CVI Accepts, published online ahead of print on 20 May 2009
Clin. Vaccine Immunol. doi:10.1128/CVI.00095-09

Evaluation of chimeric Japanese encephalitis and dengue viruses for use in diagnostic plaque reduction neutralization tests

Barbara W. Johnson[1*], Olga Kosoy[1], Elizabeth Hunsperger[2], Manuela Beltran[2], Mark Delorey[1], Farshad Guirakhoo[3], Thomas Monath[4]

[1]Diagnostic & Reference Laboratory, Arbovirus Diseases Branch, Centers for Disease Control and Prevention (CDC), Division of Vector-Borne Infectious Diseases (DVBID), 3150 Rampart Road, Fort Collins, CO 80521,USA; [2] Serology Diagnostics and Viral Pathogenesis Laboratory, Dengue Branch, CDC, DVBID; [3]Sanofi Pasteur, 1541, Avenue Marcel Merieux-D Est 2, 69280 Marcy l'Eoile, France; [4]Kleiner Perkins Caufield & Byers Pandemic & Biodefense Fund, 21 Finn Road, Harvard MA 01451, USA

* Corresponding author:

Barbara W. Johnson, PhD

Diagnostic & Reference Laboratory, Arbovirus Diseases Branch

Division of Vector-Borne Infectious Diseases (DVBID)

Centers for Disease Control and Prevention (CDC)

3150 Rampart Road, Building 401, Fort Collins, CO 80521

(970) 266-3543 (office)

FAX (970) 494-6631

e-mail: bfj9@cdc.gov

Abstract

The plaque reduction neutralization test (PRNT) is a specific serological test used to identify and confirm arbovirus infection in diagnostic laboratories and monitor immunological protection in vaccine recipients. Wild-type (wt) viruses used in the PRNT may be difficult to grow and plaque titrate, such as the dengue viruses (DENV), and/or may require BSL3 containment, such as West Nile (WN), St. Louis encephalitis, (SLE), and Japanese encephalitis (JE) viruses. These requirements preclude their use in diagnostic laboratories with only BSL2 capacity. In addition, wt JEV falls under jurisdiction of the select agent program and can be used only in approved laboratories.

The chimeric vaccine viruses ChimeriVax™-WNV and –SLEV have previously been shown to elicit antibody reactivity comparable to the parental wt WNV and SLEV viruses. ChimeriVax™ viruses provide advantages for PRNT: they grow more rapidly than most wt flaviviruses, produce large plaques, require BSL2 conditions, and are not under select agent restrictions. We evaluated the ChimeriVax™-DENV1, 2, 3, 4 and -JEV for use in PRNT on sera from DENV- and JEV-infected patients and from JEV vaccine recipients. Serostatus agreement was 100% between the ChimeriVax ™ DENV serotypes and wt prototype DENV and 97% overall with ChimeriVax™-JEV compared to prototype Nakayama JEV, 92% in a subgroup of JEV vaccine recipients and 100% in serum from encephalitis patients naturally infected with JEV. ChimeriVax™-DENV and -JEV plaque phenotype and BSL2 requirements, combined with sensitive and specific reactivity make them good substitutes for wt DENV and JEV in PRNT in public health diagnostic laboratories.

Introduction

Flaviviruses are medically important pathogens that are significant causes of disease throughout the temperate and tropical regions of the world. In Asia over 3 billion people live in areas where they are at risk of being infected with Japanese encephalitis virus (JEV) and JEV infections have become the leading cause of pediatric encephalitis in Asia, with as many as 50,000 cases and 15,000 deaths per year (49, 50). The four serotypes of dengue virus (DENV) have emerged in recent years to reach pandistribution throughout the tropics and subtropics of the Americas, Asia, and Africa, resulting in over 100 million dengue fever cases and hundreds of thousands of cases of the more severe dengue hemorrhagic fever/ dengue shock syndrome (14-17, 37).

Laboratory diagnosis of flavivirus infection is primarily serological, by detection of virus-specific immunoglobulin M (IgM) in an IgM antibody-capture enzyme-linked immunosorbant assay (MAC ELISA), ideally from paired acute and convalescent specimens, but in practice from a single acute serum or cerebral spinal fluid (CSF) specimen (6, 35, 36, 54). This method is sensitive and relatively specific. However, there is considerable cross-reactivity of antibodies elicited in the immune response to conserved regions of the flavivirus envelope protein, which may cause false-positive results in the MAC ELISA and confound diagnosis in areas where multiple flaviviruses co-circulate (45). Specificity can be improved somewhat through differential diagnosis by cross-testing specimens against multiple flaviviruses simultaneously in a standardized MAC ELISA format, but it may be difficult to distinguish between flaviviruses by MAC ELISA alone, even those from different antigenic complexes such as JEV and DENV (7, 24, 35, 42).

The plaque reduction neutralization test (PRNT) is a specific serological assay that is used in the CDC/DVBID diagnostic laboratory to confirm infection and differentiate between flaviviruses in primary flavivirus infections (4, 10, 46-48). Neutralization assays are also used to monitor protective immunity in vaccinees (26, 38). In the PRNT procedure the serological specimen (generally serum) is mixed with live virus, and if virus-specific neutralizing antibodies are present in the serum, they bind to the virus to form a complex. The mixture is then inoculated onto a monolayer of cells. Virus bound up in an antibody-virus complex is inhibited from infecting the cells, i.e. it is neutralized. Consequently, laboratories conducting these assays must have tissue culture capability, considerable technical expertise in growing and plaque titrating flaviviruses, which have a wide range of growth rates, and appropriate biosafety level laboratory conditions in which to grow the virus. JEV, West Nile virus (WNV), and St. Louis encephalitis virus (SLEV) require biosafety level 3 (BSL3) containment, which precludes their use in many public health diagnostic laboratories with only BSL2 capacity. In addition, JEV falls under jurisdiction of the select agent program and can be used only in select agent registered laboratories (11).

Acambis, Inc.(now part of Sanofi Pasteur) has developed chimeric vaccine viruses for JEV, WNV, SLEV, and the four serotypes of DENV based on the attenuated yellow fever (YF) vaccine virus 17D (YF-VAX), with the genes encoding the premembrane (prM) and envelope (E) proteins of the YF 17D virus replaced with those of heterologous flaviviruses; ie, JEV, WNV, SLEV, and DENV (3, 8, 19, 20, 39). Previously, the ChimeriVax™-WN and –SLE viruses were shown to be functionally comparable to prototype WNV and SLEV in the PRNT (44). The ChimeriVax™ viruses have many advantages over the prototype

wild-type (wt) viruses in the PRNT, most importantly for this application, they can be used under BSL2 containment. They are plaque purified and produce large-sized, relatively uniform plaques which are phenotypically similar to those of YF17D virus, but with specific reactivity to the heterologous prM-E protein insert. They grow at relatively the same rate, which has allowed the procedure to be standardized at CDC/Division of Vector-Borne Infectious Diseases (DVBID) so that when using multiple flaviviruses in differential diagnosis, the second overlay can be applied to all the PRNT plates on the same day (Table 1). This generally shortens the test duration compared to wt flaviviruses.

