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A Collaborative Study on Korean Standard JE Vaccine for Potency Assay

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Abstract The objective of this collaborative study was to establish a Korean standard of Japanese encephalitis (JE) vaccine (mouse brain-derived, formalin-inactivated) for potency assay. A candidate preparation proposed as a Korean standard was provided by GreenCross Vaccine, and six laboratories, including one national control laboratory and five manufacturers of JE vaccine, participated in the study. The potency of the candidate preparation and a reference standard obtained from Japan was estimated by mouse immunogenicity assay using the *in vitro* plaque reduction neutralization test (PRNT). The results of 72 assays conducted by the 6 laboratories showed that the overall mean potency estimate of the candidate preparation was 2.455 log median plaque reduction neutralization antibody titer per 0.5-ml dosage administered twice in mice at 7-day intervals, and that the mean potency ratio of the candidate preparation relative to the reference standard was 1.074. The potency estimates were quite variable among laboratories irrespective of the preparation. The variability of assays assessed by Z scores and coefficient of variation (CV) were in general within the level of acceptance (Z scores within ±3 and CV ≤15%). Therefore, we concluded that the candidate preparation would be suitable as a national standard for testing the potency of JE vaccine (inactivated).

Key words: Collaborative study, Japanese encephalitis vaccine, neutralization test, potency, standard

Japanese encephalitis (JE) is the most common cause of viral encephalitis in the Asia-Pacific region. Every year, more than 16,000 cases and 5,000 deaths are reported [6, 7]. Specific vaccines are the most commonly used measures for the effective control of JE. Currently, three types of JE vaccines for human use are in large-scale use: (i) a mouse brain-derived and inactivated vaccine based on Nakayama strain or Beijing-1 strain, (ii) a cell culture-derived inactivated vaccine, and (iii) a cell culture-derived live attenuated vaccine [10]. The mouse brain-derived inactivated JE vaccine is produced in several Asian countries, and the latter two types of vaccines are produced in China and widely used within the Chinese JE control program [10].

In Korea, two types of JE vaccines are currently on the market. One type is a mouse brain-derived and inactivated vaccine based on Nakayama strain. This type of vaccine is locally produced and recommended for a national JE immunization program. Another type of vaccine is a cell culture-derived live attenuated vaccine whose importation has recently been permitted. Requirements for the manufacture and control of both types of vaccines have been formulated and published in the Korea Food and Drug Administration (KFDA) Notice [3, 4].

To ensure optimal quality for human or animal patients receiving complex and sensitive medicinal substances such as vaccines, it is necessary for manufacturers and national control authorities (NCA) to make use of a set of regulations involving quality control procedures throughout the manufacturing process [1, 2]. At every stage, standardized parameters should be established in relation to common standards or references. Accordingly, WHO published guidelines on the preparation, characterization, and establishment of international and other standards and reference materials more than a decade ago [9]. A biological standard is a preparation of a biological substance including vaccine, blood product, hormone, antibiotic, or allergenic extract which has been distributed into a large number of ampoules, suitable for dispatch to user laboratories, and which has been designated a standard with defined units of activity by an appropriate authority [9].

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Most important activities for the national control laboratory (NCL) of biologics are laboratory testing/evaluation, and the establishment of national reference materials for biological substances [8]. According to the guidelines for national authorities on quality assurance for biological products, it has been recommended that appropriate national (secondary) reference materials are preferably established by the NCL after calibration against international reference materials, and made available to manufacturers. The potency of the inactivated JE vaccine for lot release in Korea should be equal or greater than the JE vaccine standard. Therefore, without the relevant standard, it is impossible to control the consistency of the JE vaccine production.

A nation-wide collaborative study on the establishment of a Korean standard of JE vaccine for potency assay was carried out for 2 years from 2000 to 2001. Since no international standard for JE vaccine (inactivated) has been available, we have used a reference standard purchased from Japan. The potency assay for JE vaccine (inactivated) consisted of a mouse immunogenicity assay with *in vitro* plaque reduction neutralization test (PRNT) procedure according to the regulatory or guideline requirements [4, 11]. Here, we report the results of the collaborative study.

