

# Human Serum With High Neutralizing Antibody Titers Against Both Zika and Dengue Virus Shows Delayed In Vitro Antibody-Dependent Enhancement of Dengue Virus Infection

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Zika virus infection in a dengue virus-naïve subject was associated with the induction of high levels of cross-reactive binding antibodies. These responses were, however, largely non-neutralizing and displayed a capacity to enhance dengue infection in vitro at significantly low dilution (1:10). In contrast, a subject who had high levels of neutralizing antibodies against both dengue and Zika viruses enhanced infection at a dilution of 1:10 000. These results suggest that high levels of dengue cross-neutralizing antibodies could potentially prevent the enhancement of dengue infection in Zika virus-convalescent individuals.

**Keywords.** antibody-dependent enhancement; cross-neutralization; dengue virus; Zika virus.

Zika virus (ZIKV) and Dengue virus (DENV) are related flaviviruses that share significant structural similarities, leading to the induction of cross-reactive antibody responses [1–3]. Previous studies have shown that ZIKV infection induces high levels of DENV cross-reactive antibodies with little or no cross-neutralization [2, 4–7], suggesting that these responses are less likely to protect from subsequent DENV infection. On the other hand, induction of cross-reactive antibodies in the absence of cross-neutralization was associated with significant enhancement of DENV infection in vivo in rhesus macaques [5]. Likewise, polyclonal serum from wild-type mice injected

with ZIKV and monoclonal antibodies isolated from a human subject previously infected with ZIKV was shown to enhance DENV infection of monocytes in vitro [8, 9].

Enhancement of infection has been well documented in humans infected with heterologous serotypes of DENV [10, 11]. Though the exact mechanisms for enhanced disease are not clear, numerous studies have implicated a role for cross-reactive antibodies that form immune complexes with virions and mediate infection of Fc-receptor-expressing cells, leading to enhancement of infection. Studies have reported higher rates of DENV infection in monocytes in the presence of diluted serum from DENV-convalescent subjects [11, 12], and passive transfer of DENV-binding antibody to nonhuman primates was associated with enhanced DENV viremia [13]. There is limited evidence to show that human subjects infected with ZIKV can similarly enhance DENV infection either in vivo or in vitro. The primary objective of this study was to characterize DENV-specific cross-reactive antibodies in 2 US travelers following infection with ZIKV and examine the capacity of sera from these individuals to enhance DENV infection in vitro.

## METHODS

### Samples

Serum samples were obtained from subjects enrolled in an observational cohort study (Deployment and Travel Related Infectious Disease Risk Assessment, Outcomes, and Prevention Strategies Among Department of Defense [DoD] Beneficiaries [TravMil]) that prospectively evaluates infectious disease risks and the effectiveness of prevention and treatment strategies in DoD beneficiaries traveling outside the continental United States for ≤6.5 months. This protocol was reviewed and approved by the Infectious Disease Institutional Review Board at the Uniformed Services University, and informed consent was obtained from each subject before sample collection. In addition, the investigators have adhered to the policies for protection of human subjects prescribed in 45 CFR 46.

### Subject #1

Subject #1 was a 30-year-old female who traveled to the Dominican Republic for 2 weeks in May 2016. No history of Japanese Encephalitis or Yellow Fever vaccination was reported. The subject experienced an illness that began with fever, night sweats, and retro-orbital headache, followed by diffuse maculopapular rash, myalgia, small joint polyarthralgia, mild sore throat, and mild conjunctivitis in the right eye during travel. Her symptom complex lasted approximately 8 days before the time of assessment in the Infectious Disease Clinic following her return. Serum was negative by Trioplex

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Real-time reverse transcription polymerase chain reaction (RT-PCR) assay for ZIKV, DENV, and Chikungunya virus, but ZIKV RNA was detected in the urine by RT-PCR on day 8 after onset of illness. Serology was confirmed using Centers for Disease Control and Prevention (CDC) Zika MAC–enzyme-linked immunosorbent assay (ELISA). A convalescent serum sample for the research study was obtained 3.5 weeks after illness onset and return from travel.