We compared serostatus and neutralizing antibody titer of the ChimeriVax™-DEN1,2,3,4 and -JE viruses to their counterpart prototype DENV and JEV used in the PRNT at CDC/DVBID. The performance of the ChimeriVax™ viruses was comparable to the prototype viruses in the PRNT, which combined with their facility of use and BSL2 classification, make them advantageous to use in diagnostic laboratories which otherwise would be unable to perform this specific confirmatory diagnostic assay.

Materials and Methods

Serum Specimens

A panel of serum specimens without personal identifiers were selected from the CDC/DVBID collection of archived samples, which had been submitted for JEV or DENV diagnostic testing or to monitor protective immunity following JEV or YFV vaccination. In addition, serum specimens from JEV-infected patients were provided by Dr. Phan Thi Nga Department of Virology, National institute of Hygiene and Epidemiology, Hanoi, Vietnam. Diagnostic specimens had been previously characterized by MAC ELISA and confirmed by PRNT using the prototype DENV and JEV, or by virus isolation. Neutralizing antibody

titer had been determined previously in the serum specimens from vaccine recipients by PRNT using the prototype viruses.

Viruses

Prototype viruses used in the PRNTs were obtained from the CDC/DVBID virus reference collection: DENV1 (Hawaii 1944); DENV2 (New Guinea C 1944); DENV3 (H -87, Philippines 1956); DENV4 (H – 241, Philippines 1956); and JEV (Nakayama 1935) (53). The ChimeriVax™-DENV1,2,3,4 and parental wt DENV strains on which the ChimeriVax™-DEN viruses are based (DENV1 PUO-359, DENV2 PUO-218, DENV3 PaH881/88, and DENV4 1228) were supplied by Acambis Inc. (Cambridge, MA, USA). (18-21, 40). The ChimeriVax™-JEV, constructed to contain the prM-E genes from JEV SA14-14-2, the live virus vaccine strain developed in China, also was supplied by Acambis Inc. (20, 57).

The prototype DEN viruses were grown in 25 cm^2 flasks with DMEM (for a closed system) on C6/36 cells at 33°C. The supernatant was harvested on the 5th day; no CPE was observed. Cellular debris was removed from the supernatant by centrifugation at 3,500 g for 30 min. Fetal bovine albumin was added to the clarified supernatant to a final concentration of 20%. The seeds were aliquoted and stored at -70°C.

The ChimeriVax™ virus seeds were prepared in Vero cells in DMEM medium with 2% FBS. The supernatant was harvested at 3-4 days, and cellular debris was removed by centrifugation at 3000 g for 30 min. The working stock ChimeriVax™ virus was stored with 20% FBS at -70°C. ChimeriVax™-JEV was passaged once in Vero cells from lyophilized JEV vaccine manufactured by Acambis Inc.; the ChimeriVax™-DENV working stock was passaged twice in Vero cells. Yellow fever virus was not used in PRNT

reported here because we have repeatedly shown that the ChimeriVax™ viruses with heterologous (e.g. DEN, JE) prM-E genes are not neutralized by YF-specific antibodies and do not induce neutralizing antibodies to YFV (39).

Plaque reduction neutralization test (PRNT): All the PRNTs were completed by one Clinical Laboratory Improvement Amendments (CLIA)-certified technician, who routinely performs neutralization assays in the CDC/DVBID arbovirus diagnostic laboratory. Each sample was tested simultaneously with the prototype and ChimeriVax™ viruses. Each test run was validated with a standardized virus-specific mouse hyperimmune ascitic fluid (MHIAF) postive control compared against the virus back titration. Because the evaluation was designed to simulate diagnostic testing conditions and protocol, in which the specimens would normally be tested once, replicate testing was not done unless noted.

Virus-specific neutralizing antibody titers were determined by 90% endpoint PRNT in 6-well plates in Vero cells using a 0.5% agarose double overlay, visualized with neutral red staining in the second overlay (4). Samples were first heat inactivated at 56°C for 30 min to destroy the complement and to inactivate adventitious viruses, so as to make fair comparison between paired samples; non-heat inactivated normal human sera added to the serum-virus mixture at a concentration of 4% provided a source of labile serum factor. Second overlays were applied on days 4-7 (Table 1).

Neutralizing antibody titer is expressed as the reciprocal of the end-point serum dilution that reduced the challenge virus plaque count by 90%, based on the back titration. In differential PRNT, in which specimens were tested simultaneously to two or more viruses, a fourfold or higher neutralizing titer to one virus compared to the others was considered virus specific, except in tests in which the neutralizing antibody reacted to all the

flaviviruses tested (7). Extensive antibody cross-reactivity between challenge viruses in the PRNT is an indication of a secondary flavivirus infection and identification of the infecting flavivirus could not be made in these specimens.

Statistical Analyses. Of primary importance was the serostatus of the sample under testing using the prototype viruses and using the ChimeriVax™ viruses. Because the serostatus outcome is binary, agreement between the prototype virus and ChimeriVax™ virus was assessed using the kappa statistic for intra class correlation (13); see, for example, op. cit. p 217). Antibody cross-reactivity to the prototype dengue viruses was compared to that of the ChimeriVax™-DEN viruses by comparing how frequently a heterologous (cross-reactive) titer indicated positivity (even if the homologous titer was fourfold greater, indicating a correct diagnosis). A non-inferiority test for proportions (34) was then used to determine whether the ChimeriVax™-DEN viruses produced more cross-reactive results than the prototype DEN viruses. A secondary question was how similar the neutralizing antibody titers were against the prototype viruses and the ChimeriVax™ viruses. For comparing antibody titers in the DENV PRNT evaluation, titers were modeled as a function of strain, test serotype, and whether the test was homologous or heterologous using generalized estimating equations (GEE). We chose to use GEEs because the titers against DENV had a complex covariance structure that could be made explicit in the GEE framework. In the JEV PRNT comparison, the titers were modeled using a linear model. A linear model not only estimates the strength of the relationship between the Chimeric and wild-type titers, but unlike a simple correlation, can identify a scaling factor between the titers.

Results

Dengue virus PRNT

The sample set used in the DENV PRNT evaluation consisted of 48 serum specimens from 33 primary DENV infections (15 DEN-1, 12 DEN-2, 3 DEN-3, and 3 DEN-4); 3 secondary DENV infections; 6 other flavivirus infections (3 WNV, 3 YFV vaccinated), and 6 flavivirus negative controls. These specimens had been characterized previously by MAC ELISA and PRNT, and/or virus isolation or viral RNA detection (4, 9, 30, 35, 36).

