MEETING REPORT

WHO informal consultation on the scientific basis of specifications for production and control of inactivated Japanese encephalitis vaccines for human use, Geneva, Switzerland, 1-2 June 2006[1]

Summary

The World Health Organization (WHO) publishes technical guidance on the quality, safety and efficacy of vaccines intended to assist national regulatory authorities and manufacturers. As part of its programme, WHO convened an informal consultation to initiate revision of the WHO recommendations on the production and control of inactivated Japanese encephalitis (JE) vaccines for human use, at its headquarters in June 2006. The attendees include experts from national regulatory authorities, national control laboratories and manufacturers from around the world as per WHO policy to include all relevant stakeholders in the standards development process. Issues pertaining to recent development with inactivated JE vaccines were presented and discussed. Participants agreed upon the scientific basis of revised specifications and the inclusion of new sections on nonclinical and clinical evaluation of inactivated JE vaccines. It was agreed that the revision will cover both existing vaccines derived from mouse brain or primary cell cultures and vaccines under development in continuous cell line cells including Vero cells.

Keywords: Japanese encephalitis; Viral vaccine; World Health Organization

1. Introduction

Japanese encephalitis (JE) is an important public health problem as it is the leading cause of viral encephalitis in Asia and more than 3 billion people live in areas where JE is transmitted. It is estimated that the JE virus causes at least 50,000 cases of clinical disease each year, mostly among children aged <10 years, resulting in about 10,000 deaths and 15,000 cases of long-term, neuropsychiatric sequelae [1]. The age groups affected are mainly children under 15 years of age but any age group may be affected. Transmission of JE is seasonal in some areas, but year-round in others. It is accepted that there is under reporting of cases mainly due to lack of surveillance and laboratory diagnosis.

Control of JE can be theoretically achieved by mosquito control, pig control or human interventions. Of these, vaccination has been proven to be the only effective long term method for control and prevention of JE. There is abundant evidence that the use of JE vaccines is both effective [2-5] and cost-effective [6,7] for controlling JE.

Vaccines currently available include inactivated vaccines produced in mouse brain, primary hamster kidney (PHK) or Vero cells, and live attenuated vaccines. Mouse brain-derived inactivated JE vaccines are produced in many Asian countries. PHK- or

[1] Disclaimer: This report contains the collective views of an international group of experts, and does not necessarily represent the decisions or the stated policy of the World Health Organization.

Vero cell-derived inactivated JE vaccines have been used in the Peoples Republic of China only. There is an increasing demand for JE vaccines in the Asian region at present. Vero cell-derived inactivated JE vaccines are under development by at least three manufacturers [2].

Manufacturers and regulatory authorities face a number of challenges in assuring the quality, safety and efficacy of new inactivated JE vaccines. These include control of animals and cell cultures used for production; nonclinical and clinical testing and evaluation; and potency assay of inactivated JE vaccines.

Dr David Wood (WHO) reminded participants in his opening remarks that the purpose of the meeting was to start the process of revising the current WHO recommendations for JE vaccines (inactivated) for human use [8]. This would include a discussion on the scientific basis of specifications for production and control of the vaccines. The WHO recommendations and guidelines for production and control of vaccines and other biological medicines are scientific and advisory in nature and intended to assist countries and manufacturers to assure the quality, safety and efficacy of biological products of public health importance.

Since the first adoption of the WHO recommendations for JE vaccines in 1987 by the WHO Expert Committee on Biological Standardization (ECBS), significant changes in the production of inactivated JE vaccines have been made. In particular, the production substrate is being changed from mouse brain to continuous cell lines. Further since the previous document had been published, there had been policy changes at WHO so that in addition to providing guidance on production and quality control (QC), the intent is now to include guidance on nonclinical evaluation and clinical evaluation.

The objectives of the meeting were (i) to review in detail the existing WHO recommendations for the production and control of inactivated JE vaccines for human use with emphasis on the scientific basis of specifications to be set out in revised recommendations; (ii) to discuss the scientific basis of nonclinical and clinical evaluation of continuous cell line derived inactivated JE vaccines; and (iii) to identify the need of development of appropriate standards and standardization of assays for inactivated JE vaccines.

2. Biological Standardization

Dr Wood summarized WHO activities and plans for assuring quality, safety and efficacy of vaccines. Participants were reminded that the goals of WHO were to facilitate the delivery of vaccines of assured quality. This is achieved by ensuring that national regulatory authorities (NRAs) are fully functional and independent of the manufacturer, and that no unresolved quality, safety or efficacy problems are present where vaccines are concerned. WHO utilizes the experience and knowledge of experts in the relevant fields in member states by bringing them together to facilitate global dissemination and exchange of information. WHO also has a mandate to develop and establish global written and measurement standards. The latter serves, for example, as the reference preparations used to benchmark potency tests.

The global written standards are technical specifications which are scientific and advisory in nature and include explanatory text. The scope is to provide guidance for NRAs and manufacturers on international regulatory expectations for the production and quality control and also for nonclinical and clinical evaluation of vaccines. They also facilitate international harmonization of vaccine licensure. These are living documents revised in response to scientific advances. The WHO recommendations for inactivated JE vaccines were published almost 20 years ago and therefore we are in urgent need of revision. Certain scientific issues had been reviewed in the meantime to support informed decision making. For example, based on animal models as well as findings from JE vaccine clinical trials, a threshold of neutralizing antibodies equal to or greater than 1:10 has recently been agreed as evidence of protection [9].

