
Nature of final product (adsorbed): _____
Preservative and nominal concentration: _____
Volume of each single human dose: _____
Number of doses per final container: _____
Virus strain: _____
Cell substrate used for production: _____

Summary of composition (*summary of the qualitative and quantitative composition of the vaccine per single human dose, including any adjuvant used and other excipients*):

_____
_____

Shelf-life approved (months): _____
Date of manufacture: _____
Expiry date: _____
Storage conditions: _____

2. Detailed information on manufacture and control

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

Summary of source materials

Identity of seed lot strain used for vaccine production

Reference number of seed lot: _____
Date(s) of reconstitution (or opening) of seed lot container(s): _____

Test for adventitious agents

Methods: _____
Result: _____
Date: _____

Identity of cell bank used for vaccine production

Reference number of cell bank: _____
Date(s) of reconstitution (or opening) of cell bank container(s): _____

Identity test

Methods: _____
Result: _____
Date: _____

Control of vaccine production

Control cell cultures

Tests on control cell cultures

Ratio of control to production cell cultures: _____
Incubation conditions:_____
Period of observation of cultures:_____
Dates observation started/ended: _____
Ratio or proportion of cultures discarded: _____
Results of observation: _____

Tests for haemadsorbing viruses

Quantity of cell tested: _____
Method used:_____
Date of start of test-1: _____
Date of end of test-1:_____
Results: _____
Date of start of test-2: _____
Date of end of test-2:_____

Results: _____

Tests for adventitious agents on supernatant culture fluids

Method used:_____
Date of start of test:_____
Date of end of test: _____
Result: _____

Identity test

Method used:_____
Date of start of test:_____
Date of end of test: _____
Result: _____

Control of single harvests

Name of the culture medium: _____
Date of inoculation:_____
Temperature of incubation:_____
Microscopic observation_____
Result: _____
Date: _____
Date of harvest:_____
Volume of harvest: _____
Yield (mg/mL): _____

Sterility tests for bacteria, fungi and mycoplasmas

Test for bacteria and fungi
Method:_____
Media: _____
Volume inoculated:_____
Date of start of test:_____
Date of end of test: _____
Result: _____

Test for mycoplasmas (if applicable)
Method:_____
Volume inoculated:_____
Date of start of test:_____
Date of end of test: _____
Result: _____

Virus titration
Method:_____
Reference lot no._____
Date: _____
Result: _____

Control of virus pool

Lot number of virus pool: _____
Date of pooling: _____
Number of harvests: _____
Volume(s), storage temperature, storage time and approved storage period:_____
_____

Purification of virus pool (may be performed after inactivation)

Purification methods: _____
Volume before purification: _____
Volume after purification:_____
Date: _____

Tests on virus pool

Virus titration
Method:_____
Reference lot no._____
Date: _____
Result:_____

Virus antigen content
Method:_____
Reference lot no._____
Date: _____
Result:_____

Specific activity
Virus antigen content:_____
Total protein content: _____
Specification: _____
Date: _____
Result:_____

Inactivation of harvest pool (may be performed before purification)

Filtration before inactivation
Filtration method: _____
Date: _____
Time of start of filtration: _____
Time of end of filtration:_____

Inactivation
Agent(s) and concentration of inactivation agent: _____
Temperature of inactivation:_____
Date of start of inactivation: _____
Virus antigen units at start of inactivation:_____
Date of taking first samples: _____
Date of completion of inactivation: _____

Virus antigen units at end of inactivation: _____

Filtration during inactivation

Filtration method: _____
Date: _____
Time of start of filtration: _____
Time of end of filtration:_____

Control of inactivated bulk

Test for effective inactivation (after removal/neutralization of inactivating agent)

Sample size tested: _____
Dates of sampling (1–4):_____
Test method: _____
Period of observation of cell cultures: _____
Period of observation of subcultures: _____
Period of observation of subcultures: _____
Period of observation of subcultures: _____
Result: _____

Sterility tests for bacteria and fungi

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

Antigen content

Method:_____
Specification: _____
Date: _____
Result: _____

Test for residual inactivating agent

Method:_____
Specification: _____
Date: _____
Result: _____

Test for residual host-cell DNA (if applicable)

Method:_____
Specification: _____
Date: _____
Result: _____

Test for residual chemicals (if applicable)

Method: _____
Specification: _____
Date: _____
Result: _____

Test for residual animal serum protein (if applicable)

Method: _____
Specification: _____
Date: _____
Result: _____

Control of final bulk

Identification (lot number): _____
Date of manufacture/blending: _____
Volume(s), storage temperature, storage time and approved storage period: _____
_____

Blending:		Prescription (per dose)	Added
	Virus antigen (IU or unit):	_____	_____
	Adjuvant:	_____	_____
	Preservative (specify):	_____	_____
	Others (chemicals):	_____	_____
	Final volume (mL):	_____	_____