Initially, each specimen was tested simultaneously against the four serotypes of the prototype DEN viruses, the four ChimeriVax™-DENV serotypes, and the four ChimeriVax™ parental DENV strains (Table 2). Because each specimen was tested in 12 X 6-well plates at a time, the samples were tested in 5 batches, with standardized positive and negative controls. The positive specimens were retested with the homologous prototype DENV, ChimeriVax™ DENV, and ChimeriVax™ parental DENV together in one replicate to confirm uniformity of the 5 tests (data not shown). Because of the limited sample volume available, and the number of different viruses against which each sample was tested, initial serum dilutions of 1:10 and working serum dilutions of 1:20 were used for the dengue test specimens. Therefore the lower limit of quantification (LLOQ) for this group was a neutralizing antibody titer of 20.

Overall serostatus agreement of each of the ChimeriVax™-DENV serotypes compared to the prototype wt DENV serotypes and ChimeriVax™ parental wt DENV was 100% (Table 2). The kappa statistic for intraclass correlation was 1, with a p-value of < 0.01, and a lower 95% confidence bound of 0.89, showing strong, positive correlation. The non-inferiority test from Liu et al. (2002), indicated that the antibody cross-reactivity between the DENV serotypes observed with the ChimeriVax™ DEN viruses was not greater than the cross-reactivity observed with the prototype DEN viruses ($p = 0.02$). It should be noted

that one of the specimens originally classified as a DENV-3 infection showed no neutralizing titer with either the chimeric or prototype DEN-3 viruses.

GEE results failed to show a statistically significant difference in neutralizing antibody titers between the ChimeriVax™-DEN viruses, the prototype DEN viruses, or the ChimeriVax™ parental DEN strain viruses (p=0.12). However, this statistical test is designed to detect a difference in titers, and therefore caution must be exercised in interpreting this finding. The fact that no statistically significant difference was found may suggest that, after adjusting for other effects, the two strains produce the same mean neutralizing antibody titers. However, on its own, it is not conclusive. We include this result in order to illustrate that not only do the ChimeriVax™-DENV and prototype DENV have similar agreement in the final diagnosis, but also to show that there is some evidence that the resultant antibody titers are similar on a log scale too.

In the three secondary DENV infections (Table 2, specimens 34-36), the infecting DENV serotype could not be differentiated by either the ChimeriVax™-DENV or prototype DENV, as the neutralizing antibody was highly reactive to all ChimeriVax™-DENV and prototype DENV serotypes.

Japanese encephalitis virus PRNT

A total of 100 serum specimens were used in the side-by-side PRNT comparison of ChimeriVax™-JEV and JEV Nakayama (Table 3). Fifty-five sera were banked specimens collected from individuals that had received JEV (Biken) or YFV vaccines and these sera had been screened previously by PRNT with JEV Nakayama to monitor protective immunity (Table 3, part A). Twenty-six had JEV-specific neutralizing titers; 29 did not have detectable JEV-specific titers. Fourteen specimens were from encephalitis patients

from Vietnam, which had been previously classified as recent JEV infections by JE MAC ELISA and PRNT (Table 3, part B). Serum specimens from persons with heterologous flavivirus infections included 9 DENV, 3 WNV, 3 YFV (1 natural infection and 2 vaccine recipients), and 8 that were classified as secondary flavivirus infections in which the infecting virus could not be determined (Table 3, part C). Ten specimens had been submitted as suspected JEV infections based on travel to JE endemic countries. In the initial diagnostic testing, these specimens had JE positive or equivocal MAC ELISA results, but were JE negative by PRNT against JEV Nakayama virus (Table 3, part D). Each specimen was tested simultaneously against JEV Nakayama and ChimeriVax™ -JEV in three groups (Table 3, set A, set B, and sets C,D) and each group included standardized positive and negative controls. The initial serum dilution was 1:5 and the LLOQ was 10.

With respect to indicating a positive or negative result, agreement between the prototype JEV Nakayama and ChimeriVax™ JE viruses was high, at 96% (96/100). The kappa statistic for intraclass correlation was 0.92 ($p < 0.01$) with a lower 95% confidence bound of 0.76. In the vaccinated subgroup (n = 55), agreement between JEV Nakayama and ChimeriVax™-JEV was 92% (Table 3, part A). Of those with a detectable neutralizing antibody titer against JEV Nakayama (n = 26), 50% (13/26) had a higher titer against JEV Nakayama, 7.6% (2/26) had a higher titer against ChimeriVax™-JEV, and 42.3% (11/26) had equivalent titers. Serostatus agreement between JEV Nakayama and ChimeriVax™-JEV was 100% within the subgroup of persons with naturally acquired JEV infections from Vietnam (Table 3, part B). Of these 78.6% (11/14) had higher titers against ChimeriVax™-JEV, none had a higher titer to JEV Nakayama, and in 21.4% (3/14) the titers were equivalent. When $\log_2$ (ChimeriVax™-JEVtiter/10) is regressed on $\log_2$ (prototype JEV

titer/10) and strain, the titer with ChimeriVax™-JEV is, on average, approximately two logs higher than the titer with JEV Nakayama for the JEV-infected group ($p<0.01$; 95% confidence interval of 1.69 to 3.03). Additionally, in the JEV vaccinated group, the $\log_2$ (ChimeriVax™-JEVtiter/10) is slightly less than the $\log_2$ (prototype JEV titer/10) (a 95% confidence interval for their ratio is 0.82 to 0.97) (Figure 1).

Discussion

Diagnostic testing for arbovirus infections has increased in the United States since the introduction of WNV in 1999, and globally through laboratory-based surveillance projects designed to aid countries in making decisions on implementation of vaccine and/or arbovirus control programs. Combined with the increase in the number of tests, laboratories are also expanding the repertoire of diagnostic assays that they routinely carry out in order to improve detection. Virus-specific MAC ELISA has been the primary serological test for detecting arbovirus infection when a single acute serological specimen is obtained. The MAC ELISA is sensitive, as IgM antibody is produced early in infection and detectable within a few days from illness onset, and is more virus-specific than IgG antibody (27, 28, 36, 54-56). However, in flavivirus infections antibodies elicited against conserved epitopes on the immunogenic envelope protein may cross-react with other flaviviral antigens in the MAC ELISA, which can cause false-positive results (45). PRNT is a specific, quantitative assay, which measures the amount of virus-specific neutralizing antibody present in the serum, and is used to confirm infection and differentiate cross-reactive MAC ELISA results.

Acute serological specimens received in the CDC/DVBID arbovirus diagnostic laboratory are initially tested by MAC ELISA against selected arboviruses circulating in a

geographical area to which the patient may have been exposed (4, 32, 35, 36, 46). Positive or equivocal MAC ELISA results are confirmed by PRNT. (An equivocal MAC ELISA result with a negative PRNT titer is considered negative.) In differential diagnosis, in which the specimens are tested for multiple flaviviruses, cross-reactive results in the MAC ELISA are resolved by PRNT.

Neutralization assays are also used to monitor protective immunity in JEV vaccine recipients. WHO vaccine guidelines consider a neutralizing antibody titer of 10 in $PRNT_{50}$ protective (26, 53). The more stringent $PRNT_{90}$ is used in the CDC/DVBID diagnostic laboratory for both monitoring immune protection and confirmatory testing of clinical specimens, as low-level cross-reactivity may result in false positives in the $PRNT_{50.}$ This is especially important in making a differential diagnosis between closely related, co-circulating flaviviruses, such as between WNV and SLEV or JEV and WNV.