In addition to the guidance documents specific for vaccine products, WHO also has several existing general guidance documents for vaccines. Working groups are set up as required to consider technical risk reduction strategies for specific issues such as residual DNA from cell substrates. The availability of such guidance facilitates harmonization of vaccine licensure as well as global regulatory convergence, e.g. guidance on transmissible spongiform encephalopathies (TSE) [10].

Global measurement standards facilitate the comparison of results from different laboratories and support harmonization of international regulations and also define the international unit (IU). These reference standards facilitate the development of in vitro biological diagnostics, vaccines and therapeutics, and are recognized by other international organizations e.g. World Trade Organization (WTO).

Dr Wood charged the group to identify issues which need to be considered for JE. This would include consideration of standardization issues. Although there is no international reference preparation for inactivated JE vaccine, there is an ongoing initiative in WHO South-East Asia Region (SEAR) to develop a regional reference preparation for inactivated JE vaccine. With respect to antibody standards, a candidate international reference preparation for anti-JEV antibody was assessed but not considered suitable for purpose by the 56th ECBS (2005) as the use of the candidate preparation did not result in an improvement of between-laboratory results. In assays of sera from vaccinees immunized with inactivated vaccines manufactured from different virus seeds, a wide variation in titres was seen, especially when differing challenge viruses were used for the assays.

The WHO recommendations on production and quality control of vaccines are used as the basis of national regulations in many countries and also serve as the technical specifications against which compliance is assessed for the purposes of prequalification (PQ) of vaccine supply by United Nations (UN) agencies. Sources of vaccines used in national immunization programme (NIP) vary but those procured by UN agencies all go through PQ schemes. At present, there are no JE vaccines inactivated or live, which are prequalified.

WHO also has programs to strengthen NRAs which are based on a 5-step capacity building programme. The capacity building involves benchmarking, NRA assessment, development of plans to address gaps, implementation of the plan, and monitoring and re-evaluation of the NRA. Six critical functions for vaccine regulatory system in a

country are evaluated through on-site NRA assessments. The number of functions required to be met by different NRAs depend on the source of vaccines, i.e. if countries have local vaccine producers, all 6 functions must be in place; procuring countries which must have 4 functions in place and countries whose vaccines are supplied by UN agencies must have 2 in place.

In addition, NRAs in developing countries face new challenges as the initial licensure of vaccines in high disease-burden countries is a regulatory strategy being used for some vaccines. In the past vaccines were always licensed first in the country of manufacture, often a developed country. In response to these needs, WHO is developing new support mechanisms for regulatory authorities through the establishment of the Developing Countries Vaccine Regulators Network (DCVRN). A Network of Sentinel Countries for the post marketing surveillance of new vaccines is also under development with rotavirus vaccines being used as a model for developing this network.

The Global Advisory Committee on Vaccine Safety (GACVS) has also been established to address issues relating to vaccine safety.

The use of JE vaccines in NIP is addressed in the WHO position paper on JE vaccines which has recently been revised [1]. There is a separate guidance on live JE virus vaccines [11] but this meeting focused only on the inactivated vaccines. Recombinant subunit JE vaccines are also being developed but are still at the preclinical stage. General guidelines for recombinant vaccines are available and therefore it was agreed that the document under development should focus on vaccines closest to clinical use.

3. Clinical Development

Dr Yuko Muraki (Biken, Japan) described the development of Vero cell-derived JE vaccines at Biken. This vaccine contains no preservative, is a single dose presentation and is freeze-dried

The production of vaccine in Vero cells reduces the risk of adventitious viruses being introduced into the production process compared to vaccines produced in mice. The QC tests for freedom from adventitious viruses for the Vero cell-derived vaccine include (i) tests on seed virus, which is based on the virus seed lot system; (ii) tests on the Vero cells, which are identified by the isoenzyme analysis; and (iii) tests on serum and trypsin used for the cell culture process. The cell culture medium is examined by the sterility tests, tested for freedom from abnormal toxicity (guinea pig), and by the endotoxin test. The QC tests are specified for each stage of production including master cell bank (MCB), working cell bank (WCB), master and working seed viruses, cell culture, virus suspension, inactivated virus suspension, purified concentrate, final bulk, and final lot which is freeze-dried. Additional tests for Vero cell vaccine include, on the purified virus concentrate, test for viral antigen content by enzyme-linked immunosorbent assay (ELISA), tests for cellular DNA, and on the final lot test, for animal serum content, consistency of fill and osmolality test.

A phase III clinical study of Vero-JE vaccine has been undertaken. The study involved administration of the two initial inoculations and then a booster a year later.

Sera were collected 1 month after second dose. Seroconversion rate of both groups was 100% . Seroconversion rate of Vero-JE vaccine group was not inferior to that of control comparator group (mouse brain-derived JE vaccine). No serious adverse events were observed. However a few common adverse events such as local reaction (redness, swelling) and fever (mainly mild to moderate) were reported. Following a booster dose given 6 to 24 months after the second dose of the primary inoculations, log geometric mean titer (GMT) of serum antibody rose from 2.65 to 4.08 as measured by a 50% plaque reduction neutralization ($PRNT_{50}$) assay.