MATERIALS AND METHODS

Candidate Standard Preparation

The preparation proposed as a candidate standard for mouse brain-derived formalin-inactivated JE vaccine (Code No. KFDA 01/002) was provided by GreenCross Vaccine. The candidate material was a batch of *in house* reference material prepared in 1996 (GC Final Lot No. X004) of which the final bulk (GC Final Bulk No. B6003) was composed of the Nakayama strain of JEV in suckling mice. The final lot was prepared in a freeze-dried state in a 3-ml vial, and was tested for sterility, identity, residual moisture, stability, and protein content. The precision of filling was 1.10±0.02 g/vial, which was acceptable for the specification limits established by the manufacturer. The stability of the final lot was checked for up to 6 months at 37°C, and the lot retained minimum potency (greater than that of reference standard) until 2 months at 37°C, which passed the specification limit required by WHO [11].

For the comparative assessment of potency of the candidate standard, a standard preparation was purchased from the National Institute of Infectious Disease (NIID), Japan (Dr. T. Takasaki, Toyama 1-23-1, Shinjuku-ku, Tokyo, 1642-8640). This is a Japanese standard for JE vaccine (inactivated) for potency assay (Lot No. 189).

Participants

Six laboratories including one NCL and five QC laboratories of JE vaccine manufacturers participated in the study. Each laboratory was randomly coded to ensure anonymity.

Study Design

Participants were asked to perform 12 independent mouse immunogenicity assays for the candidate preparation in parallel with the standard preparation according to the detailed procedure provided by the KFDA. In each assay, participants were requested to immunize two groups of 4-week-old, outbred female ICR mice (12/group) intraperitoneally with 0.5 ml of 1:32× 10 ml-reconstituted standard and 1:16× 2 ml-reconstituted candidate preparations. Participants were requested to immunize the animal twice at 7-day intervals, and then to pool antisera of each group collected at day 14 post-immunization, to inactivate at 56°C for 30 min, and to store at -70°C. Participants were asked to perform three independent PRNT, using primary chicken embryonic (CE) cells and the dilutions (1:160×, 1:320×, and 1:640×) of the pooled antisera of each group. JEV Nakayama-NIH strain was used as challenge virus for the PRNT assay. Participants were asked to provide a complete report including raw data and calculations of the potency of the candidate Korean standard (KFDA 01/002) relative to a Japanese standard (NIID Lot No. 189) on standard result forms written in the Microsoft Excel software program per individual assay, as well as detailed technical information on the procedure used.

Validity Criteria

The mean number of plaques of the challenge virus control (n=12) included in each PRNT should be 50 to 150 per dish. In addition, the number of plaques of the challenge virus control above and below the mean number of plaques should not significantly deviate from the mean number of plaques at the level of alpha=0.05 by chi-square test.

Calculation of Potency and Potency Ratio

For each assay, the potency of each preparation was estimated from the mean response of three independent PRNT using CE cells, and expressed as geometric mean titer (GMT), which corresponds to 'the mean of the Log 50% virus plaque reduction neutralization antibody titer per dosage administered at 7-day intervals' (mean Log $PRNT_{50}$ /2× [0, 7] dosage). The potency ratio for the candidate preparation was calculated by dividing the potency of the candidate preparation with that of the standard preparation included in parallel in each assay.

Table 1. Overall statistics of potency estimates of reference standard and candidate standard preparations by mouse immunogenicity assay using the *in vitro* plaque reduction neutralization test.