PRNT using wt flaviviruses can be technically difficult, as many flaviviruses grow slowly and have pinpoint-sized plaque phenotypes, or may be comprised of mixed populations, or quasispecies, which grow at different rates and produce plaques of varying sizes. The difficulties are compounded in differential PRNT when specimens are tested against multiple flaviviruses simultaneously, such as with the four DENV serotypes reported here. The prototype DENV-2 NCG and DENV-4 H241 produce medium-sized plaques and grow relatively quickly, with second overlays applied on days 6 and 4, respectively. However, DENV-1 Hawaii44 and DENV-3 H-87 grow more slowly and produce diffuse plaques of varying sizes which are difficult to visualize even 2 days following the second overlay application at 7 days. Thus using the four prototype DEN viruses in the PRNT requires application of the second agarose overlay on three different days (Table 1). The ChimeriVax™-DENV viruses grow at relatively the same rate, generally more rapidly than the prototypes, so that in

differential PRNT using multiple ChimeriVax™ viruses the second overlays can all be applied on the same day, which simplifies the overlay procedure and shortens the test (Table 1). They also produce larger, more uniform plaques which are more readily visualized soon after neutral red staining compared to the prototype DEN viruses.

JEV prototype Nakayama strain produces medium-sized, easy-to-read plaques, following application of the second agarose overlay at 4 days. However, the American Committee on Arthropod-borne Viruses has classified wt JEV as a BSL3 agent which must be used under BSL3 containment (23). In addition, wt JEV is under U.S. Department of Agriculture select agent restrictions and can be used only in registered laboratories (11). The ChimeriVax™ viruses require only BSL2 containment and are not under select agent restrictions, and can be used in most public health laboratories with tissue culture capabilities. For these reasons we evaluated the ChimeriVax™- JEV and -DENV to determine if they could replace the prototype JEV and DENV in routine diagnostic neutralization assays.

Prototype DENV and ChimeriVax™-DENV were compared in differential PRNTs, in which the specimens were tested against all four DENV serotypes simultaneously, in a panel of 48 serum specimens from primary and secondary DEN infections and suspected flavivirus-infection cases (Table 2). The parental strains of DENV from which the ChimeriVax™-DENV viruses were constructed were also included in the evaluation to control for any DENV strain differences. Although neutralizing antibody reactivities to the parental DENV strains have been characterized in ChimeriVax™-DENV vaccine trials, they have not been used as challenge viruses in PRNT with diagnostic specimens. Isolated from human classical dengue fever cases in Thailand (DENV-1 PUO359, DENV-2 PUO218, and DENV-3 PaH881/88) and Indonesia (DENV-4 1228), these parental DENV

strains were difficult to plaque titrate (18, 21). They grew slowly, with the second agarose overlay applied on day 6 or 7, and produced very small plaques. The lower neutralizing antibody titers using DENV-1 PUO359 and DENV-2 PUO218 compared to ChimeriVax™-DENV-1 and -2 may have been due to the unfamiliarity of using these strains in PRNT. However, serostatus agreement between the ChimeriVax™ parental DEN and ChimeriVax™ DEN viruses and the prototype DEN viruses was 100%, indicating that the anti-DENV neutralizing antibodies in the test specimens effectively reacted with ChimeriVax™ parental DENV strains, and therefore the ChimeriVax™-DEN viruses.

ChimeriVax™-JEV was compared to the prototype JEV Nakayama strain in a group of 100 serum specimens from JEV or YFV vaccinees, a JEV outbreak in Vietnam, and suspected JEV- or other flavivirus-infection cases. Serostatus agreement between ChimeriVax™-JEV and JEV Nakayama was 92% among the 55 JEV vaccine recipients (Table 3, part A). In this subset there were 26 specimens with neutralizing antibody titers against JEV Nakayama. ChimeriVax™-JEV failed to detect neutralizing antibody in 2 of these specimens, although the titers were also low in both specimens against JEV Nakayama. Generally, the neutralizing antibody titers were higher to JEV Nakayama than ChimeriVax™-JEV in the vaccine recipient subset. (Table 3 and Figure 1). This was not unexpected, as studies monitoring immunological protection by PRNT in JEV vaccine trials have shown that using JEV strains homologous to the JEV vaccine result in higher titers compared to heterologous JEV strains (12, 26, 39). Further, cross-neutralization studies have demonstrated that there are significant differences in neutralization titers between strains within a flavivirus species, based on the degree of homology between the strain used to produce polyclonal antibody and the reference strain used as the challenge

virus in the PRNT (1, 2, 7). Serum specimens in this subset were from individuals that had been vaccinated with the Biken™ JEV vaccine, which is an inactivated vaccine prepared from the Nakayama strain. Therefore, it would be expected that serum from the vaccine recipients would be more reactive to the homologous JEV Nakayama strain than to the ChimeriVax™-JEV which contains the prM-E genes from a different strain (SA14-14-2) (12, 25, 33, 39, 51).

In general, the neutralizing antibody titers in the vaccinee subset were low with both JEV Nakayama and ChimeriVax™-JEV, and no neutralizing antibody titer was detected with either the JEV Nakayama or ChimeriVax™-JEV in 29 of 55 of the subset of vaccinees (Table 3 part A; specimens 27-55). Annual monitoring of the protective immunity among vaccine recipients who are otherwise seldom exposed to flaviviruses, such as residents of the United States, has shown that neutralizing antibody titers may be very low to undetectable, although the person may still be protected (26, 53). ChimeriVax™-JEV may be less sensitive in detecting neutralizing antibody titer in JEV Biken™ vaccinees in which the titer is close to the detection threshold. However, it is notable how little difference there was between the titers measured with JEV Nakayama and ChimeriVax™-JEV. ChimeriVax™-JEV has been optimized at CDC/DVBID for use in neutralization assays to monitor immune protection in JEV vaccinees by lowering the LLOQ, which increased serostatus agreement to 100% compared to JEV Nakayama (data not shown).

In the subset of specimens from the JE outbreak in Vietnam (n = 14), other flavivirus primary (n = 13) and secondary (n = 8) infections, and suspected flavivirus infections (n = 10) ChimeriVax™-JEV showed high serostatus agreement with JEV Nakayama. In these groups of specimens the neutralizing titers were significantly higher to ChimeriVax™-JEV

than to JEV Nakayama (Table 3 and Figure 1). JEV Nakayama, isolated in Japan in 1935, and SA 14-14-2, an attenuated strain of SA-14 which was isolated in China in 1954, are both grouped in JEV genotype III based on phylogenetic analysis (1, 2, 12, 22, 38, 39, 51, 52). Historically, isolates from Vietnam have also clustered into genotype III, with slightly higher homology to SA-14 than to Nakayama. However, JEV isolates from JE outbreaks in Vietnam in 2001 and 2002 showed a shift to genotype I (41). The specimens from Vietnam used in this evaluation were obtained from JEV outbreaks in 2004 and 2005. Whether the difference between antibody reactivity in these sera to ChimeriVax™-JEV and JEV Nakayama was due to greater nucleotide sequence homology of the parental ChimeriVax™-JEV strain (SA 14-14-2) to the JEV strains circulating in Vietnam than to JEV Nakayama cannot be determined, as a JEV isolate was not obtained from these specimens (40).