The passage history of the primary seed virus was discussed and it was agreed that the testing of seed virus will be dependent on passage history particularly if mouse brain derived virus seeds are used.

The selection of clinical trials site was also discussed in relation to cross-reactivity with other flaviviruses and also antibody responses against other genotypes. Strains of JE virus belong to a single serotype but there are 5 genotypes. However it is accepted that vaccine from one genotype protects against infection with all other genotypes. Post-registration monitoring should be done to assess vaccine effectiveness.

Dr Shailesh Dewasthaly (Intercell, Austria) described the clinical development of a Vero cell-derived JE vaccine by Intercell. This vaccine is based on JEV strain SA14-14-2 produced in Vero cells, purified, inactivated and alum-adjuvanted. Phase I and II studies were done in USA by Walter Reed Army Institute of Research. Nonclinical studies undertaken included reproductive and developmental toxicology studies in rats as this vaccine will be marketed for travelers and may be administered to women of child bearing potential. Passive transfer study in mice to correlate PRNT titers and protection by two different strains of JEV is ongoing, and it was reported that general safety testing and mouse immunogenicity testing of every batch is performed before release. Clinical trials have been conducted in non-endemic countries (Europe, USA and Australia). A course of vaccination comprises 2 doses as compared to another inactivated JE vaccine which is administered in 3 doses. The phase III program involved 6 studies with 4,900 subjects in total.

The clinical studies required for licensure have involved: (i) a non-inferiority study against a licensed mouse-brain derived inactivated JE vaccine; (ii) a safety/consistency study with placebo control; and (iii) long term immunogenicity studies with sera taken 6, 12 and 24 months after final immunization. Additional studies have involved the concomitant administration of other vaccines. As the vaccine will be licensed for use in travellers, studies on rapid immunization and following booster doses are also pursued. Protective effect has only been assessed using passive transfer studies in mice in which challenge with different viruses are planned.

Based on this presentation, issues which need to be considered in the revision of the requirements included the need for reproductive toxicology studies if the vaccine was likely to be used in women of child bearing potential.

Dr Chenglin Xu (Beijing Tiantan Biological Products, BTBP, China) described the experience with Vero cell-derived inactivated JE vaccines produced by Tiantan in China. This is an inactivated Vero cell-derived vaccine which contains the P3 strain of

virus. Vero cells show a high susceptibility to JEV, and the P3 virus strain shows good proliferation in cultured cells. The virus antigen prepared by <10 passages in Vero cells provides good protection against intra-cerebral challenge in mice. However, the P3 strain with >10 passages in Vero cells has been shown to result in weakening of potency as measured by median infectious dose (ID_{50}) test in mice. The P3 primary seed was derived from mouse brain at passage 53 in mice. The primary seed was passaged once in Vero cells to give the master virus seed and then once more to give the working virus seed P3V2. This process was followed in order to minimize the presence of mouse brain protein and potential adventitious agents.

The manufacturing process involved inoculation of virus in Vero cells cultured in roller bottles, the clarification of the pooled culture media from the virus harvests, inactivation by formaldehyde, concentration, purification by protamine precipitation, sucrose gradient density zonal centrifugation, and sucrose removal by filtration. Virus antigen is stabilized by human albumin after which it is diluted, filled and lyophilized.

Phase I trials were undertaken with a vaccine formulated to contain 6.8 μg antigen per dose. This vaccine was well tolerated in a small group of adults but in subsequent studies in 60 children aged between 7-8 years, there were febrile reactions which occurred after 6 h of vaccination. This was higher than seen in control groups and the second dose was not administered. After administrating a single dose, 90% seroconversion was observed.

Phase II studies were undertaken to investigate the relationship between the antigen dosage and tolerability, immunogenicity and the febrile reactions in children. A lower dose was administered and 3 groups randomly immunized with doses of 1.7 μg and 0.85 μg and the PHK-derived inactivated vaccine was used as active control.

Antibody titers were assayed by a PRNT assay and the mean neutralizing antibody titers were expressed as GMTs of the highest dilution of serum, which caused a 50% reduction of plaque number of the diluted challenge virus in the absence of antiserum. The seroconversion rate was calculated on the basis of a neutralizing antibody titer higher than 1:10 indicating a positive for neutralizing test.

Only mild febrile reactions were observed at the lower doses which were no different to controls. More than 90% of subjects were seroconverted, 100% after boosting, and the overall mean PRNT titer rose from 16 to 89 after boosting which was higher than control groups.

The 0.85 μg dose was evaluated in phase III studies. Febrile reaction data and antibody titres were similar to those seen in the phase II studies. Immunopersistence was monitored up to 60 months after the first dose was administered (47 months after booster shot). Antibody titres with GMT 1:41 were observed at 47 months after booster shot. The vaccine was licensed in China in 1998 and 5 million doses have been produced per year. Ten million doses have been used and adverse event rates per million are reported as 0.8 cases for urticaria and 22.3 cases for fever >38.5 °C.

During the discussion, questions on the source of the Vero cells and the passage levels of MCB and WCB used by various companies were raised by Dr Ivana Knezevic (WHO). Manufacturers present in the meeting indicated that Vero cells were

purchased from the American Type Culture Collections with initial passage levels ranging p108 to p121.

