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A collaborative study of an alternative *in vitro* potency assay for the Japanese encephalitis vaccine

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ABSTRACT

The use of inactivated Japanese encephalitis (JE) vaccines has been ongoing in East Asia for 40 years. A mouse immunogenicity assay followed by a Plaque Reduction Neutralization (PRN) Test (PRNTest) is currently recommended for each lot release of the vaccine by many national authorities. We developed an alternative *in vitro* ELISA to determine the E antigen content of the Japanese encephalitis virus to observe the 3Rs strategy. A collaborative study for replacing the *in vivo* potency assay for the Japanese encephalitis vaccine with the *in vitro* ELISA assay was confirmed comparability between these two methods.

The study demonstrated that an *in vitro* assay could perform faster and was more convenient than the established *in vivo* PRNTest. Moreover, this assay had better precision and reproducibility compared with the conventional *in vivo* assay. Additionally, the content of antigen determined using the *in vitro* ELISA correlated well with the potency of the *in vivo* assay. Furthermore, this method allowed discrimination between individual lots. Thus, we propose a progressive switch from the *in vivo* assay to the *in vitro* ELISA for JE vaccine quality control.

1. Introduction

Japanese encephalitis (JE) is the cause of viral encephalitis in most Asian countries and parts of the western Pacific (Fischer et al., 2008; Halstead and Jacobson, 2008; Fischer et al., 2009; Wilder-Smith and Freedman, 2009; Misra and Kalita, 2010). JE has a case-fatality ratio of approximately 20–30%, and 30–50% of survivors have neurologic or psychiatric sequelae (Solomon et al., 2000; WHO, 2006). The JE vaccine is the only method available to prevent this disease, as no specific antiviral agent or other medication currently exists (Gould et al., 2008). Thus, the mass vaccination of children has led to a decrease in the number of JE cases in some countries (Arai et al., 2004; Gupta et al., 2008). Therefore, the importance of the vaccine is paramount. The following three types of JE vaccines are licensed in the world: inactivated mouse brain-derived vaccines, inactivated Vero cell culture-derived vaccines and live-attenuated primary hamster kidney cell culture-derived vaccines (Halstead and Thomas, 2011).

The World Health Organization recommends that a quality control test for each individual lot of licensed vaccine be performed by the manufacturer and by a national control laboratory prior to approving its release onto the market (WHO, 1992; WHO, 2010). It is essential to ensure the consistent quality of each lot, as vaccines are biological products used in a healthy population, and the safety and efficacy of the vaccine have a drastic impact on many individuals (Griffiths, 1996). Furthermore, vaccines have an inherent potential for variability. Therefore, independent testing that is based on a validated methodology is essential to ensure the consistent quality of each lot (Hendriksen et al., 1998).

Currently, the potency test used as a lot release test for the inactivated JE vaccine is an *in vivo* assay that compares the amount of vaccine necessary to induce neutralizing antibodies in mice equated with the amount of reference preparation necessary to produce the same effect (WHO, 1998; Ashok and Rangarajan, 2000; MHW, 2006; WHO, 2007; MFDS, 2011). However, there are inher-

Abbreviations: JE, Japanese encephalitis; PRN, plaque reduction neutralization; ELISA, enzyme-linked immunosorbent assay; CEC, chick embryo cell; EMEM, Eagle's minimum essential medium; CV, coefficient of variation.

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ent problems with this assay. First, this method is time consuming, as there is a required period of time for the murine immunization and the preparation of the plaque reduction neutralization assay (Hendriksen, 2007). In addition, there are animal ethical issues and deviations of test results from the condition of the animal or the cultured cells or variability due to the proficiency of the technician. Moreover, the global trend is to discourage the use of experimental animals; thus, many developed countries are reducing the number of animals utilized in quality control assays of vaccines through the development of *in vitro* assays (De Mattia et al., 2011; Isbrucker et al., 2011; Shin et al., 2011). In Korea, it is estimated that the number of mice used in the national lot release tests for JE vaccines are approximately 2000 per year, with more than 10,000 mice used annually when including the manufacturers' quality control tests.

The Ministry of Food and Drug Safety (MFDS) developed an *in vitro* enzyme-linked immunosorbent assay (ELISA) potency test for the JE vaccine to solve the above problems and to join the global trend in a 3Rs strategy (Refinement, Reduction and Replacement of animal testing) (Hendriksen, 2002). Our previous study suggested that an alternative *in vitro* method for the measurement of the amount of the JE E-antigen be used, which consisted of an ELISA kit that included neutralizing monoclonal capture and detection of antibodies mapped to specific epitopes (Kim et al., 2012).

Herein, we describe a study performed by the MFDS to assess the feasibility of replacing the *in vivo* assay with the *in vitro* ELISA for the lot release test, including a comparison between these two tests and collaborative tests with the manufacturers.

