

1 Evaluation of chimeric Japanese encephalitis and dengue viruses for use in diagnostic

2 plaque reduction neutralization tests

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1 **Abstract**

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

10 The chimeric vaccine viruses ChimeriVax™-WNV and -SLEV have previously been  
11 shown to elicit antibody reactivity comparable to the parental wt WNV and SLEV viruses.  
12 ChimeriVax™ viruses provide advantages for PRNT: they grow more rapidly than most wt  
13 flaviviruses, produce large plaques, require BSL2 conditions, and are not under select agent  
14 restrictions. We evaluated the ChimeriVax™-DENV1, 2, 3, 4 and -JEV for use in PRNT on  
15 sera from DENV- and JEV-infected patients and from JEV vaccine recipients. Serostatus  
16 agreement was 100% between the ChimeriVax™ DENV serotypes and wt prototype  
17 DENV and 97% overall with ChimeriVax™-JEV compared to prototype Nakayama JEV,  
18 92% in a subgroup of JEV vaccine recipients and 100% in serum from encephalitis patients  
19 naturally infected with JEV. ChimeriVax™-DENV and -JEV plaque phenotype and BSL2  
20 requirements, combined with sensitive and specific reactivity make them good substitutes  
21 for wt DENV and JEV in PRNT in public health diagnostic laboratories.

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## 1 **Introduction**

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

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

1       The plaque reduction neutralization test (PRNT) is a specific serological assay that is  
2 used in the CDC/DVBID diagnostic laboratory to confirm infection and differentiate  
3 between flaviviruses in primary flavivirus infections (4, 10, 46-48). Neutralization assays  
4 are also used to monitor protective immunity in vaccinees (26, 38). In the PRNT procedure  
5 the serological specimen (generally serum) is mixed with live virus, and if virus-specific  
6 neutralizing antibodies are present in the serum, they bind to the virus to form a complex.  
7 The mixture is then inoculated onto a monolayer of cells. Virus bound up in an antibody-  
8 virus complex is inhibited from infecting the cells, i.e. it is neutralized. Consequently,  
9 laboratories conducting these assays must have tissue culture capability, considerable  
10 technical expertise in growing and plaque titrating flaviviruses, which have a wide range of  
11 growth rates, and appropriate biosafety level laboratory conditions in which to grow the  
12 virus. JEV, West Nile virus (WNV), and St. Louis encephalitis virus (SLEV) require  
13 biosafety level 3 (BSL3) containment, which precludes their use in many public health  
14 diagnostic laboratories with only BSL2 capacity. In addition, JEV falls under jurisdiction  
15 of the select agent program and can be used only in select agent registered laboratories  
16 (11).

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

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

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

## 15 **Materials and Methods**

### 16 **Serum Specimens**

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

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

### 3 **Viruses**

4 Prototype viruses used in the PRNTs were obtained from the CDC/DVBID virus reference  
5 collection: DENV1 (Hawaii 1944); DENV2 (New Guinea C 1944); DENV3 (H -87,  
6 Philippines 1956); DENV4 (H – 241, Philippines 1956); and JEV (Nakayama 1935) (53).  
7 The ChimeriVax™-DENV1,2,3,4 and parental wt DENV strains on which the  
8 ChimeriVax™-DEN viruses are based (DENV1 PUO-359, DENV2 PUO-218, DENV3  
9 PaH881/88, and DENV4 1228) were supplied by Acambis Inc. (Cambridge, MA, USA).  
10 (18-21, 40). The ChimeriVax™-JEV, constructed to contain the prM-E genes from JEV  
11 SA14-14-2, the live virus vaccine strain developed in China, also was supplied by Acambis  
12 Inc. (20, 57).

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

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

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

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

12 Virus-specific neutralizing antibody titers were determined by 90% endpoint PRNT in  
13 6-well plates in Vero cells using a 0.5% agarose double overlay, visualized with neutral red  
14 staining in the second overlay (4). Samples were first heat inactivated at 56°C for 30 min to  
15 destroy the complement and to inactivate adventitious viruses, so as to make fair  
16 comparison between paired samples; non-heat inactivated normal human sera added to the  
17 serum-virus mixture at a concentration of 4% provided a source of labile serum factor.  
18 Second overlays were applied on days 4-7 ( Table 1).

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

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

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

## 22 **Results**

### 23 **Dengue virus PRNT**



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

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

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

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

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

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

#### 17 **Japanese encephalitis virus PRNT**

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

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

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

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

## 6 **Discussion**

7 Diagnostic testing for arbovirus infections has increased in the United States since the  
8 introduction of WNV in 1999, and globally through laboratory-based surveillance projects  
9 designed to aid countries in making decisions on implementation of vaccine and/or  
10 arbovirus control programs. Combined with the increase in the number of tests, laboratories  
11 are also expanding the repertoire of diagnostic assays that they routinely carry out in order  
12 to improve detection. Virus-specific MAC ELISA has been the primary serological test for  
13 detecting arbovirus infection when a single acute serological specimen is obtained. The  
14 MAC ELISA is sensitive, as IgM antibody is produced early in infection and detectable  
15 within a few days from illness onset, and is more virus-specific than IgG antibody (27, 28,  
16 36, 54-56). However, in flavivirus infections antibodies elicited against conserved epitopes  
17 on the immunogenic envelope protein may cross-react with other flaviviral antigens in the  
18 MAC ELISA, which can cause false-positive results (45). PRNT is a specific, quantitative  
19 assay, which measures the amount of virus-specific neutralizing antibody present in the  
20 serum, and is used to confirm infection and differentiate cross-reactive MAC ELISA  
21 results.