4. Current Issues

4.1 Cell substrates

Dr Anil Chawla (Panacea Biotec, India) outlined issues relating to cell substrate characterization. He reminded participants that "cell substrate" refers to 'microbial cells or cell lines derived from human or animal sources that possess the full potential for generation of the desired biotechnological/biological products for human in vivo or ex vivo use'. These may be primary cells or tissues (used without passage in tissue culture); diploid cells (cells with a finite lifespan and passage in tissue culture) or continuous cell lines (immortal, neoplastic cells with unrestricted passage in tissue culture which are non-tumorigenic).

Current control approaches includes production, identification and characterization of the cell substrate, validation of the manufacturing process for removal and/or inactivation of adventitious agents; and testing of the bulk and final product to assure safety. The objectives of cell line characterization are to confirm the identity and purity of the cell substrate and to provide a high level of confidence in its safety as a component in the manufacture of biologicals. Dr Chawla emphasized that all the tests required to prove the suitability of the cell line must be performed according to good laboratory practice (GLP) and GMP standards using validated test procedures and according to international guidelines. The cell line characterization includes documentation of the history and general characterization of cell line; the cell bank system; and quality control and safety testing

General characteristics of the cell line include growth pattern, morphological appearance, stability from MCB to end-of-production, cell culture identity, microbial sterility (bacterial, fungal, mycoplasma), general virology (freedom from viral contaminants, in vitro test), freedom from inapparent viruses (in vivo test), retrovirology, and tumorigenicity.

The cell bank system assures the constant and adequate supply of cell substrate for use over the entire life span of the product. The generation of cell banks is of importance and procedures used to avoid microbial contamination and cross-contamination by other cell types and procedures that allow the cell bank containers to be traced (documentation/labeling system).

Points to consider in the characterization of cell lines used to produce biologicals are in vitro cell culture tests (which may include inoculation of a minimum of 3 species, i.e. the same species and tissue type as that used in production, human diploid cells and monkey kidney cells); In vivo assays (such as adult mice, suckling mice, embryonated hens' eggs, guinea pigs, and rabbits); transmission electron microscopy and polymerase chain reaction (PCR)-based product-enhanced reverse transcriptase (PERT) assay for retroviruses may also be performed. Comprehensive testing regimens for detection of known and unknown adventitious viruses in novel vaccine

cell substrates should be designed to minimize the risk of virus contamination in vaccines, thereby assuring product safety.

Dr Scott Lambert (WHO) informed the group that a working group on cell substrates is in the process of reviewing the WHO cell substrate guidelines [12,13]. Cells may undergo changes when they are passaged under specific conditions in order for virus to grow well, and these procedures should be documented.

4.2 Potency testing

Dr Ichiro Kurane (National Institute of Infectious Diseases, NIID, Japan) outlined the potency assay for JE vaccine in the current WHO recommendations and studies undertaken in Japan of the test now used by the manufacturers and NCL. The current test in the WHO recommendations includes immunization of mice with 0.5 ml of two doses of the test vaccine and the reference vaccine, intraperitoneally at 7-day intervals into at least 10 mice of 4 weeks of age. Seven days following the second injection, each animal is bled. The serum is pooled at each dilution of vaccine and then inactivated at 56 °C for 30 min: it may then be stored at -20 °C. The serum is appropriately diluted and mixed with an equal volume of challenge virus, containing about 200 plaque forming unit (PFU)/0.4 ml. The mixture is kept at 37 °C for 90 min for neutralization. The virus suspension is then diluted and inoculated on to chicken embryo (CE) or BHK-21 cells. The infected cells are overlaid with 1% agar or methyl cellulose.

Issues to be considered in the revision include the immunization protocol (single or multiple dilutions), age and strain of mice, number of mice, number of injections, number of days apart, when to collect blood, and whether to assay pooled sera or individual serum. Issues relating to the neutralization test on the sera include cells (CE, BHK-21, or Vero cells), how to propagate virus (suckling mice or cells), how to dilute serum samples (2-fold, 4-fold), which overlay (1% agar or methyl cellulose), how many days of incubation, how to stain cells with which dye (neutral red or crystal violet), calculation of $PRNT_{50}$, how to compare neutralization (NT) titers. Studies undertaken at NIID included an investigation of the effect of age using DDY mice. NT titers were compared among 4, 6 and 8 week-old mice. No significant differences were detected.

Pooling of sera prior to assay for antibodies was investigated and, although there were apparent differences in NT titers among serum samples from immunized mice, the titer obtained from a pooled serum seems to represent a group of individual serum sample. As it is practically difficult to assay individual samples, the assay of a pool of serum appears reasonable.

Neutralization assay results were examined and it was observed that antibody titers were consistent in 3 assays on 3 pooled sera by 3 operators on Vero cells. Direct comparison of the neutralizing titers obtained in Vero cell and CE cell neutralization assays among 6 different laboratories showed a significant correlation between these two assay systems in a statistical analysis. Therefore in Japan, manufacturers have changed over from CE to Vero cells. Multiple dilutions of serum were assayed and the numbers of plaques obtained were counted. The reduction percent at each serum dilution was calculated. The reduction percent was typed to a worksheet on a

computer software program (Bioassay Assist, NIID). $PRNT_{50}$ was automatically determined and index of a tested vaccine to the reference was calculated by the linear regression analysis program. The new method was assessed by 5 manufacturers in Japan plus NIID and it was found to be satisfactory.