Infection with one flavivirus does not necessarily confer protective immunity against infection by a heterologous flavivirus (43). Consequently, secondary flavivirus infections are common in individuals who live in areas where multiple flaviviruses co-circulate. PRNT may not be a virus-specific diagnostic assay in sera from patients with secondary flavivirus infections, as antibodies from the primary and secondary infection may both react to the challenge flavivirus, whether it is the infecting virus or not. This antibody cross-reactivity results in high titers to all the flaviviruses used in the differential PRNT, or in a titer which is higher to the primary flavivirus infection than to the infecting flavivirus, indicating the phenomenon of 'original antigenic sin' (29, 43). A differential diagnosis cannot be made by PRNT in these cases, even if there is a fourfold difference between titers. However, these results are diagnostically informative to indicate evidence of a

secondary flavirus infection (7). The four DENV serotypes are considered four separate flavivirus species, and consistent with the secondary flavivirus infection PRNT profile, the neutralizing antibody titers were high to all the prototype DENV and ChimeriVax™-DENV serotypes in sera from secondary dengue infections (Table 2, samples 34-26) (5, 31). In these types of specimens, identification of the infecting DENV serotype would not be possible by PRNT using either the prototype DEN or ChimeriVax™-DEN viruses.

ChimeriVax™-WNV and -SLEV are powerful diagnostic tools which have proved useful in resolving cross-reactive results between WNV and SLEV in the MAC ELISA. As reported here, the ChimeriVax™-DENV and -JEV have also been shown to have comparable performance to the prototype DEN and JE viruses currently in use in the PRNT. The BSL2 classification, uniform growth rates, and easy-to-read plaque phenotypes of the ChimeriVax™ viruses facilitates performing PRNT in public health diagnostic laboratories, particularly in differential diagnosis. Addition of ChimeriVax™-DENV and –JEV to the reagent repertoire for use in the PRNT, and distribution by CDC to public health laboratories, will further enhance diagnostic laboratory capacity.

Acknowledgments:

We would like to thank Dr. Phan Thi Nga from the Department of Virology at the National Institute of Hygiene and Epidemiology in Hanoi,Vietnam for providing the specimens used in the JEV PRNT.

References

1. **Ali, A., and A. Igarashi.** 1997. Antigenic and genetic variations among Japanese encephalitis virus strains belonging to genotype 1. Microbiol Immunol **41:**241-52.
2. **Ali, A., A. Igarashi, L. R. Paneru, F. Hasebe, K. Morita, M. Takagi, W. Suwonkerd, Y. Tsuda, and Y. Wada.** 1995. Characterization of two Japanese encephalitis virus strains isolated in Thailand. Arch Virol **140:**1557-75.
3. **Arroyo, J., C. A. Miller, J. Catalan, and T. P. Monath.** 2001. Yellow fever vector live-virus vaccines: West Nile virus vaccine development. Trends Mol Med **7:**350-4.
4. **Beaty, B., C. Calisher, and R. Shope.** 1995. Arboviruses, p. 189-212. *In* E. Lennette, D. Lennette, and E. Lennette (ed.), Diagnostic procedures for viral, rickettsial, and chlamydial infections, 7th ed. American Public Health Association, Washington, DC.
5. **Burke, D., and T. P. Monath.** 2001. Flaviviruses, p. 1043-1125. *In* D. Knipe and P. Howley (ed.), Fields Virology, 4th edition, 4 ed, vol. 1. Lippincott Williams and Wilkins, Philadelphia.
6. **Burke, D. S., A. Nisalak, and M. A. Ussery.** 1982. Antibody capture immunoassay detection of japanese encephalitis virus immunoglobulin m and g antibodies in cerebrospinal fluid. J Clin Microbiol **16:**1034-42.
7. **Calisher, C. H., N. Karabatsos, J. M. Dalrymple, R. E. Shope, J. S. Porterfield, E. G. Westaway, and W. E. Brandt.** 1989. Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. J Gen Virol **70 (Pt 1):**37-43.

8. **Chambers, T. J., A. Nestorowicz, P. W. Mason, and C. M. Rice.** 1999. Yellow fever/Japanese encephalitis chimeric viruses: construction and biological properties. J Virol **73:**3095-101.

9. **Chao, D. Y. D.-Y., B. S. B. S. Davis, and G. J. G.-J. J. Chang.** 2007. Development of multiplex real-time reverse transcriptase PCR assays for detecting eight medically important flaviviruses in mosquitoes. Journal of clinical microbiology **45:**584-589.

10. **Dulbecco, R., M. Vogt, and A. G. Strickland.** 1956. A study of the basic aspects of neutralization of two animal viruses, western equine encephalitis virus and poliomyelitis virus. Virology **2:**162-205.

11. **Federal Register.** 2001. Requirements for facilities transferring or receiving select agents. Final rule. Fed Regist **66:**45944-5.

12. **Ferguson, M., S. Johnes, L. Li, A. Heath, and A. Barrett.** 2008. Effect of genomic variation in the challenge virus on the neutralization titres of recipients of inactivated JE vaccines--report of a collaborative study on PRNT50 assays for Japanese encephalitis virus (JE) antibodies. Biologicals **36:**111-6.

13. **Fleiss, J. L.** 1981. Statistical Methods for Rates and Proportions, 2nd ed. John Wiley & Sons, New York.

14. **Gubler, D. J.** 2006. Dengue/dengue haemorrhagic fever: history and current status. Novartis Found Symp **277:**3-16; discussion 16-22, 71-3, 251-3.

15. **Gubler, D. J.** 1998. Epidemic dengue and dengue hemorrhagic fever: a global public health problem in the 21st century, p. 1-14. *In* W. Scheld, D. Armstrong, and J. Hughes (ed.), Emerging Infections, vol. 1. ASM Press, Washington, DC.

16. **Gubler, D. J.** 2002. The global emergence/resurgence of arboviral diseases as public health problems. Arch Med Res **33:**330-42.

17. **Gubler, D. J.** 1998. The global pandemic of dengue/dengue haemorrhagic fever: current status and prospects for the future. Ann Acad Med Singapore **27:**227-34.

18. **Guirakhoo, F., J. Arroyo, K. V. Pugachev, C. Miller, Z. X. Zhang, R. Weltzin, K. Georgakopoulos, J. Catalan, S. Ocran, K. Soike, M. Ratterree, and T. P. Monath.** 2001. Construction, safety, and immunogenicity in nonhuman primates of a chimeric yellow fever-dengue virus tetravalent vaccine. J Virol **75:**7290-304.

