Zika Virus and the World Health Organization Criteria for Determining Recent Infection Using Plaque Reduction Neutralization Testing

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Abstract. The recent Zika virus (ZIKV) epidemic swept across Latin America and the Caribbean, where dengue virus (DENV) is endemic. The antigenic similarities of these closely related flaviviruses left researchers and clinicians with challenges to interpret serological tests. Thirty-six women attending a prenatal clinic in Honduras and with positive DENV IgM enzyme-linked immunoabsorbent assays (ELISAs) were screened with a ZIKV immunoglobulin M ELISA, reverse transcription polymerase chain reaction for ZIKV and DENV 1–4, and plaque reduction neutralization tests (PRNTs) for ZIKV and DENV-2. Plaque reduction neutralization test results were interpreted using the World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC) criteria. Using the WHO criteria of a PRNT90 titer ≥ 20 and a 4-fold difference between ZIKV and DENV titers, we determined that 69.4% of samples had a recent ZIKV infection, compared with 5.6% using CDC criteria. The interpretation of ZIKV PRNTs in a DENV-endemic region is highly dependent on the choice of interpretation criteria.

Zika virus (ZIKV) is an arbovirus of the genus *Flaviviridae* that is transmitted by mosquitoes of the genus *Aedes*, most notably *Aedes aegypti* and to a lesser extent *Aedes albopictus*.¹ In recent decades ZIKV has emerged onto the global scene, spreading out of Africa to Southeast Asia, the Pacific Islands, and most recently to the Americas.² Although typically resulting in a nondescript febrile illness, it was observed in the most recent outbreaks in South America that ZIKV can result in severe neuropathology in the offspring of women who are infected while pregnant.^{3,4}

Dengue virus (DENV) is another arbovirus of the genus *Flaviviridae*. Similar to ZIKV, DENV is transmitted by *Ae. aegypti* and *Ae. albopictus* and is historically endemic to most the regions in which ZIKV has emerged.¹ The co-circulation of these viruses and their close antigenic relationship has posed a major problem for their serological diagnosis after acute infection.^{5–8} Historically, during convalescence DENV has been diagnosed using serological techniques such as enzyme-linked immunoabsorbent assay (ELISA) and the gold standard plaque reduction neutralization test (PRNT). Unfortunately, most serological methods, such as ELISA, have been shown to be highly unspecific due to the cross-reactivity of the currently available ZIKV and DENV antibodies and antigens used in these tests.⁹ Likewise, it has been suggested that PRNTs are also unspecific due to the cross-reactivity of circulating human DENV antibodies to ZIKV found in people living in DENV-endemic areas, as well as inconsistent selection and interpretation of PRNT90 titer values.^{6–8}

In this report, we examine the potential for PRNTs to differentiate recent ZIKV infections from DENV infections in a sub-set of samples taken at the height (July–September) of the 2016 ZIKV outbreak as part of a study among pregnant women in Tegucigalpa, Honduras.¹⁰ This study was reviewed and

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Venous blood was collected from pregnant women during their routine prenatal visits and tested at Centro de Investigaciones Genéticas, Escuela de Microbiología, and the National Autonomous University of Honduras using an ELISA to detect IgM to DENV (Standard Diagnostics, Gyeonggi-do, Republic of Korea). To assess the best means to determine recent ZIKV infection, samples from the first 200 enrolled women who tested positive with the DENV ELISA were selected for further testing at Tulane University's School of Public Health and Tropical Medicine ($N = 36$). Selected samples were tested using the following: A TaqMan RT-PCR assay for ZIKV, a TaqMan multiplex RT-PCR assay for all four serotypes of DENV, an ELISA for IgM to ZIKV (InBios, Seattle, WA), and PRNTs to detect neutralizing antibodies to both ZIKV and DENV-2, historically the most common DENV serotype circulating in Central America.¹¹ The ZIKV ELISAs were carried out according to the manufacturer's instructions. RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and run with an iTaq™ Universal Probes One-Step

TABLE 1

Plaque reduction neutralization test results of DENV IgM enzyme-linked immunoabsorbent assay-positive samples tested for neutralizing antibodies to ZIKV and DENV-2 interpreted using both the WHO and CDC criteria ($N = 36$)

PRNT interpretation ($N = 36$)	WHO criteria*		CDC criteria†	
	No.	%	No.	%
Recent ZIKV infection	25	69.4	2	5.6
Recent DENV-2 infection	1	2.8	1	2.8
Recent unspecified flavivirus infection	10	27.8	33	91.7
Negative	0	0.0	0	0.0

CDC = Centers for Disease Control and Prevention; DENV = dengue virus; PRNT = plaque reduction neutralization test; WHO = World Health Organization; ZIKV = Zika virus.

* A PRNT90 titer of ≥ 20 and a 4-fold difference between ZIKV and DENV-2 titers.¹⁶

† A PRNT90 titer of ≥ 10 with the corresponding DENV-2 or ZIKV PRNT90 titer < 10 .¹⁷

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TABLE 2

Plaque reduction neutralization test results of samples tested for neutralizing antibodies to ZIKV and DENV-2 interpreted using both the WHO and CDC criteria and stratified by ZIKV IgM status

PRNT interpretation	ZIKV IgM positive (N = 17)				ZIKV IgM negative (N = 19)			
	WHO criteria*		CDC criteria†		WHO criteria*		CDC criteria†	
	No.	%	No.	%	No.	%	No.	%
Recent ZIKV infection	15	88.2	1	5.9	10	52.6	1	5.3
Recent DENV-2 infection	1	5.9	1	5.9	0	0.0	0	0.0
Recent unspecified flavivirus infection	1	5.9	15	88.2	9	47.4	18	94.7
Negative	0	0.0	0	0.0	0	0.0	0	0.0

CDC = Centers for Disease Control and Prevention; DENV = dengue virus; PRNT = plaque reduction neutralization test; WHO = World Health Organization; ZIKV = Zika virus.