A comparison of the previous test undertaken in Japan and the current test is summarized below:

Original method	New method
• Using chick embryonic cells	• Using Vero cells (9013)
• Immunization of mice with a single dilution of the reference or a tested vaccine	• Immunization of mice with graded multiple (4) dilutions of the reference or a tested vaccine
• Determination of $PRNT_{50}$ titers based on a chart	• Determination of $PRNT_{50}$ titers at respective vaccine dilutions.
• Comparison of $PRNT_{50}$ titers between a tested vaccine and the reference	• Comparison of $PRNT_{50}$ titers between a tested vaccine and the reference using a computer program (Bioassay Assist)
• No inferiority comparison	• Potency index of the tested vaccine to the reference is calculated

Issues raised during discussion were the challenge virus and it was suggested that the homologous virus strain be used. The dose administered is generally a dilution of the human dose and the strain of mice may affect NT titres. Different ways of calculating and expressing potency were also discussed e.g. titres, calculation of median effective dose (ED_{50} — vaccine dilution required to immunize 50% mice), or probit model which may be used if individual animal sera are assayed and seroconversion determined.

5. Regulatory Perspectives

5.1 China

Dr Guanmu Dong (National Institute for the Control of Pharmaceutical and Biological Products, NICPBP) reported that since the peak of >150,000 JE cases in China during epidemics in 1966 and 1971, the number of cases had reduced to around 10,000 cases in 2000. The number of cases has steadily reduced since then and this may be due to change of living habit of people who increasingly live separately from domestic animals, improved sanitation throughout the country, improved quality of inactivated vaccine by purification as well as the widespread introduction of live JE vaccine and the availability of JE vaccine without charge for immunization enabling increased coverage.

Inactivated vaccines historically used in China included a P3 strain produced in mouse brain. This gave high adverse reaction rates. Another P3 vaccine produced in chicken embryo fibroblast (CEF) cells was not efficacious and a PHK cell vaccine was then produced. The live attenuated SA14-14-2 vaccine is in widespread use in China and 60-70 million doses produced each year by 3 manufacturers, with 15 million doses being exported.

Although the use of inactivated vaccines in China is declining because of the availability of the live attenuated vaccine, around ten institutes or companies are developing the vaccine with P3 virus strain on Vero cell lines. Three of these vaccines have production licenses and a further three are in clinical trials.

In 2002, Beijing Institute of Biological Products obtained the production license for a purified inactivated Vero cell vaccine using the P3 strain. This vaccine is purified by ultracentrifugation. However, the yield was not very satisfactory with only about 3 million doses produced per year since that time. In 2005 Lanzhou Institute of Biological Products has procured the production license of a vaccine derived from PHK cell culture with P3 strain which was purified by chromatography, but they have not produced vaccine on a regular basis. In 2005, Zhejiang Tian Yuan Co. Ltd., procured a license to produce chromatography-purified, inactivated JE vaccine derived from PHK cell culture with SA14-14-2 live attenuated virus strain. This company plan to produce 1-2 million doses in 2006

QC tests required in China for inactivated JE vaccines include a sterility test and virus titration on each single harvest, for which the virus titer shall be not less than 7.5 log LD_{50}/ml. Tests on bulk are sterility test and test for inactivation in animals or cell cultivation. Tests on final bulk are sterility test and tests on final product include identity test, inspection of final containers, pH, free formaldehyde content, thiomersal content, potency test, thermostability test (appropriate for freeze-dried vaccines only), sterility test, abnormal toxicity test, residual bovine serum protein, and cell DNA.

5.2 India

Dr Keshaw Shrivastaw (Central Drugs Laboratory, CDL) described the production and control of vaccine at the Central Research Institute, Kasauli, India. This vaccine is produced on suckling mice brain (Nakayama strain). Formalin inactivation was undertaken at the bulk stage for 45 days at 4 ^{o}C. This is the only licensed vaccine but others are under development. Research work suggest that the efficacy of vaccine produced with local Indian strains is similar to that of Nakayama NIH strain.

All regulatory requirements in the Indian Pharmacopoeia (IP) are based on the WHO recommendations and the Japanese Pharmacopoeia (JP). The vaccine is used in India and is considered highly stable with good potency present even after 4.5 years and immunogenic even after 7 years. Indian manufacturers are now considering replacing the use of CEF cells with Vero cells in the potency test. Potency assay by single dilution method is giving satisfactory result. The reference standard was obtained from Japan in 1985 and still gives a satisfactory potency. The trend analysis of potency data from 1985 to 2005 shows that the reference vaccine is stable. An in house standard has now been developed.

5.3 Japan

Dr Kurane outlined the production of JE vaccine in Japan. The JE virus strain used for the production currently is Beijing-1 although before 1989, Nakayama strain was used. Currently Nakayama strain is used only for the vaccine to be exported. Five vaccine manufactures produce mouse brain-derived, inactivated JE vaccine and three are

developing Vero cell-derived, inactivated JE vaccine. Two are in phase III trials and one at the preclinical stage.

There has been a suspension of the recommendation for the universal use of mouse brain-derived JE vaccines in Japan since May 30, 2005, when the Ministry of Health, Labor and Welfare (MHLW) requested all local health departments in Japan to temporarily suspend the recommendation for the universal use of mouse brain-derived JE vaccines currently used in the country until licensure of theoretically-safer vaccines are available in the future.