19. **Guirakhoo, F., K. Pugachev, J. Arroyo, C. Miller, Z. X. Zhang, R. Weltzin, K. Georgakopoulos, J. Catalan, S. Ocran, K. Draper, and T. P. Monath.** 2002. Viremia and immunogenicity in nonhuman primates of a tetravalent yellow fever-dengue chimeric vaccine: genetic reconstructions, dose adjustment, and antibody responses against wild-type dengue virus isolates. Virology **298:**146-59.

20. **Guirakhoo, F., Z. X. Zhang, T. J. Chambers, S. Delagrave, J. Arroyo, A. D. Barrett, and T. P. Monath.** 1999. Immunogenicity, genetic stability, and protective efficacy of a recombinant, chimeric yellow fever-Japanese encephalitis virus (ChimeriVax-JE) as a live, attenuated vaccine candidate against Japanese encephalitis. Virology **257:**363-72.

21. **Guirakhoo, R., R. Weltzin, T. Chambers, Z.-X. Zhang, K. Soike, M. Ratterree, J. Arroyo, K. Georgakopoulos, J. Catalan, and T. Monath.** 2000. Recombinant chimeric yellow fever-dengue type 2 virus is immunogenic and protective in nonhuman primates. Journal of Virology **74:**5477-5485.

22. **Halstead, S. B., and J. Jacobson.** 2003. Japanese encephalitis. Adv Virus Res **61:**103-38.

23. **HHS.** 2007. Biosafety in Microbiological and Biomedical Laboratories, 5th ed. U.S Government Printing Office, Washington, DC.

24. **Hogrefe, W. R., R. Moore, M. Lape-Nixon, M. Wagner, and H. E. Prince.** 2004. Performance of immunoglobulin G (IgG) and IgM enzyme-linked immunosorbent assays using a West Nile virus recombinant antigen (preM/E) for detection of West Nile virus- and other flavivirus-specific antibodies. J Clin Microbiol **42:**4641-8.

25. **Holbrook, M. R., and A. D. Barrett.** 2002. Molecular epidemiology of Japanese encephalitis virus. Curr Top Microbiol Immunol **267:**75-90.

26. **Hombach, J., T. Solomon, I. Kurane, J. Jacobson, and D. Wood.** 2005. Report on a WHO consultation on immunological endpoints for evaluation of new Japanese encephalitis vaccines, WHO, Geneva, 2-3 September, 2004. Vaccine **23:**5205-11.

27. **Johnson, A. J., D. A. Martin, N. Karabatsos, and J. T. Roehrig.** 2000. Detection of anti-arboviral immunoglobulin G by using a monoclonal antibody-based capture enzyme-linked immunosorbent assay. Journal of Clinical Microbiology **38:**1827-31.

28. **Johnson, A. J., A. J. Noga, O. Kosoy, R. S. Lanciotti, A. A. Johnson, and B. J. Biggerstaff.** 2005. Duplex microsphere-based immunoassay for detection of anti-West Nile virus and anti-St. Louis encephalitis virus immunoglobulin m antibodies. Clinical and diagnostic laboratory immunology **12:**566-574.

29. **Johnson, B. W., O. Kosoy, D. A. Martin, A. J. Noga, B. J. Russell, A. A. Johnson, and L. R. Petersen.** 2005. West Nile virus infection and serologic

response among persons previously vaccinated against yellow fever and Japanese encephalitis viruses. Vector Borne Zoonotic Dis **5:**137-45.

30. **Johnson, B. W., B. J. Russell, and R. S. Lanciotti.** 2005. Serotype-specific detection of dengue viruses in a fourplex real-time reverse transcriptase PCR assay. J Clin Microbiol **43:**4977-83.

31. **Kuno, G., G. J. Chang, K. R. Tsuchiya, N. Karabatsos, and C. B. Cropp.** 1998. Phylogeny of the genus Flavivirus. J Virol **72:**73-83.

32. **Lanciotti, R. S., and T. F. Tsai.** 2007. Arboviruses, p. 1486-1500. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), Manual of Clinical Microbiology, 9th ed, vol. 2. ASM Press, Washington, DC.

33. **Lindsey, H. S., C. H. Calisher, and J. H. Mathews.** 1976. Serum dilution neutralization test for California group virus identification and serology. Journal of Clinical Microbiology **4:**503-10.

34. **Liu, J. P., H. M. Hsueh, E. Hsieh, and J. J. Chen.** 2002. Tests for equivalence or non-inferiority for paired binary data. Stat Med **21:**231-45.

35. **Martin, D. A., B. J. Biggerstaff, B. Allen, A. J. Johnson, R. S. Lanciotti, and J. T. Roehrig.** 2002. Use of immunoglobulin M cross-reactions in differential diagnosis of human flaviviral encephalitis infections in the United States. Clinical & Diagnostic Laboratory Immunology **9:**544-9.

36. **Martin, D. A., D. A. Muth, T. Brown, A. J. Johnson, N. Karabatsos, and J. T. Roehrig.** 2000. Standardization of immunoglobulin M capture enzyme-linked immunosorbent assays for routine diagnosis of arboviral infections. Journal of Clinical Microbiology **38:**1823-6.

37. **Monath, T. P.** 1994. Dengue: the risk to developed and developing countries. Proc Natl Acad Sci U S A **91:**2395-400.

38. **Monath, T. P.** 2002. Japanese encephalitis vaccines: current vaccines and future prospects. Curr Top Microbiol Immunol **267:**105-38.

39. **Monath, T. P., K. McCarthy, P. Bedford, C. T. Johnson, R. Nichols, S. Yoksan, R. Marchesani, M. Knauber, K. H. Wells, J. Arroyo, and F. Guirakhoo.** 2002. Clinical proof of principle for ChimeriVax: recombinant live, attenuated vaccines against flavivirus infections. Vaccine **20:**1004-18.

40. **Monath, T. P., K. Soike, I. Levenbook, Z. X. Zhang, J. Arroyo, S. Delagrave, G. Myers, A. D. Barrett, R. E. Shope, M. Ratterree, T. J. Chambers, and F. Guirakhoo.** 1999. Recombinant, chimaeric live, attenuated vaccine (ChimeriVax) incorporating the envelope genes of Japanese encephalitis (SA14-14-2) virus and the capsid and nonstructural genes of yellow fever (17D) virus is safe, immunogenic and protective in non-human primates. Vaccine **17:**1869-82.

41. **Nga, P. T., M. del Carmen Parquet, V. D. Cuong, S. P. Ma, F. Hasebe, S. Inoue, Y. Makino, M. Takagi, V. S. Nam, and K. Morita.** 2004. Shift in Japanese encephalitis virus (JEV) genotype circulating in northern Vietnam: implications for frequent introductions of JEV from Southeast Asia to East Asia. J Gen Virol **85:**1625-31.

42. **Niedrig, M., K. Sonnenberg, K. Steinhagen, and J. T. Paweska.** 2007. Comparison of ELISA and immunoassays for measurement of IgG and IgM antibody to West Nile virus in human sera against virus neutralisation. J Virol Methods **139:**103-5.