Preparation	Weighted estimate				Unweighted estimate			
	N	Mean	SD	Range	N	Mean	SD	Median
Standard (S)	214	2.295	0.273	1.584-3.070	72	2.295	0.255	2.324
Candidate (C)	214	2.454	0.326	1.744-3.182	72	2.455	0.314	2.485
Ratio (C/S)	214	1.074	0.124	0.766-1.608	72	1.074	0.118	1.063

Statistical Analysis

Z-scores and coefficient of variation (CV) were calculated to evaluate the variability of assay. Z scores were calculated using standardization formula for each assay: (potency estimate of individual assay-overall mean potency estimate)/overall standard deviation (SD) of potency estimate. The CV (%) was calculated using: (mean potency estimate/SD)×100. For the comparison of laboratory mean potency estimate of each preparation among laboratories, analysis of variation (ANOVA) and multiple means comparison using the least significance difference (LSD) method were performed using Statistical Package for the Social Sciences (SPSS) version 10.0. For the comparison of potency estimates between preparations, a studentized t test was performed.

RESULTS

Assay Data

The six participants contributed data from a total of 72 mouse immunogenicity assays of which 3 replicate PRNTs/assay were included for the candidate preparation in parallel with the standard preparation. All the study data were checked thoroughly for clerical and technical errors, including transcription errors in completing the result forms and keying errors in entering the data into the computer. Data items which appeared to be clearly suspect were queried with the participant, and the final set of data files was reviewed carefully against the original result forms.

Deviations from the study protocol were not found, however several errors in entering the data into the computer and consulting the titer table (tabulated by 10 to 90% plaque reduction rate and a range of serum dilution) were found in all laboratories.

Assay Validity

All of the PRNT data were checked for validity according to the validity criteria described in Materials and Methods. Deviations from the validity criteria were found in two occasions out of total 216 PRNTs. Both occasions were noticed in laboratory F. In one occasion, the mean number of plaques in the virus control was below 50, and in the other occasion, the number of plaques of the challenge virus control above and below the mean number of plaques significantly deviated from the mean number of plaques at the level of alpha=0.05 by chi-square test. From the tests of validity, the two PRNT data provided by laboratory F were excluded from the estimation of the potency and potency ratio.

Potency Estimates and Ratios

The overall weighted mean±SD and range of the potency estimates of the standard and candidate preparations from 214 PRNTs were 2.295±0.273 [1.584-3.070] and 2.454±0.326 [1.744-3.182] log $PRNT_{50}$/2x [0, 7] dosage, respectively. The overall weighted mean±SD and range of the potency ratio for the candidate preparation relative to the standard preparation were 1.074±0.124 [0.766-1.608] (Table 1).

The overall unweighted mean±SD of the potency estimates of the standard and candidate preparations from 72 mouse immunogenicity assays were 2.295±0.255 and 2.455±0.314 log $PRNT_{50}$/2x [0, 7] dosage, respectively. The overall mean±SD of the potency ratio for the candidate preparation was 1.074±0.118 (Table 1).

Assay Variability

The potency estimates of standard and candidate preparations for individual mouse immunogenicity assay were obtained from the mean response of three independent PRNTs per assay, and are presented in Fig. 1 to obtain graphical impression of the distribution of the overall assay results. The distribution of potency estimates of the standard and candidate preparations appeared to show a similar pattern without any obvious outliers. In the distribution of potency ratios of the candidate preparation, there were two cases of outliers observed in the assay numbers 09 and 10 of laboratory F.

The variability within and between assays for potency estimates and ratios were evaluated using Z score and CV (%), respectively. The XY plots using the Z score and CV (%) showed generally good reproducibility in the potency estimates (Figs. 2A and 2B). In the potency ratios of the candidate preparation relative to the standard preparation, the assay numbers 09 and 10 of laboratory F showed just above +3 of the Z score, and the assay number 02 of laboratory E showed greater than 15% of the CV (%) (Fig. 2C).