#### **Subject #2**

Subject #2 was a 28-year-old male who traveled to Puerto Rico for 2 weeks in July 2016. The subject was vaccinated against Japanese Encephalitis in 2015, and no prior Yellow Fever vaccination was reported. He experienced fever, fatigue, itching, headache, rash, diarrhea, arthralgia, conjunctivitis, and enlarged cervical lymph nodes starting 2 days before his return. His symptoms were ongoing at the time of presentation to the Infectious Disease Clinic 1 day following his return from travel. ZIKV RNA was detected in both serum and urine by Trioplex Real-time RT-PCR assay on day 3 after the onset of illness. Serology was confirmed using CDC Zika MAC-ELISA. A convalescent serum sample for the research study was obtained 4 weeks after illness onset and return from travel.

Serum samples collected from 4 subjects enrolled in the TravMil study whose sera was negative for ZIKV and DENV antibodies by plaque reduction neutralization test (PRNT) were included as negative controls in the present study. Trioplex Real-time RT-PCR assay was not performed as the negative control subjects were asymptomatic, and serum was collected after return from travel.

#### **ELISA**

Relative levels of ZIKV- and DENV-2-specific IgM and IgG in acute serum were determined using whole ZIKV and DENV-2 virus in a sandwich ELISA-based assay. Briefly, ELISA plates were coated with anti-flavivirus monoclonal antibody (clone 4G2) overnight at 4°C. After washing 3x with 1x PBS-Tween, the plates were blocked with 1x PBS-BSA overnight at 4°C. Whole ZIKV or DENV-2 virus diluted in 1x PBS-Tween was added to each well after washing 3x and incubated for 1 hour at 37°C. Serum samples diluted 1:100 in 1x PBS-Tween were added to each well and incubated for 1 hour at 37°C. No serum control wells were set up simultaneously as negative controls, and all samples were set up in duplicate. The plates were washed 3x, and horseradish peroxidase (HRP)–conjugated anti-IgG or IgM detection antibody (IBL-America, Minneapolis, MN) was added to each well. After incubating at room temperature for 30 minutes, the plates were developed with tetramethylbenzidine (TMB). The reaction was stopped after 15 minutes with TMB stop solution (IBL-America, Minneapolis, MN), and the plates were analyzed at 450 nm using an ELISA plate reader. The relative optical density (OD) was determined after subtracting the

OD of the no-serum negative controls from the OD of the samples. The assay cutoff was set at an OD of 0.1 for both IgM and IgG based on the OD of serum samples from flavivirus-negative subjects (n = 4).

#### **Plaque Reduction Neutralization Test**

Plaque reduction neutralization test assays were performed as described previously [5].

#### **Antibody-Dependent Enhancement Assay**

Antibody-dependent enhancement (ADE) assays were performed as described previously [5, 14].

#### **Data Analysis**

Enhancement of DENV infection was examined by flow cytometry, and the data were analyzed using Flowjo 9.8 software.