This emergency action by the Ministry was prompted by a recent recommendation by the Advisory Committee on Vaccine Injury Compensation in Japan. The committee, consisting of clinicians, researchers and legal experts, considered whether a recently-reported case of near-fatal acute disseminated encephalomyelitis (ADEM) could be causally-related to the administration of inactivated JE vaccine. Even though there was no biological evidence or scientific confirmation, the committee concluded there was a theoretical risk.

Twenty one cases of ADEM were reported to MHLW after vaccination with mouse brain-derived inactivated JE vaccines over an 8-year period from 1996-2004, i.e. 1 per 1.5 to 2 million inoculations. It is, however, assumed that there are 60-120 cases of ADEM per year of whatever cause in children in Japan.

The current policy of the Ministry on immunization is to temporarily suspend the use of vaccines even when there is a slight doubt about its safety until the concern is completely excluded.

In spite of suspension of its nationwide use, the current JE vaccine can be given to children at a higher risk of JE virus infection and some companies are still producing from stored bulks.

5.4 Korea, Rep.

Mr Kyungil Min (Korea Food and Drug Administration, KFDA) reported that following introduction of mouse brain vaccine in Korea in 1982 the incidence of disease has reduced. There have been few cases of JE in the last 10 years. Vaccines licensed and produced in Korea include 6 locally produced liquid mouse brain derived Nakayama strain JE vaccines (inactivated). There are 2 manufacturers of bulk vaccine and 6 manufacturers of final product. In addition the live attenuated SA14-14-2 lyophilized JE vaccine is imported. The minimum requirements are documented in the Korean Pharmacopeia. Twenty eight lots of JE vaccines (inactivated) (1,945,561 doses) were released in 2005 and 317,473 doses exported. Ten lots (300,000 doses) of JE vaccine (live attenuated) were imported.

The post-marketing surveillance data is available for 4,438 subjects who had received inactivated JE vaccine between April 1 and August 31, 2001. These were healthy children aged between 12 months and 14 years. This was a large scale, multi-institutional study; all the adverse events within 1 month after administration were recorded electronically. Local events recorded were pain, erythema, rash, oedema,

itch, irritability and systemic events fever, headache, vomiting, diarrhea and crying. Numbers of adverse events recorded were low.

GreenCross Corp. is currently developing a cell culture-derived JE (inactivated) vaccine in Korea using the Beijing strain. The feasibility of using various cell cultures are under evaluation including Vero and Sf9. Production in serum-free media and on several culture system (e.g. adherent, suspension or bioreactor culture), are under investigation. The potency of cell culture-derived JE vaccine is being assessed in preclinical tests.

5.5 Sri Lanka

Dr Omala Wimalaratne (Medical Research Institute, MRI) described the status of JE immunization in Sri Lanka. All licensed vaccines are imported (Biken, Japan — Nakayama strain; GreenCross Corp., Korea — Nakayama strain; and Denkaseiken, Japan — Beijing strain). The vaccine produced by the Government Pharmaceutical Organization (GPO), Thailand (Beijing strain) has been in use in the NIP since 2003. The PHK cell culture (live) SA-14-14-2 produced by the Chengdu Institute of Biologicals, China is licensed and will be introduced when a safety monitoring study is completed. The number of doses of inactivated JE vaccines used has increased since 2003, when 809,769 doses were used, 603,615 in 2004 and 1,134,072 in 2005. The target group is 1 to 10 years of age and 4 doses of vaccine recommended. JE vaccine was introduced in a phased manner in different districts.

No vaccine batches were withdrawn and the same batches were used in Thailand with no adverse events reported. Lot release is undertaken by the NCL largely based on protocol review of documents submitted by the manufacturer with each batch of vaccine. Vaccine testing (for appearance, innocuity and safety) is done when necessary.

5.6 Thailand

Mrs Teeranart Jivapaisarnpong (Ministry of Public Health) described the current status of JE vaccination in Thailand. JE vaccines are used in the expanded program on immunization (EPI) programs and administered at 18 months, 1 month after the first dose, and 1 y after the second dose. The vaccines used in Thailand are a local product (produced by GPO) liquid vaccine, Beijing strain and imported vaccines, Nakayama strain liquid vaccine and Beijing strain (lyophilized). All are inactivated JE vaccines derived from mouse brain. The number of lots and doses of JE vaccines released in 2005 was 72 lots (1,851,867 doses) of GPO vaccine and 7 lots (66,960 doses) of Nakayama and 1 lot (74,001 doses) of lyophilized form Beijing vaccine. None of JE vaccine batches has been rejected or withdrawn.

For licensing of imported products, certificate of free sale from the producing country is required as well as clinical study data (mostly phase III) for all products. There are regular GMP inspections for local products but no GMP inspection for imported products by the Thai NRA. However, a GMP Certificate from the NRA of producing country is required for the registration process.

Local products have been examined in serological studies during the clinical trial conducted in Thailand. It was found that the NT antibody titer against local strain has no significant difference with that against Nakayama or Beijing vaccine strains.

After registration and before distributing to the market, all vaccines have to pass the lot release procedure based on summary protocol review and testing. For imported products only testing for appearance is undertaken but local product is tested for appearance, sterility, abnormal toxicity, pyrogen, endotoxin content (≤ 5 endotoxin unit/ml), pH (6.8-7.4), protein content (≤ 80 µg/ml), formaldehyde content(≤ 100 µg/ml), thiomersal content (≤ 0.012% w/v), and potency.