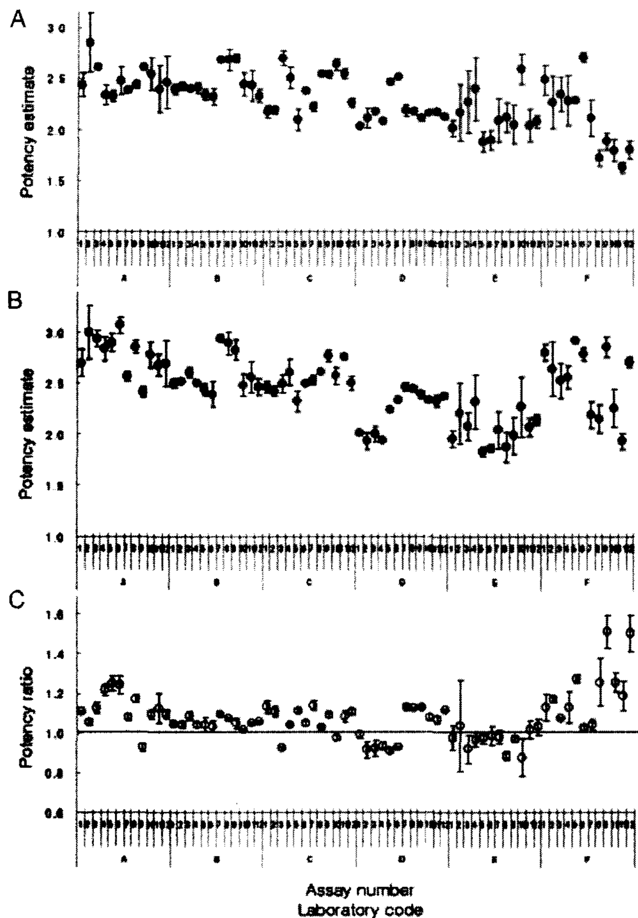

Fig. 1. Distribution of potency estimates.
A: Reference standard (NIID Lot No. 189, Japan). B: Korean candidate standard (KFDA 01/002). C: Potency ratio of the candidate standard relative to the reference standard. The potency of both preparations was estimated by *in vitro* plaque reduction neutralization test (PRNT) using pools of antisera obtained from 12 mice immunized twice at 7-day intervals. A filled circle represents geometric mean titer of PRNT repeated 3 times (panels A and B). An open circle represents mean potency ratio of the candidate standard relative to the reference standard (panel C). Error bars represent the standard deviation (panels A, B, and C). The dotted line represents the equipotency of the candidate standard and the reference standard (panel C). A through F on the X axis represent laboratory code, and 1 through 12 represent assay number in each laboratory.

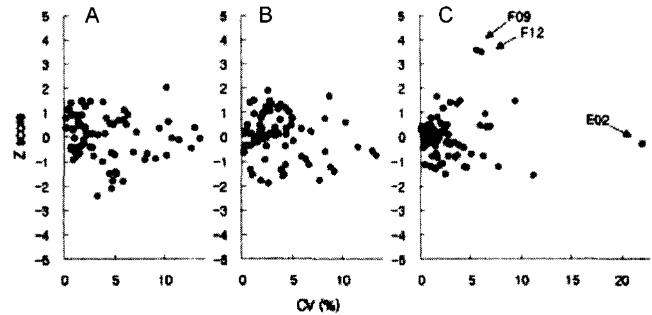

Fig. 2. Assay variability.
A: Z score and CV (%) of potency estimate for reference standard (NIID Lot No. 189, Japan). B: Z score and CV (%) of potency estimate for candidate standard preparation (KFDA 01/002). C: Z score and CV (%) of potency ratio of the candidate standard relative to the reference standard. Z scores were calculated using standardization formula: (individual assay mean-overall assay mean)/(overall assay SD). CV (%) was calculated using mean/SD×100. F09, F12, and E02 represent laboratory code with assay number.

Laboratory Variability

Detailed values of individual laboratory mean potency estimates of the standard and candidate preparations along with SD and CV (%) are listed in Table 2. The ANOVA and LSD test results were used to evaluate the variability among laboratories, and CV (%) was used for the variability within each laboratory.

The laboratory mean potency estimates were significantly different among laboratories irrespective of the preparation ($p<0.05$). In the case of the standard preparation, laboratories A, B, and C were grouped together, while laboratories D, E, and F formed a different group. In the case of the candidate preparation, laboratories were grouped into three categories (A, B=C=F, and D=E).

When laboratory mean potency estimates between preparations were compared, laboratories A, C, and F showed the potency estimates of candidate preparation greater than those of standard preparation, while laboratories B, D, and E showed statistical equipotency between the preparations.

