Incorporation of IgG Depletion in a Neutralization Assay Facilitates Differential Diagnosis of Zika and Dengue in Secondary Flavivirus Infection Cases

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ABSTRACT Zika virus (ZIKV) has emerged as a major global public health concern due to its link as a causative agent of human birth defects. Laboratory diagnosis of suspected ZIKV infections by serological testing of specimens collected a week or more after symptom onset primarily relies on detection of anti-ZIKV-specific IgM antibodies by enzyme-linked immunosorbent assay coupled with detection of ZIKV-specific neutralizing antibody by neutralization tests. A definitive diagnosis based on serological assays is possible during primary ZIKV infections; however, due to the cross-reactivity of antibodies elicited during flaviviral infections, a definitive diagnosis is not always possible, especially among individuals who have previously been exposed to closely related flaviviruses, such as dengue virus (DENV). Here, we investigated the neutralizing IgM antibody profiles of 33 diagnostic specimens collected from individuals with suspected primary and secondary flaviviral infections acquired when visiting areas experiencing active ZIKV transmission in 2015 and 2016. Specimens collected between 1 day and 3 months postexposure were tested for ZIKV and dengue virus type 1 (DENV1) and type 2 (DENV2) by the plaque reduction neutralization test (PRNT) before and after IgG depletion. We found that IgG depletion prior to neutralization testing had little effect in differentiating samples from individuals with secondary infections taken less than 3 weeks postexposure; however, IgG depletion significantly reduced the cross-reactive neutralizing antibody titers and increased the percentage of cases discernible by PRNT from 15.4% (95% confidence interval [CI], 4.3 to 42.2%) to 76.9% (95% CI, 49.7 to 91.8%) for samples collected between roughly 3 and 12 weeks postexposure. These results highlight the potential of IgG depletion to improve the specificity of PRNT for better confirmation and differential diagnosis of flavivirus infections.

KEYWORDS Zika virus, confirmatory testing, dengue virus, flavivirus, immunoglobulin M, neutralization test, neutralizing antibodies, serological testing

Zika virus (ZIKV) is a mosquito-borne virus (genus *Flavivirus*, family *Flaviviridae*) mainly transmitted by the *Aedes aegypti* mosquito. ZIKV was first isolated in 1947 from the blood of a febrile sentinel rhesus monkey during a study of yellow fever in the Zika Forest, Uganda (1). Other notable viruses in this genus make up several serocomplexes, including the dengue virus (DENV), the tick-borne encephalitis virus (TBEV), the Japanese encephalitis virus (JEV), and the yellow fever virus (YFV) serocomplexes. ZIKV is categorized in the Spondweni serocomplex, of which only ZIKV and Spondweni virus (SPONV) are members. ZIKV causes a mild febrile illness with symptoms very similar to the symptoms caused by infection with DENV. There is only transient cross-protection among the 4 DENV serotypes (DENV serotype 1 [DENV1] to DENV4); hence, individuals can be infected with multiple types of DENV in their lifetimes. Given their common mosquito vector, ZIKV has recently emerged and spread across many areas where DENV

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is endemic. Therefore, in addition to secondary infection by DENV (for example, DENV1 and then DENV2), cases of secondary infection by ZIKV following a prior DENV infection have emerged in many areas. While ZIKV and DENV1 to DENV4 are not in the same serocomplex, they do share 53 to 57% amino acid sequence identity in the envelope (E) protein, the viral structural protein responsible for eliciting the majority of flavivirus cross-reactive antibodies (2–6). Many studies have shown that antibodies isolated from persons with previous DENV exposure can cross-neutralize ZIKV *in vitro*; however, this cross-neutralization may not be long-lived (7–9).

Until recently, ZIKV was known to cause only a mild illness, with very few cases being reported since its discovery. In April 2007, ZIKV caused an epidemic of disease in Yap State, Federated States of Micronesia, after which the virus spread across the South Pacific for the next 8 years before being identified as the causative agent of an outbreak of disease in March 2015 in Bahia, Brazil (10–13). Since that time, ZIKV has been confirmed to be transmitted by sexual contact, from mother to fetus, and possibly through blood transfusions (14–19). It has also been associated with microcephaly and other congenital brain abnormalities in some infants born to mothers infected during pregnancy and has triggered cases of Guillain-Barré syndrome in infected patients (10, 12, 20–24).