Post-marketing surveillance is undertaken and the cases of AEFI of JE vaccine are mostly rash and urticaria with none resulting in death. Every suspected lot of vaccine linked to serious cases of AEFI was reassessed for quality and all were found to pass the requirements.

Potency testing of JE vaccine is in 4-week old ICR male mice (14 to 18 gm). Twenty mice/dilution are used with 8 mice being held as negative controls. The vaccine dilution used is as follows: 3 dilutions (1/16, 1/32, 1/64). The reference vaccine contains the Beijing strain, p184 from NIID, Japan. The $PRNT_{50}$ antibody titres are determined in Vero cell culture and the relative potency calculated by comparing the antibody titers of the test vaccine sample against reference vaccine.

6. Discussion on the Need to Revise Current WHO Recommendations

6.1 Manufacturing recommendations

Dr Robin Levis (Food and Drug Administration, FDA) led the discussions on the proposed outline of the revised requirements and issues raised which will be addressed as a new draft is developed. Topics considered were:

- Inclusion of neural tissue as a substrate — include in draft and re-consider at next meeting when the status of vaccines under development may be clearer
- The issue of the quality of mice used in production — to be included
- Recombinant vaccines should be mentioned in introduction but detailed specifications are not yet warranted
- Manufacturing/containment — biosafety issues (include statement as in rabies requirements). It was proposed that for infectious area of JE vaccine production, biosafety level 2 may be recommended
- Cell bank — substrates/availability of qualified cells; requirement to qualify and characterize any cells obtained as a manufacturing substrate/adventitious agent testing dependent on passage history; citation of cell substrate guidelines (currently under review)
- TSE for cell substrate — to be consistent with current WHO guidance
- Passage history of virus seeds — issues to be covered include tissue origin / traceability of passaging / passage of virus seeds on Vero cells / need to sequence genome of working virus seed bank/specifications for testing of seed virus will be dependent on passage history
- Validation of inactivation procedures/viral clearance studies

- Test of virus content in primary harvests — necessity for requirement
- The requirement for the minimal virus content in the primary virus harvest (paragraph 4.3.2) — not necessary / relevant
- Residual protein — specification for total protein (consider inclusion of a test for myelin basic protein for mouse brain-derived vaccines)
- Residual DNA — consistency with other WHO guidelines
- Potency assay — single dose or multiple does, validated $PRNT_{50}$ assay/virus strain
- Identity testing by methods other than currently specified
- Stability testing — real-time stability to establish shelf life and thermal stability (to be consistent with WHO guidance under development)
- Standardization of references — strain of virus, stability of reference

6.2 Nonclinical evaluation

Dr Dewasthaly (Intercell, Austria) reviewed issues relating to the nonclinical evaluation of vaccines and in particular JE vaccines. Several agencies have issued guidance on the matter including WHO, FDA and EMEA. Nevertheless, even though guidances are available, most nonclinical development plans are considered on a case-by-case basis.

The general aims of nonclinical studies for vaccines are to assess the biological activity and safety (characteristics) of the product. These studies must be undertaken in relevant species that shows immune response and are preferably undertaken on material representative of that used in clinical trials. Changes in the manufacturing procedures might require a nonclinical assessment. Pharmacological assessment is limited to immunogenicity (pharmacodynamic) testing and pharmacokinetics studies are not required i.e. biodistribution, persistence, excretion in general for vaccines. The immunogenicity of a vaccine can be measured by quantitation of the immune response or by measuring the protective efficacy of the vaccine. For JE virus the role of antibody in protection is well studied and neutralization assays are considered more reliable than the virus binding assays like ELISA. The dose response relationship should be demonstrated and immunogenicity assays are usually recommended by almost all national authorities as a product release criteria for every batch.

Challenge studies in small animals like mice should be also performed at least once during development. The use of more than one challenge viruses should be considered to demonstrate protection from a strain other than the homologous virus. Passive transfer of human antibodies followed by challenge with JE virus might be done to correlate antibody titers with protection.

Toxicology studies on vaccines must reflect the clinical dosage (amount, route dose etc.). Usually complete human dose is recommended although in limitation of volume in mice require smaller doses to be administered. Since inactivated JE vaccines require more than one dose a repeat dose toxicology study might be considered. If a vaccine is to be indicated for use in women of child bearing age, reproductive and developmental toxicology studies are recommended. However, these are not required if the vaccine is only to be recommended for use in children. It is now accepted by some authorities that attempts should be made to decrease the use of thiomersal in the

vaccines. Any preservative and novel adjuvant, excipient requires additional toxicological analysis