43. **Porterfield, J. S.** 1986. Antibody-dependent enhancement of viral infectivity. Advances in Virus Research **31:**335-55.

44. **Pugachev, K. V., F. Guirakhoo, F. Mitchell, S. W. Ocran, M. Parsons, B. W. Johnson, O. L. Kosoy, R. S. Lanciotti, J. T. Roehrig, D. W. Trent, and T. P. Monath.** 2004. Construction of yellow fever/St. Louis encephalitis chimeric virus and the use of chimeras as a diagnostic tool. Am J Trop Med Hyg **71:**639-45.

45. **Roehrig, J. T.** 2003. Antigenic structure of flavivirus proteins. Advances in Virus Research **59:**141-175.

46. **Roehrig, J. T.** 2000. Arboviruses, p. 356-373. *In* S. Spector, R. Hodinka, and S. Young (ed.), Clinical Virology Manual, third ed. ASM Press, Washington, DC.

47. **Roehrig, J. T., J. Hombach, and A. D. Barrett.** 2008. Guidelines for Plaque-Reduction Neutralization Testing of Human Antibodies to Dengue Viruses. Viral Immunol **21:**123-32.

48. **Russell, P. K., A. Nisalak, P. Sukhavachana, and S. Vivona.** 1967. A plaque reduction test for dengue virus neutralizing antibodies. J Immunol **99:**285-90.

49. **Solomon, T.** 2006. Control of Japanese encephalitis--within our grasp? N Engl J Med **355:**869-71.

50. **Solomon, T., N. M. Dung, R. Kneen, M. Gainsborough, D. W. Vaughn, and V. T. Khanh.** 2000. Japanese encephalitis. J Neurol Neurosurg Psychiatry **68:**405-15.

51. **Solomon, T., H. Ni, D. W. Beasley, M. Ekkelenkamp, M. J. Cardosa, and A. D. Barrett.** 2003. Origin and evolution of Japanese encephalitis virus in southeast Asia. J Virol **77:**3091-8.

52. **Tsai, T. F., Y. X. Yu, L. L. Jia, R. Putvatana, R. Zhang, S. Wang, and S. B. Halstead.** 1998. Immunogenicity of live attenuated SA14-14-2 Japanese encephalitis vaccine--a comparison of 1- and 3-month immunization schedules. J Infect Dis **177:**221-3.

53. **WHO.** 2007. Guidelines for plaque reduction neutralizing testing of human antibodies to dengue viruses.

54. **WHO.** 2003. WHO-recommended standards for surveillance of selected vaccine-preventable diseases.

55. **Wong, S. J., R. H. Boyle, V. L. Demarest, A. N. Woodmansee, L. D. Kramer, H. Li, M. Drebot, R. A. Koski, E. Fikrig, D. A. Martin, and P. Y. Shi.** 2003. Immunoassay targeting nonstructural protein 5 to differentiate West Nile virus infection from dengue and St. Louis encephalitis virus infections and from flavivirus vaccination. J Clin Microbiol **41:**4217-23.

56. **Wong, S. J., V. L. Demarest, R. H. Boyle, T. Wang, M. Ledizet, K. Kar, L. D. Kramer, E. Fikrig, and R. A. Koski.** 2004. Detection of human anti-flavivirus antibodies with a west nile virus recombinant antigen microsphere immunoassay. J Clin Microbiol **42:**65-72.

57. **Yu, Y.-X., P. F. Wu, J. Ao, L. H. Liu, and H. M. Lim.** 1981. Selection of a better immunogenic and highly attenuated live vaccine virus strain of JE. I. Some biological characteristics of SA14-14-2 mutant. . Chinese Journal of Microbiology and Immunology **1:**77-84.

Table 1 $PRNT_{90}$ double agarose overlay procedure: number of days following application of first overlay to application of second overlay

Virus	Prototype virus strain	Days to second overlay	ChimeriVax™ virus	Days to second overlay	ChimeriVax™ Parental strain	Days to second overlay
DEN-1	Hawaii44	7	ChimeriVax™-D1	4	PUO-359	7
DEN-2	NGC44	6	ChimeriVax™-D2	4	PUO-218	6
DEN-3	H-87	7	ChimeriVax™-D3	4	PaH881/88	7
DEN-4	H-241	4	ChimeriVax™-D4	4	1228	7
JEV	Nakayama	4	ChimeriVax™-JE	4	SA-14-14-2	ND

ND, not done.

1 Table 2 Comparison of neutralizing antibody titers[a] using prototype DENV, ChimeriVax™DENV, and

2 ChimeriVax™ parental DENV strains in $PRNT_{90}$

	Prototype DENV				ChimeriVax™- DENV				ChimeriVax™-DENV parental			
	DEN-1	DEN-2	DEN-3	DEN-4	DEN-1	DEN-2	DEN-3	DEN-4	DEN-1	DEN-2	DEN-3	DEN- 4
DENV-1 infection (n = 15)												
1	1280	-	-	-	5120	40	-	-	160	-	-	-
2	1280	40	-	-	640	40	20	-	160	-	20	-
3	640	-	-	-	80	20	-	-	160	-	-	-
4	160	-	-	-	320	-	-	-	20	-	-	-
5	160	-	-	-	160	-	-	-	160	-	-	-
6	160	-	-	-	80	-	-	-	80	-	-	-
7	320	-	-	-	2560	40	40	20	320	-	-	-
8	1280	-	40	20	320	80	80	20	640	-	40	-
9	40	-	-	-	40	-	-	-	40	-	-	-
10	320	-	-	-	640	-	-	-	160	-	-	-
11	320	-	-	20	640	20	20	-	320	-	20	-
12	640	40	20	20	1280	80	40	-	640	-	20	-
13	80	ND	ND	ND	160	ND	ND	ND	160	ND	ND	ND
14	40	-	-	-	640	20	-	-	160	-	-	-
15	20	-	-	-	80	20	-	-	40	-	-	-
DENV-2 infection (n = 12)												
16	20	320	-	-	-	640	-	-	-	160	-	-
17	40	160	-	-	-	320	-	-	-	80	-	-
18	40	640	-	-	-	640	20	-	-	320	-	-
19	160	640	20	-	40	640	40	40	20	320	20	-
20	40	320	-	-	-	320	-	-	-	80	-	-
21	-	80	-	-	-	160	-	-	-	80	-	-
22	-	160	-	-	-	320	-	-	-	320	-	-
23	20	320	-	20	-	320	20	-	-	160	20	-
24	20	320	-	-	-	1280	-	-	20	160	-	-
25	-	160	-	-	-	640	-	-	-	40	-	-
26	-	320	-	-	-	1280	-	-	-	320	-	-
27	-	640	-	-	-	1280	-	-	-	320	-	-
DENV-3 infection (n= 3)												
28	-	-	-	-	-	-	-	-	-	-	-	-
29	-	-	40	-	-	-	40	-	-	-	80	-
30	20	-	160	-	20	-	320	-	-	-	160	-
DENV-4 infection (n = 3)												
31	-	-	-	1280	-	-	-	160	-	-	-	320
32	-	20	-	320	-	20	-	160	-	-	-	320
33	-	40	-	5120	-	20	-	2560	-	-	-	5120
Secondary DEN infection (n = 3)												
34	1280	640	640	640	640	1280	320	640	1280	320	640	80
35	5120	2560	5120	5120	5120	10240	2560	5120	5120	1280	10240	1280
36	10240	5120	10240	40960	5120	20480	5120	40960	10240	2560	20480	10240
WNV infection (n = 3)												
37-38	-	-	-	-	-	-	-	-	-	-	-	-
39	-	-	-	-	-	-	-	-	-	-	-	-
YFV vaccinated (n = 3)												

