Laboratory and Epidemiology Communications

Estimation of Focus Reduction Neutralization Test for Measurement of Neutralizing Antibody Titer against Japanese Encephalitis Virus

Kanako Watanabe*, Chika Hirokawa, Miyako Kon, Tsutomu Tamura and Makoto Nishikawa

Virology Section, Niigata Prefectural Institute of Public Health and Environmental Sciences, Niigata 950-2144, Japan

Communicated by Ichiro Kurane

(Accepted August 21, 2008)

Japanese encephalitis (JE) is the main cause of viral encephalitis in many Asian countries. Approximately 50,000 cases of JE are reported annually in the world, with a high fatality rate and frequent residual neuropsychiatric sequelae in survivors (1). In Japan less than 10 cases have been reported annually since 1992 (2). It is generally accepted that the decrease in the number of JE patients has been caused by multiple factors: nationwide vaccination of children, environmental sanitation measures for insect control and relocation of swine farms to be separated from urban areas. Vaccination is considered to be the most important control measurement. It is believed that the presence of neutralizing antibody assures protection from JE. Thus, the assessment of neutralizing antibody is important for estimating the protective immunity against JE virus.

The 50% plaque reduction neutralization test (PRNT50) using chick embryo cells or Vero cells has been the standard method for assessing JE virus neutralizing antibody titers. This method is subject to technical difficulties in some situations. It requires large amounts of Vero cells and a safety cabinet and incubator with a high capacity. This limits the number of samples that can be examined at one time. The 50% focus reduction neutralization test (FRNT50) has been introduced as an alternative method. In FRNT50 the amounts of cells required as well as the necessary capacity of the safety cabinet and incubator are reduced. In the present study, FRNT50 was evaluated for determining JE virus neutralizing antibody titer in comparison with PRNT50.

Serum samples were diluted at 1:10 and heated at 56°C for 30 min to inactivate complement. Heat-inactivated, 1:10 diluted sera were further serially diluted 2-fold from 1:10 to 1:320 and used in an assay for neutralizing the antibody to JE virus.

PRNT50 assays were performed as described below (3). One hundred microliters of serially diluted serum was mixed with an equal volume of 200 plaque-forming units of JE virus, JaGAr01 strain. Serum-virus mixtures were incubated at 37°C for 1 h and placed on Vero 9013 cell monolayers in 6-well plates. The plates were incubated for 1 h at 37°C in 5% CO2. The cells were then overlaid with maintenance medium containing 1% methylcellulose (Wako, Tokyo, Japan). The plates were incubated at 37°C in 5% CO2 for 4 days. After 4 days of incubation, the cells were fixed with 10% neutral formalin for 60 min at room temperature and washed with tap water. The cells were stained by 0.3% methylene blue for 60 min at room temperature and washed with tap water. The antibody titers were expressed as reciprocals of the highest serum dilution showing a 50% plaque reduction or greater compared with control values.

FRNT50 assays were performed as described below (4). Twenty-five microliters of serially diluted serum was mixed with an equal volume of 100 focus-forming units of JE virus, Beijing-1 strain. Serum-virus mixtures were incubated at 37°C for 1 h and placed on Vero Osaka cell monolayers in 96-well plates. The plates were incubated at 37°C in 5% CO2 for 46 h. The maintenance medium was removed, and the cells were washed with phosphate-buffered saline (PBS). The cells were fixed 2 times with 100% ethyl alcohol at room temperature for 5 min. Peroxidase anti-peroxidase (PAP) staining was performed. (i) The cells were overlaid with rabbit monoclonal anti-JE virus antibody at 1:50 dilution. The plates were incubated at 37°C for 30 min and then washed with PBS. (ii) The cells were overlaid with anti-rabbit immunoglobulin G (MP Biomedicals; Cappel, Irvine, Calif., USA) at 1:500 dilution. The plates were incubated at 37°C for 30 min, and the cells were washed with PBS. (iii) The cells were overlaid with the PAP-conjugated anti-rabbit complex (Dako, Glostrup, Jpn. J. Infect. Dis., 61, 2008

*Corresponding author: Mailing address: Environment and Public Health Inspection Division, Health, Social Welfare and Environmental Administration Department, Nagaoka Regional Promotion Bureau, Niigata Prefecture, 2711-1 Maeda, Kawasaki, Nagaoka, Niigata 940-0861, Japan. Tel: +81-258-33-4939, Fax: +81-258-33-4933, E-mail: watanabe.kanako@pref.niigata.lg.jp
Denmark) at 1:1,000 dilution. The plates were incubated at 37°C for 30 min, and the cells were washed with PBS. (iv) Finally, 3,3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, Mo., USA) was added to the wells. The plates were incubated at room temperature for 30 min. After the completion of the reaction, the cells were washed with tap water, and the foci were counted. The antibody titers were expressed in the same fashion as in PRNT50 assays.

A total of 321 human serum samples were tested for JE virus neutralizing antibody titer by PRNT50 and FRNT50 assays (Table 1, Fig. 1). The presence and absence of neutralizing antibody were consistent between PRNT50 and FRNT50 assays for 302 of 321 samples (94.1%) (Table 1). The results were different between these two methods for 19 of 321 samples (5.9%). Neutralizing antibody titers determined by FRNT50 were within 4-fold differences compared with those determined by PRNT50 for 286 of 302 positive samples (94.7%). Seven of the 19 positive samples were determined to be negative by FRNT50, but were found to be positive with a titer of 10 by PRNT50. Ten of these 19 samples demonstrated plaque reduction of nearly 50% at 1:10 dilution as determined by PRNT50 and were determined to be negative.

The results indicate that neutralizing antibody titers determined by FRNT50 were consistent with those determined by PRNT50. The FRNT50 method can reduce both the total amounts of Vero cells and the necessary working space, and consequently enables the assessment of a large number of samples at one time. The results indicate that FRNT50 is a reliable method for measuring serum JE virus neutralizing antibody titers, and suggest that FRNT50 is more practical than PRNT50 in certain laboratory settings.

Table 1. Comparison of the results by 50% plaque reduction neutralization test (PRNT50) and 50% focus reduction neutralization test (FRNT50) for measuring Japanese encephalitis virus neutralizing antibody.

<table>
<thead>
<tr>
<th>Results of Japanese encephalitis virus neutralizing antibody</th>
<th>FRNT50</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRNT50 Positive (≥10)</td>
<td>133 (41.4%)</td>
</tr>
<tr>
<td>PRNT50 Negative (&lt;10)</td>
<td>0</td>
</tr>
<tr>
<td>PRNT50 Positive (≥10)</td>
<td>19 (5.9%)</td>
</tr>
<tr>
<td>PRNT50 Negative (&lt;10)</td>
<td>169 (52.7%)</td>
</tr>
</tbody>
</table>

We thank Dr. C. K. Lim, Dr. T. Takasaki, and Dr. I. Kurane (Department of Virology I, National Institute of Infectious Diseases) for providing anti-JE virus (Beijing-1 strain) monoclonal antibodies in rabbit.

REFERENCES