Dr Dewasthaly described studies on the Intercell vaccine which is based on the SA14-14-2 attenuated strain. The original SA14-14-2 strain was adapted to primary dog kidney (PDK) cells and various studies including monkey neurovirulence studies were performed on this virus. The strain was further adapted to Vero cells. The E protein gene was sequenced analysed to show equivalence and studies in mice demonstrated the attenuated phenotype. The immunogenicity of the vaccine following two doses of vaccine was demonstrated by the immunization of mice with different amounts of the Intercell JE vaccine IC51 followed by assay of serum in PRNT assays. A 50% plaque reduction titre of 1 in 10 was indicated as seroconversion titer, and was used to determine seroconversion rate. Experiments to show both active and passive cross protection (using human vaccinee's sera) against Beijing and SA14 strains are ongoing. The challenge studies are done by the intra-peritoneal route. Two studies were undertaken by Intercell vaccine in which immunized rats were mated at different times following immunization during the time at which antibodies were produced. The rats were monitored for clinical signs, body weight, food consumption and antibody titre in sera were measured. The pups were examined and necropsy undertaken at specific time-points. During the discussion of issues specific for JE vaccine which should be highlighted in the draft, it was emphasized that materials for non-clinical studies should be representative of material in initial clinical trials, and that cross protection and passive transfer studies should be performed. It was agreed that reproductive toxicity studies depend on product development goal.

6.3 Clinical evaluation

Dr Eric Tauber (Intercell, Austria) reviewed the clinical evaluation of JE vaccines. As there are already licensed vaccines efficacy cannot be demonstrated in placebo controlled trials and, because of the low incidence of disease due to the present vaccine coverage in endemic areas, it had been calculated that one would need 250,000 subjects to reproduce the Hoke study in which efficacy had been demonstrated [14].

Efficacy has previously been demonstrated by the passive transfer of human post-infection sera to at-risk subjects and correlated with detectable neutralizing antibodies in recipients [15]. In addition this has been demonstrated in many studies done in animals. It is now agreed that a surrogate for the efficacy of JE vaccines is the demonstration of neutralizing antibodies as measured by $PRNT_{50}$ assays [9]. It was emphasized that EIA should not be used in such studies as cross-reactions with other flavivirus, e.g. tick-borne encephalitis, are found. It was agreed that a titre equal to or greater than 1 in 10 is indicative of seroconversion.

The demonstration of non-inferiority to current vaccines is an endpoint for licensure trials. Seroconversion rates and GMTs should be calculated. The parameters and margins for non-inferiority, a 10% margin is usually acceptable so if old vaccine gives 95% seroconversion, 85% seroconversion with a new vaccine is likely to be acceptable. However different authorities may have different opinions.

PRNT assays of sera from vaccine recipients in clinical trials should be carried out under GLP. Consideration should be given to the selection of the challenge virus strain, particularly if vaccines are derived from different strains. Nevertheless one strain is generally considered sufficient as antibodies to JE virus have been shown to be cross-reactive to all genotypes and vaccines based on the genotype 3 (Nakayama or Beijing or SA14-14-2) are effective worldwide in countries where other genotypes circulate.

Other issues are immunological memory, effect of booster doses, interaction with concomitant vaccines and long term immunogenicity which should be demonstrated for at least 2 years although some regulatory authorities may require up to 5 years. Placebo control trials may be undertaken in non-endemic areas although these are not possible in endemic areas.

The clinical trial population must be representative for target population (age, sex, pre-existent immunity, ethnic origin). The number of subjects should be large enough to allow detection for symptoms of high and medium frequency (i.e. at least 1,000 to 2,000 subjects) and needs to be tailored to previous experience with the vaccine. However post marketing surveillance may address some issues

In the discussion Dr Kurane reminded participants that a previous WHO meeting on clinical endpoints agreed that head to head comparisons of licensed and new vaccines and that if they were derived from different virus strains, a neutral virus or, preferably, viruses homologous respectively to vaccine strains should be used. Antibody titres are not only affected by the homology of the vaccine strain and the challenge virus but he considered titres also could be affected by the presence of defective interfering (DI) particles. However, checks for DI particles in JE virus preparations are not usually done.

The need to undertake blind studies was also discussed and although this is preferable, there may be practical difficulties, for example if sites of administration are different or if labels and packages are different.

Dr Kurane indicated that validation of $PRNT_{50}$ assays for JE virus may not be as difficult for JE virus as for dengue vaccines as the JE vaccine is monovalent. It is likely however that responses to JE vaccine differ in Caucasians and Asians. However, the need for national studies is up to national agencies.

7. Global Needs in Research and Collaboration

Participants agreed that the potency test for JE vaccine should be reviewed and studies organized to assess the effect of multiple dilutions against single dilution and the cell line used for assay of the sera. Dr Ferguson informed the group that a Vero cell bank which is suitable for use in PRNT assays of dengue and JE viruses was available from NIBSC. Dr Ferguson emphasized that this cell bank is not suitable for vaccine production. Dr Kurane offered to share the NIID software for probit analysis which is suitable for use when a dose-response curve is generated.

Dr Kurane agreed to draft a protocol for a study which would facilitate the introduction of the assay currently performed in his laboratory to other laboratories. The availability of reference vaccines was also discussed further and Mrs Jivapaisarnpong informed that the SEAR initiative to evaluate a regional reference could be extended to include WHO Western-Pacific Region (WPR). The study to evaluate the Japanese potency test method could be combined with the study to assess the suitability of the regional reference candidate. This project would concentrate on vaccines derived from the Beijing strain. Dr Kurane said that he could supply the Beijing strain to any laboratories which required it. Vaccines to be included in the study might be the current Japanese reference, the candidate regional reference, a typical vaccine, and the Korean JE vaccine reference. Participants would likely include NCLs in India, Thailand, Korea, Japan, China, and Japanese manufacturers.

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