## **RESULTS**

### **DENV Cross-Reactive Antibodies Are Readily Detectable in Serum After ZIKV Infection**

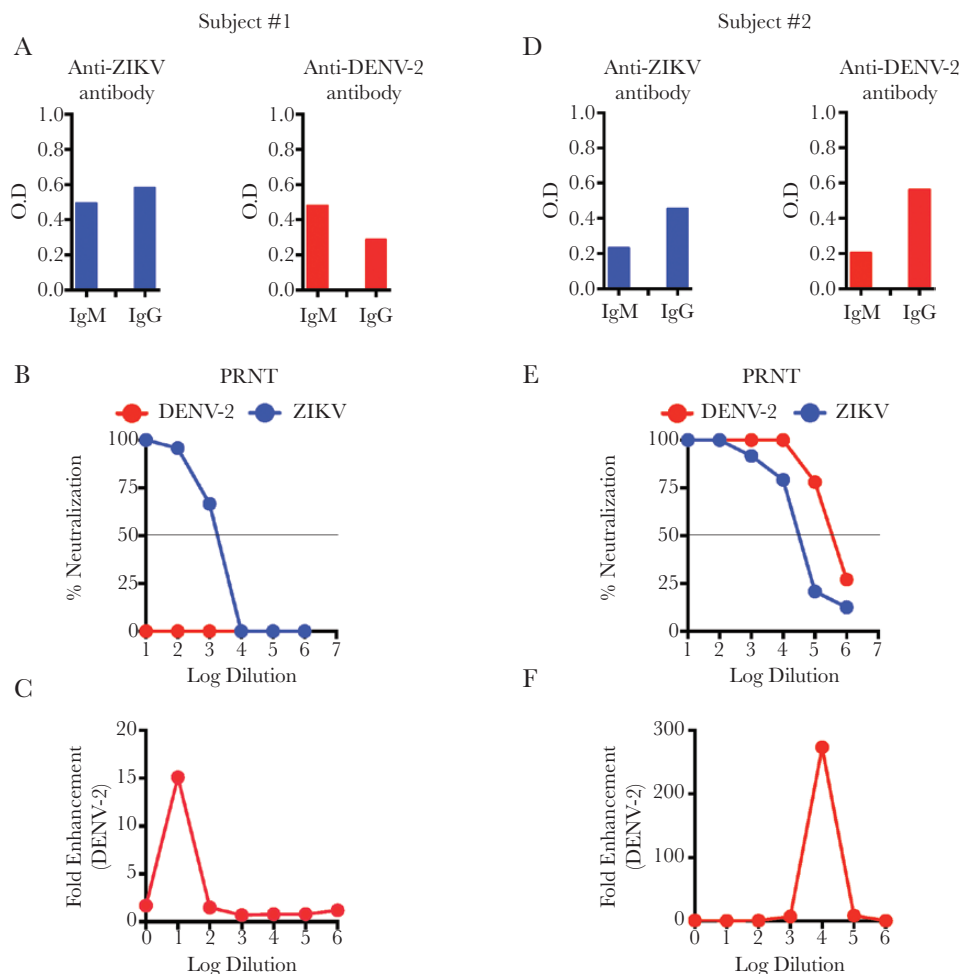
We first examined the relative levels of ZIKV and DENV-2-binding antibodies (bAb) using whole virus with the intent of determining envelope-specific antibody responses (Figure 1A and D). Subject #1 had similar levels of anti-ZIKV and DENV-2 IgM, where anti-ZIKV IgG levels were higher than anti-DENV IgG. In contrast, both anti-ZIKV and DENV-2 serum IgM levels were 2x lower in Subject #2 than Subject #1. Interestingly, though anti-ZIKV IgG levels were similar to those of Subject #1, anti-DENV-2 serum IgG levels were higher than Subject #1, suggesting that Subject #2 higher levels of DENV cross-reactive antibodies.

### **Subject #1 Induced High Levels of ZIKV-Specific Neutralizing Antibodies That Did Not Cross-Neutralize DENV**

To determine if the differences in DENV-specific IgG levels between the 2 subjects were due to differences in induction of cross-reactive responses or prior DENV infection, we assessed their ZIKV- and DENV-2-specific neutralizing antibody responses using the PRNT assay (Figure 1B and E). Our results showed that Subject #1 had a ZIKV PRNT<sub>50</sub> titer of 1:1000 with no cross-neutralizing antibody responses against DENV-2. In contrast, Subject #2 had higher PRNT<sub>50</sub> titers against both ZIKV and DENV-2, suggesting that this subject was exposed to both ZIKV and DENV.