Sterility tests for bacteria and fungi

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

Potency test

In vivo assay (may be performed at final bulk stage)

Species, strain, sex and weight specifications: _____
Number of mice tested: _____
Dates of vaccination, bleeding: _____
Date of assay: _____
Lot 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 (with 95% fiducial limits): _____

If an in vitro assay is used

Method: _____
Specification: _____
Date: _____
Result: _____

Adjuvant content

Method: _____
Specification: _____
Date: _____
Result: _____

Preservative content (if applicable)

Method: _____
Specification: _____
Date: _____
Result: _____

Control of final lot

Lot number: _____
Date of filling: _____
Type of container: _____
Filling volume: _____
Number of containers after inspection: _____
Number and percentage of containers rejected: _____

Appearance

Method: _____
Specification: _____
Date: _____
Result: _____

Identity test

Method: _____
Specification: _____
Date: _____
Result: _____

Sterility tests for bacteria and fungi

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

Potency test

In vivo assay (may be performed at final bulk stage)

Species, strain, sex and weight specifications: _____
Number of mice tested:_____
Dates of vaccination, bleeding:_____
Date of assay: _____
Lot 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 (with 95% fiducial limits): _____

If an in vitro assay is used

Method:_____
Lot number of reference and assigned potency: _____
Specification: _____
Date: _____
Result:_____

Preservative content (if applicable)

Method:_____
Specification: _____
Date: _____
Result:_____

Endotoxin content

Method:_____
Specification: _____
Date: _____
Result:_____

pH

Method:_____
Specification: _____
Date: _____
Result:_____

Adjuvant content

Method:_____
Specification: _____
Date: _____
Result:_____

Degree of adsorption

Method:_____
Specification: _____
Date: _____

Result: _____

Residual antibiotics (if applicable)

Method: _____
Specification: _____
Date: _____
Result: _____

Extractable volume

Method: _____
Specification: _____
Date: _____
Result: _____

3. Certification by the manufacturer

Name of head of production and/or quality control (typed) _____

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

I certify that lot no. _____ of enterovirus 71 vaccine (inactivated), whose number appears on the label of the final containers, meets all national requirements and satisfies Part A[1] of the WHO Recommendations to assure the quality, safety and efficacy of enterovirus 71 vaccines (inactivated).[2]

Signature _____

Name (typed) _____

Date _____

4. Certification by the NRA/NCL

If the vaccine is to be exported, attach the model NRA/NCL Lot Release Certificate for enterovirus 71 vaccines (inactivated) (as shown in Appendix 2), a label from a final container and an instruction leaflet for users.

[1] With the exception of provisions on distribution and transport, which the NRA may not be in a position to assess.
[2] WHO Technical Report Series, No. XXXX, Annex 3.

Appendix 2

Model NRA/NCL Lot Release Certificate for enterovirus 71 vaccines (inactivated)

This certificate is to be provided by the NRA or NCL of the country in which the vaccine has been manufactured, on request by the manufacturer.

Certificate no._____

The following lot(s) of enterovirus 71 vaccine (inactivated) 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 enterovirus 71 vaccines (inactivated),[5] and comply with WHO good manufacturing practices for pharmaceutical products: main principles;[6] WHO good manufacturing practices for biological products;[7] and the WHO Guidelines for independent lot release of vaccines by regulatory authorities.[8]

The release decision is based on_____[9]

Final lot number_____

Number of human doses released in this final lot_____

Expiry date_____

[1] Name of manufacturer.
[2] Country of origin.
[3] If any national requirements have not been met, specify which one(s) and indicate why the release of the lot(s) has nevertheless been authorized by the NRA or NCL.
[4] With the exception of provisions on distribution and transport, which the NRA or NCL may not be in a position to assess.
[5] WHO Technical Report Series, No. XXXX, Annex 3.
[6] WHO Technical Report Series, No. 986, Annex 2.
[7] WHO Technical Report Series, No. 999, Annex 2.
[8] WHO Technical Report Series, No. 978, Annex 2.
[9] Evaluation of the product-specific summary protocol, independent laboratory testing and/or specific procedures laid down in a defined document, and so on as appropriate.

The certificate may also include the following information:

- name and address of manufacturer;
- site(s) of manufacturing;
- trade name and/or common name of product;
- marketing authorization number;
- lot number(s) (including sub-lot numbers and packaging lot numbers if necessary);
- type of container;
- number of doses per container;
- number of containers or lot size;
- date of start of period of validity (for example, manufacturing date);
- storage conditions;
- signature and function of the person authorized to issue the certificate;
- date of issue of certificate.

The Director of the NRA/NCL (or other appropriate authority)

Signature _____

Name (typed) _____

Date _____