40-42	-	-	-	-	-	-	-	-	-	-	-	-
All flavivirus negative (n = 6)												
43-48	-	-	-	-	-	-	-	-	-	-	-	-
Positive controls[b]												
DEN-1	640 (5120	(80)	(160)	(80)	2560 (2560)	(80)	(320)	(160)	1280 (320)	(20)	(40)	(-)
DEN-2	(320)	1280 (1280)	(80)	(160)	(160)	2560 (2560)	(160)	(80)	(-)	640 (80)	(-)	(-)
DEN-3	(320)	(160)	1280 (1280)	(160)	(320)	(160)	640 (2560)	(80)	(80)	(40)	1280 (320)	(-)
DEN-4	(160)	(80)	(80)	5120 (5120)	(160)	(80)	(80)	2560 (2560)	(-)	(-)	(-)	1280 (80)
Negative control[c]	-	-	-	-	-	-	-	-	-	-	-	-

[a]Neutralizing antibody titer is expressed as the reciprocal of the end-point serum dilution that neutralized the challenge virus plaque count by 90%. LLOQ = 20; only positive titers (≥20) are shown; hyphen = no detectable titer (<20). A ≥ 4-fold neutralizing titer difference between antibody to the homologous DENV serotype and antibody to the other DENV serotypes indicates a DENV serotype-specific antibody response.

[b]Mouse hyperimmune ascitic fluid (MHIAF) produced against a single DENV serotype. The neutralizing antibody titers in parentheses are the results of the initial evaluations of the MHIAFs using each of the four DENVserotypes as challenge viruses. These titers are shown to illustrate the level of cross-reactivity of the MHIAF to the other DENV serotypes.

[c]Normal human control serum.

ND, not done due to insufficient sample volume.

Table 3. Comparison of neutralizing antibody titers[a] using prototype JEV (Nakayama) and ChimeriVax™-JEV in $PRNT_{90}$

Specimen type	JEV Nakayama	ChimeriVax™-JEV
A. JEV vaccinated (n=55)		
1	1280	1280
2	640	320
3	640	1280
4-5	160	160
6	160	320
7	160	80
8-9	160	40
10-11	80	40
12	40	40
13	40	20
14	20	20
15-16	20	-
17-20	40	10
21-26	10	10
27-55	-	-
B. JEV infection (n=14)		
56	2560	20480
57	5120	10240
58	1280	10240
59	2560	5120
60	640	5120
61	640	2560
62	640	1280
63-64	320	1280
65-66	160	1280
67-68	640	640
69	320	320
C. Other flavivirus infections (n= 21)		
DENV infection (n=9)		
70-78	-	-
WNV infection (n=1)		
79	-	-
YFV infection (n=3)		
80-82	-	-
Past or secondary flavivirus infection (n=8)		
83	320	640
84	-	10
85-90	-	-

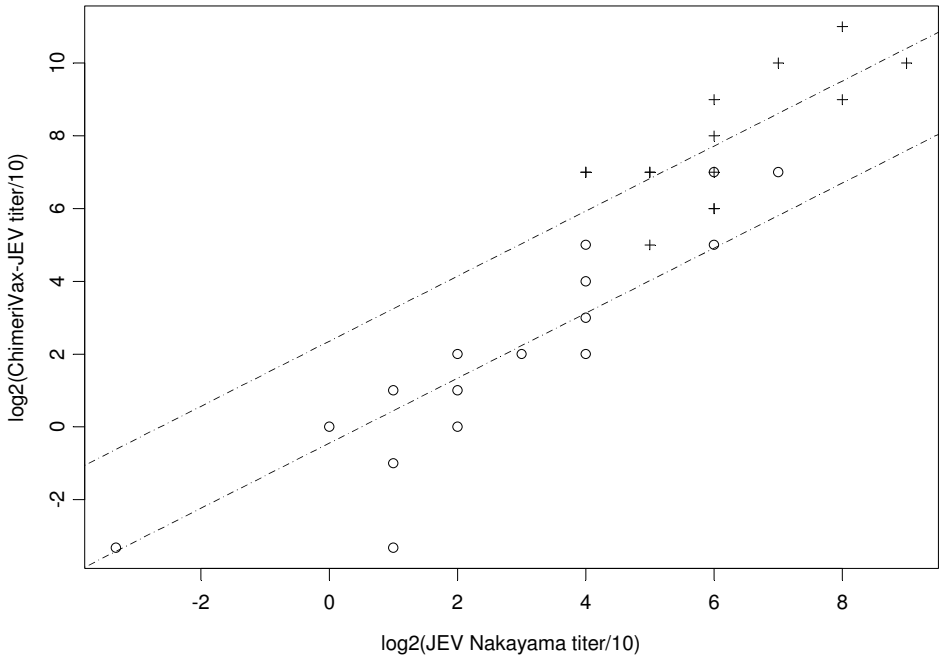
D. All flavivirus negative (n=10)		
91-99	-	-
100	-	20
Positive control[b] (JEV vaccine serum)	1280	320
Negative control[c]	-	-

[a]Neutralizing antibody titer is expressed as the reciprocal of the end-point serum dilution that neutralized the challenge virus plaque count by 90%. LLOQ = 10; only positive titers (≥10) are shown; hyphen = no detectable titer (<10) .

[b] Mouse hyperimmune ascitic fluid produced against JEV Nakayama strain.

[c] Normal human control serum.

Figure 1. Linear regression of $\log_2$ ChimeriVax™-JEVtiter/10 (y-axis) on $\log_2$ prototype JEV titer/10 (x-axis). JEV vaccinated samples are represented by circles; samples with natural JEV infections are represented by plus signs. The slopes of the regression lines through the two groups is slightly less than one (a 95% CI is 0.82 to 0.97). There is no statistical difference between the two slopes (p=0.89). However, the difference between the regression lines is statistically significant (p<0.01, 95% CI for difference on the log scale is 1.69 to 3.03). Together, these results suggest that, on average, titers against ChimeriVax™-JEV and JEV Nakayama are approximately equal in the vaccinated group, but in the naturally infected group the titers against ChimeriVax™-JEV are about 2 logs greater than those against JEV Nakayama.



1