2. Materials and methods

2.1. Vaccine samples

Two different inactivated liquid JE vaccines (labeled Samples A and B) commercially available in Korea containing the Nakayama strain derived from mouse brain were used for all tests with four participants. To obtain the reduced-potency vaccine samples, a vaccine lot was treated by incubating aliquots at 4 °C, 25 °C, 37 °C and 56 °C for 3 weeks.

2.2. Standard

The standard for this study was a Korean national reference standard (code: 07/022) with an assigned titer of 2.661 log PFU/vial of antigen. This standard contained the inactivated and lyophilized Nakayama strain was established in 2007 by MFDS similarly to the Korean commercial vaccine. This was used to assess both the *in vitro* ELISA and the immunogenicity, via the *in vivo* animal PRN test, of the vaccine samples.

2.3. In vivo *immunogenicity potency assay*

The *in vivo* potency assay was performed for two different commercial vaccines in accordance with the Korean Biologics Specification and Test Methods. Briefly, each group of 10, 4-week-old ICR (Institute of Cancer Research) strain mice was immunized subcutaneously on days 0 and 7 with 0.5 mL of standard or vaccine sample diluted in phosphate buffered saline. One week later, the mice were bled from the heart, and serum pooled from each mouse was prepared.

The neutralizing antibody titer was measured with a 50% plaque-reduction end-point assay test in 60 mm culture dishes containing a monolayer of primary chick embryo cells (CECs), as reported previously (Oya et al., 1967; Gowal et al., 2010). These sera were diluted 1: 320 in 1 x Eagle's minimum essential medium (EMEM) with 5% fetal bovine serum (FBS) and were mixed with 100 PFU of the challenge virus followed by incubation for 90 min at 36 ± 1 °C. The incubated samples were added onto 4 culture dishes containing the CEC monolayer, and the cells were covered with 0.9% agarose overlay medium containing 1 x EMEM with 7% FBS. Following incubation for 48 h at 37 °C, the covered cells were overlaid again with agarose containing 1 x EMEM and 0.02% neutral red. After incubation for 1–2 days at 37 °C, the plaque numbers were counted, and the 50% reduction in potency was calculated as: Z = (Y-50)/47.7622 + log X, where Z: NT-Ab titer (log10), Y: plaque reduction rate (%), Y = 10–90% and X: reciprocal of serum dilution used (Abe et al., 2003; Borges et al., 2008). The plaque numbers of 10 culture dishes inoculated with 100 PFU of the challenge virus were counted concurrently to confirm the titer of the virus. The result has the unit of logPFU/mL

2.4. In vitro *ELISA*

Anti-JEV ELISA kits were produced by JENO Biotech Inc., a Korean diagnostic medicine manufacturer. The kits consisted of an ELISA 96-well plate with 2 μg/mL of capture mAb for the JEV E-protein immobilized on the plate, wash solution (phosphate buffered saline containing 0.1% Tween 20), dilution buffer (wash solution containing 0.1% bovine serum albumin), Horseradish peroxidase (HRP)-labeled detector mAb, TMB (3,3′,5,5′-tetramethylbenzidine) substrate, stop solution (2N sulfuric acid) and positive (1: 80 dilutions of national reference standard with tris-buffered saline and Tween 20 containing 0.1% bovine serum albumin) and negative controls (1 × EMEM) (Kim et al., 2012).

The ELISA was performed as directed by the manufacturer's manual. Briefly, serial ten-fold dilutions of the national reference standard and the commercial JEV vaccines with dilution buffer were added to the microplate (final volume 100 μL). The microplate was incubated with these samples, including the positive and negative controls, at 37 ± 1 °C for 1 h ± 5 min. After incubation, the microplate was washed three times with wash solution and was incubated with HRP-labeled detector mAb for 1 h ± 5 min at 37 ± 1 °C (final volume 100 μL). After washing three times, the microplate was incubated in the dark with 100 μL/well of TMB substrate. After 15 min, the reaction was stopped with 50 μL/well of stop solution. The absorbance was measured at 450 nm (reference wavelength 650 nm) using an ELISA plate reader (Molecular Devices, USA). The result has the unit of ELISA Unit (E.U.)/mL.

2.5. *Method validation of* in vitro *ELISA*

2.5.1. *Specificity*

Dilution buffer, negative control, positive control, national reference standard and two commercial JE vaccines were assayed to confirm specificity for the E antigen of JEV. The national reference standard and the two JE vaccines were diluted to 1: 80 with dilution buffer.

2.5.2. *Linearity*

Six concentrations of the value ranging from 1: 10–1: 320 were tested with three independent determinations to obtain the slope and correction coefficient (R^2).

2.5.3. *Accuracy*

The estimation of the accuracy was performed using the linearity result. The national reference standard was used to artificially determine the ELISA Unit (E.U.) to 1331 mE.U./mL.

2.5.4. *Precision*

The precision was established to assess assay repeatability and intermediate precision. To determine the repeatability, three concentrations ranging from 1: 40–1: 160 were tested with three wells