### **Serum From Subject #1 and Subject #2 Displayed Differential Capacity for In Vitro Enhancement of DENV Infection**

We previously reported that prior exposure to ZIKV significantly enhanced DENV viremia in rhesus macaques that was associated with high levels of DENV cross-reactive antibody responses [5]. To assess if ZIKV infection in humans show a similar phenotype, we examined the capacity for serum from both subjects to enhance DENV infection in vitro using K562 cells, a cell line that expresses the low-affinity FcγRIII receptor that binds to IgG immune complexes.



**Figure 1.** Zika virus infection induces Dengue virus (DENV) cross-reactive IgM and IgG in serum that enhance DENV infection. Relative levels of anti-Zika and anti-Dengue-2 IgM and IgG in serum from (A) Subject #1 and (D) Subject #2 were determined using Zika and Dengue-2 virus-specific enzyme-linked immunosorbent assay. The assay cutoff was set at an OD of 0.1 for both IgM and IgG based on the optical density (OD) of serum samples from flavivirus-negative subjects ( $n = 4$ ). Zika and Dengue-2 virus-specific PRNT<sub>50</sub> titers in serum from (B) Subject #1 and (E) Subject #2 were determined using the plaque reduction neutralization test (PRNT). Antibody-dependent enhancement of Dengue-2 infection in K562 cells using sera from (C) Subject #1 and (F) Subject #2.

Serum from Subject #1 was found enhance infection of K562 cells by DENV-2 at a dilution of 1:10 (Figure 1C). In contrast, Subject #2 demonstrated enhancement at a dilution of 1:10 000 (Figure 1F), suggesting that the presence of higher levels of DENV-specific neutralizing antibodies likely prevents enhancement at lower dilutions.

## DISCUSSION

Structural similarities between ZIKV and DENV have been associated with the induction of highly cross-reactive antibodies [1–3]. Though studies in nonhuman primates [5] and mice [8] have shown the potential for ZIKV-induced cross-reactive responses to enhance DENV infection, there are limited data to support these findings in humans. Our results show that serum from ZIKV-infected human subjects was capable of mediating the ADE of DENV infection in vitro. Large epidemiological studies are needed to determine if these findings

directly translate to the potential for higher risk of severe dengue, but they add to the evidence from animal studies that ZIKV-induced antibodies could potentially enhance DENV infection; monoclonal antibodies [9] or polyclonal serum [8] has been shown to enhance DENV infection in vitro, whereas ZIKV-convalescent nonhuman primates experienced enhanced DENV viremia following infection with DENV [1–3].

As has been reported previously, serum from a DENV-naïve ZIKV-infected human subject showed limited cross-neutralizing antibody responses against DENV [2, 4–7]; PRNT<sub>50</sub> titers were <1:10 (Figure 1B), suggesting that the in vitro enhancement of DENV infection was likely associated with cross-reactive binding antibodies in the absence of detectable neutralizing activity against DENV. Data from Subject #2 were found to support this argument, as enhancement of DENV infection occurred at a dilution of 1:10 000 in the presence of high levels of DENV-specific neutralizing antibodies as compared with a

dilution of 1:10 for serum from Subject #1, who had no detectable cross-neutralizing antibodies against DENV. Previous studies using heterologous DENV challenge in nonhuman primates have reported an increase in the titer of maximally enhancing dilution of serum that correlated with higher levels of neutralizing activity against DENV [15]. These findings raise the exciting possibility that high levels of neutralizing antibodies, if induced against both ZIKV and DENV simultaneously, could potentially prevent enhancement of DENV infection and at the same time protect from ZIKV infection. Additional studies are needed to test this hypothesis.

Taken together, these data provide additional evidence that DENV cross-reactive antibodies induced by ZIKV could potentially enhance DENV infection. Further epidemiological evidence is needed to determine if prior exposure to ZIKV in DENV-naïve humans enhances subsequent DENV infection in vivo.

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**Authors' contributions.** W.G.V. performed the experiments and analyzed the data; W.J.V., T.L., T.H.B., and J.J.M. designed the study and wrote the manuscript. H.C.Y. and A.K. supervised the collection of samples and edited the manuscript.

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