* A PRNT90 titer of ≥ 20 and a 4-fold difference between ZIKV and DENV-2 titers.¹⁶

† A PRNT90 titer of ≥ 10 with the corresponding DENV-2 or ZIKV PRNT90 titer < 10 .¹⁷

Kit (Bio-Rad, Hercules, CA) using previously published primer/probe sets.^{12,13} Dengue virus-2 and ZIKV PRNTs were conducted according to previously published protocols.^{14,15} Zika virus MEX-I-44 isolated in Tapachula, Mexico, in 2016 was obtained from The University of Texas Medical Branch, Galveston, TX, and cultured to passage eight in Vero cells. Sanofi Pasteur's ChimeriVax[®]-D2 was obtained from the Centers for Disease Control and Prevention (CDC), Fort Collins, CO, and cultured to passage four in Vero cells for use in the DENV-2 PRNT. A PRNT90 cutoff was used for interpretation. A PRNT90 titer is the dilution of a sample at which a 90% reduction in possible plaques is observed. The maximum number of potential plaques was obtained for each run using a corresponding back-titration, and each PRNT90 value was calculated using the linear equation derived from at least two dilutions for each sample.

Plaque reduction neutralization test results were interpreted using both the World Health Organization (WHO) and the CDC criteria.^{16,17} The WHO criteria classifies recent infection as samples with PRNT90 titer values ≥ 20 and a 4-fold difference between ZIKV and DENV PRNT90 titers, whereas the CDC criteria classifies recent infection as samples with PRNT90 titer values of ≥ 10 with all other possible flavivirus PRNT90 titers < 10 .^{16,17}

The results showed that nearly half of the DENV IgM ELISA-positive samples were positive for IgM to ZIKV and that only one woman was ZIKV RT-PCR positive. Analysis of the PRNT results of DENV IgM ELISA positive samples using the WHO criteria revealed 25 (69.4%) recent ZIKV infections, one (2.8%) recent DENV-2 infection, and 10 (27.8%) recent unspecified flavivirus infections (Table 1). Analyzing the same PRNT results using the CDC criteria revealed only two (5.6%) recent ZIKV infections and 33 (91.7%) recent unspecified flavivirus infections (Table 1).

Analysis of the PRNT results of the samples that were also ZIKV IgM ELISA-positive using the WHO criteria revealed 15 (88.2%) recent ZIKV infections, one (5.9%) recent unspecified flavivirus infection, and one (5.9%) recent DENV-2 infection (Table 2). Analysis of the same results using the CDC criteria revealed only one (5.9%) recent ZIKV infection, one (5.9%) recent DENV-2 infection and 15 (88.2%) recent unspecified flavivirus infections (Table 2). According to the WHO criteria, 10 (52.6%) of the 19 samples negative for ZIKV IgM were also positive on ZIKV PRNTs (Table 2).

Consistent with the endemicity of DENV in Honduras all of the samples tested demonstrated plaque neutralization of DENV and/or ZIKV. Based on the more conservative CDC criteria for the interpretation of PRNTs these samples would be classified as indeterminate recent flavivirus infections for

the purposes of not missing a potential ZIKV case. However, we have shown that if the WHO guidelines for interpreting ZIKV and DENV PRNT results are used, PRNTs distinguish between these recent infections in most cases. The WHO criteria take into account the very large difference between PRNT titers we observed for ZIKV and DENV and might be more useful in DENV-endemic areas and for epidemiological studies. When log transformed, the medians and interquartile ranges of the PRNT90 values for ZIKV and DENV illustrate these large differences and how exclusive not using a 4-fold difference criteria with a low PRNT90 threshold can be (Figure 1). It is also interesting to note that according to the WHO criteria, most samples with negative ELISA results for ZIKV IgM were confirmed as ZIKV by PRNT, suggesting strong cross-reactivity of the DENV IgM ELISA and a relatively low sensitivity of the ZIKV IgM ELISA. Several other explanations for the incongruous PRNT and ZIKV IgM ELISA results include waning levels of IgM if the ZIKV infections were older or cross-reactivity of IgM related to secondary flavivirus exposure.

One limitation of our study is that we only obtained a single blood test at the first prenatal visit, and we were thus unable to precisely date the infections, especially considering only three of the 36 samples came from women exhibiting symptoms consistent with ZIKV infection. Early positive RT-PCR results would have allowed us to more accurately determine the etiology and timing of the infection. Only one of our samples was

FIGURE 1. The medians of the log-transformed PRNT90 titer values with interquartile ranges for ZIKV and DENV-2 PRNTs on study samples. For reference, the dashed line indicates the Centers for Disease Control and Prevention PRNT90 threshold of 10. DENV = dengue virus; PRNT = plaque reduction neutralization test; ZIKV = Zika virus.

ZIKV RT-PCR-positive, suggesting that the others were from convalescent women. However, these samples were taken at the height of the ZIKV epidemic in Honduras and within weeks of ZIKV being introduced to the sampling area and therefore are samples from relatively recent infections, so although we did not have to worry about waning IgM levels, it is unknown how long-term convalescence will affect the specificity, reactivity, and cross-reactivity of the apparent neutralizing antibodies in these samples.

Despite this, these data show that the usefulness of PRNT results is highly dependent on the interpretation criteria used and the reason for the testing. More research is needed to determine which criteria are valid. Until then, reporting according to both the WHO and CDC criteria would be a prudent approach in the epidemiological study of the unforeseen clinical sequelae that may be attributable to the recent ZIKV epidemic.

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