All laboratories showed <15% of CV with the exception of one case in the potency estimates of the standard

Table 2. Comparison of potency estimates of reference standard and candidate standard preparations by laboratories.

Laboratory code	No. of assays	Standard (S)			Candidate (C)			Mean ratio (C/S)	Comparison between preparations (p value)
		Mean*	SD	CV (%)	Mean*	SD	CV (%)		
A	12	2.485^{a}	0.146	5.9	2.784^{a}	0.185	6.6	1.124	S<C (0.0002)
B	12	2.460^{a}	0.142	5.8	2.592^{b}	0.185	7.1	1.053	S=C (0.0635)
C	12	2.396^{a}	0.204	8.5	2.543^{b}	0.128	5.0	1.066	S<C (0.0458)
D	12	2.191^{b}	0.144	6.6	2.232^{c}	0.200	9.0	1.021	S=C (0.5702)
E	12	2.131^{b}	0.203	9.5	2.053^{c}	0.158	7.7	0.967	S=C (0.3051)
F	12	2.108^{b}	0.339	16.1	2.524^{b}	0.320	12.7	1.122	S<C (0.0052)
Overall	72	2.295	0.255	11.1	2.455	0.314	12.8	1.074	S<C (0.0010)

*Means with the same letter within the column had no significant difference ($\alpha=0.05$).

preparation (16.1%). The highest CV (%) was observed in laboratory F for both preparations.

DISCUSSION

This is the first report to describe a collaborative study on the potency estimates of reference materials for JE vaccine (inactivated). The present collaborative study was primarily undertaken to characterize a candidate standard of JE vaccine in parallel with a reference standard in terms of variability of assay and laboratory by the historical single dilution assay system with the reduced number of animals by taking advantage of well established *in vitro* titration of JE antibodies, using PRNTs which can reliably provide responders close to 50% within the designated level of serum dilution from 1:160× to 1:640×. Therefore, the potency estimates with a certain level of assay precision obtained by this approach would be quite reliable.

A biological reaction by itself cannot be used to define the potency of one preparation alone, since responses of biological assays vary from day to day, person to person, and laboratory to laboratory. To define the potency of a biological material, the assay must be calibrated using a suitable reference standard, and this is often carried out by comparing the biological response to a dilution series of a standard to those of test samples. The potency of any biological product should be expressed relative to a well-defined reference preparation. Therefore, the selection and validation of a suitable reference preparation play a key role in assay validation and specification setting.

The potency specification limit for JE vaccine (inactivated) for lot release in the current regulatory or guideline requirements are defined as the potency of JE vaccine that shall be equal to or greater than that of a standard [4, 11]. Historically in Korea, a single dilution dose assay system with *in vitro* titration of JE antibodies using PRNTs for testing the potency of JE vaccine (inactivated) has been adopted. Although the single dilution dose assay cannot provide intra-assay uncertainty estimate of potency (i.e., 95% confidence limit of mean potency), it can be used to demonstrate that a test vaccine is above an acceptable level of potency, so that it may well be adequate for lot release testing where a history of consistent production exists [12]. This type of assay gives considerable savings in the number of animals used, especially where many batches are produced with relatively smaller sizes.

The results of the present study showed that the potency estimates of both the standard and candidate preparations produced very similar characteristics of precision within and/or between assays and/or laboratories with a generally acceptable level within ±3 of Z score and 15% of CV. The potency estimates of the candidate preparation were equivalent to or higher than those of the Japanese reference standard. From the overall mean potency estimate of 72 assays obtained in this study, the following potency of the candidate standard (KFDA 01/002) for JE vaccine potency assay was tentatively proposed: 2.455 log $PRNT_{50}$/2× [0, 7] dosage. A further characterization using multiple dilution dose assay to estimate more accurate potency is currently under study.

As a conclusion, the availability of a common national standard for JE vaccine (inactivated) will be of a great value in the production of the standardized, uniform quality of JE vaccine (inactivated).

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