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

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

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

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

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

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

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

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

9 ChimeriVax™-JEV was compared to the prototype JEV Nakayama strain in a group of  
10 100 serum specimens from JEV or YFV vaccinees, a JEV outbreak in Vietnam, and  
11 suspected JEV- or other flavivirus-infection cases. Serostatus agreement between  
12 ChimeriVax™-JEV and JEV Nakayama was 92% among the 55 JEV vaccine recipients  
13 (Table 3, part A). In this subset there were 26 specimens with neutralizing antibody titers  
14 against JEV Nakayama. ChimeriVax™-JEV failed to detect neutralizing antibody in 2 of  
15 these specimens, although the titers were also low in both specimens against JEV  
16 Nakayama. Generally, the neutralizing antibody titers were higher to JEV Nakayama than  
17 ChimeriVax™-JEV in the vaccine recipient subset. (Table 3 and Figure 1). This was not  
18 unexpected, as studies monitoring immunological protection by PRNT in JEV vaccine  
19 trials have shown that using JEV strains homologous to the JEV vaccine result in higher  
20 titers compared to heterologous JEV strains (12, 26, 39). Further, cross-neutralization  
21 studies have demonstrated that there are significant differences in neutralization titers  
22 between strains within a flavivirus species, based on the degree of homology between the  
23 strain used to produce polyclonal antibody and the reference strain used as the challenge

1 virus in the PRNT (1, 2, 7). Serum specimens in this subset were from individuals that had  
2 been vaccinated with the Biken™ JEV vaccine, which is an inactivated vaccine prepared  
3 from the Nakayama strain. Therefore, it would be expected that serum from the vaccine  
4 recipients would be more reactive to the homologous JEV Nakayama strain than to the  
5 ChimeriVax™-JEV which contains the prM-E genes from a different strain (SA14-14-2)  
6 (12, 25, 33, 39, 51).

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

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



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

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

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

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

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- 22  
23

- 1 Table 1 PRNT<sub>90</sub> double agarose overlay procedure: number of days following application of first overlay  
 2 to application of second overlay

<b>Virus</b>	<b>Prototype virus strain</b>	<b>Days to second overlay</b>	<b>ChimeriVax™ virus</b>	<b>Days to second overlay</b>	<b>ChimeriVax™ Parental strain</b>	<b>Days to second overlay</b>
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

3 ND, not done.  
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1 Table 2 Comparison of neutralizing antibody titers<sup>a</sup> using prototype DENV, ChimeriVax™DENV, and  
 2 ChimeriVax™ parental DENV strains in PRNT<sub>90</sub>

	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
<b>DENV-1 infection (n = 15)</b>												
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	-	-	-
<b>DENV-2 infection (n = 12)</b>												
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	-	-
<b>DENV-3 infection (n= 3)</b>												
28	-	-	-	-	-	-	-	-	-	-	-	-
29	-	-	40	-	-	-	40	-	-	-	80	-
30	20	-	160	-	20	-	320	-	-	-	160	-
<b>DENV-4 infection (n = 3)</b>												
31	-	-	-	1280	-	-	-	160	-	-	-	320
32	-	20	-	320	-	20	-	160	-	-	-	320
33	-	40	-	5120	-	20	-	2560	-	-	-	5120
<b>Secondary DEN infection (n = 3)</b>												
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
<b>WNV infection (n = 3)</b>												
37-38	-	-	-	-	-	-	-	-	-	-	-	-
39	-	-	-	-	-	-	-	-	-	-	-	-
<b>YFV vaccinated (n = 3)</b>												

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

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

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

10 <sup>c</sup>Normal human control serum.

11 ND, not done due to insufficient sample volume.

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1 Table 3. Comparison of neutralizing antibody titers<sup>a</sup> using prototype JEV (Nakayama) and ChimeriVax™.

2 JEV in PRNT<sub>90</sub>

Specimen type	JEV Nakayama	ChimeriVax™-JEV
<b>A. JEV vaccinated (n=55)</b>		
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>B. JEV infection (n=14)</b>		
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
<b>C. Other flavivirus infections (n= 21)</b>		
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	-	-

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**D. All flavivirus negative (n=10)**

91-99	-	-
100	-	20
Positive control <sup>b</sup> (JEV vaccine serum)	1280	320
Negative control <sup>c</sup>	-	-

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1 <sup>a</sup>Neutralizing antibody titer is expressed as the reciprocal of the end-point serum dilution that neutralized  
2 the challenge virus plaque count by 90%. LLOQ = 10; only positive titers ( $\geq 10$ ) are shown; hyphen = no  
3 detectable titer ( $< 10$ ).

4 <sup>b</sup> Mouse hyperimmune ascitic fluid produced against JEV Nakayama strain.

5 <sup>c</sup> Normal human control serum.

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

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