The laboratory diagnosis of ZIKV relies on the detection of viral RNA or anti-ZIKV-specific immunoglobulin M (IgM) by enzyme-linked immunosorbent assay (ELISA) followed by confirmation by the plaque reduction neutralization test (PRNT), an assay in which virus-specific neutralizing antibody is measured quantitatively. The predominant target of flavivirus-neutralizing antibody is the envelope (E) glycoprotein, responsible for virus-cell membrane attachment and virus-mediated cell membrane fusion. PRNT measures the ability of virus-specific antibody to inhibit these functions, thus inhibiting viral infectivity *in vitro*, resulting in reduced amounts of viral plaques measured by PRNT. Anti-ZIKV IgM is typically detectable by day 4 to 7 after symptom onset and can persist for 12 weeks or more, while virus-neutralizing antibody is detectable concurrently with IgM but persists for years after infection, and its presence is indicative of a past infection in the absence of vaccination (13). Immunoglobulin G (IgG) to flaviviruses can be virus serotype specific, serocomplex cross-reactive, and flavivirus group cross-reactive, while IgM has been shown to be more virus specific (25–29).

Though a definitive diagnosis based on serology or molecular assays is typically possible during primary ZIKV infections (without prior flavivirus exposure), in secondary flaviviral infections, a definitive diagnosis based on serology results alone is challenging to make due to the high flavivirus cross-reactive antibody response (12, 13). In fact, flavivirus cross-reactive antibodies can result in higher titers of neutralizing antibody to the virus from the previous infection, making PRNT results uninterpretable and inaccurate (13). We hypothesize that the greater portion of cross-reactive neutralizing antibodies in secondary infections is IgG. By depleting these highly cross-reactive IgG antibodies from the specimen and thereby measuring only the virus-neutralizing response of IgM, the IgM-PRNT may be more specific to the recent virus infection and allow the more accurate differentiation of recent and past flaviviral infections. To test this hypothesis, we depleted IgG from a panel of 33 diagnostic serum specimens from individuals with primary and suspected secondary ZIKV and DENV infections and compared the PRNT results before and after IgG depletion.

MATERIALS AND METHODS

Viruses and cells. The chimeric viruses West Nile virus (WNV)/DENV1, WNV/DENV2, and WNV/ZIKV used for the PRNT were engineered and prepared in-house using reverse genetic technology with a strategy similar to that previously described for chimeric flaviviruses based on cDNA clones (30, 31). Briefly, the chimeras were constructed using a fast-growth WNV NY99 genetic replication vector expressing the viral surface structural proteins premembrane and envelope (prM-E) from DENV1 16007, DENV2 16681, or ZIKV PRVABC59 (32–34). The recombinant viral RNA was transcribed from the chimeric viral cDNA constructs and transfected into C6/36 cells or Vero cells as described before (31). The chimeric viruses recovered from the transfected cells were amplified once in cell culture to generate a working virus stock for the study. Full-length genome sequences were verified for all of the chimeric virus working seeds. Before using the chimeric viruses in this study, we demonstrated with three flavivirus-infected

FIG 1 Ratios of mean neutralization titers of the wild-type (WT) reference virus over its corresponding WNV chimera. Neutralization tests based on a 90% virus plaque or focus reduction were performed with either WT reference viruses (ZIKV PRVABC59, DENV1 16007, DENV2 16681) or fast-growth chimeric viruses (WNV/ZIKV, WNV/DENV1, WNV/DENV2) and three panels of human immune serum samples: panel 1 is in white ($n = 15$), panel 2 is in gray ($n = 24$ for DENV1, $n = 25$ for DENV2), and panel 3 has diagonal lines ($n = 22$ for DENV1, $n = 23$ for DENV2). Average ratios of the neutralization test titers (for the WT virus to the chimera) are shown with standard errors. The dashed line at a ratio of 0.5 represents a 2-fold decrease in the PRNT₉₀ titer of WT viruses compared to that for the chimeric viruses.

human serum panels that WNV/DENV1, WNV/DENV2, and WNV/ZIKV functioned equivalently (within a 2-fold neutralization titer difference, on average) to their cognate wild-type (WT) strains DENV1 16007, DENV2 16681, and ZIKV PRVABC59, respectively, in PRNT (Fig. 1). In the past several years, CDC diagnostic labs have used these chimeric viruses to shorten and synchronize the PRNT for DENV and ZIKV, decreasing the assay time from 5 to 9 days (for ZIKV, 5 days; for DENVs, 7 to 9 days) to 3 days for all viruses.

Diagnostic specimens. We obtained ethics approval for use of previously collected human diagnostic specimens from the CDC's Human Subjects Institutional Review Board (CDC IRB number 6773). Thirty-three specimens from patients with a possible recent flaviviral infection were randomly selected and acquired from CDC's Arboviral Diseases Branch diagnostic laboratory. These samples included specimens collected from asymptomatic pregnant women (designated with an A after the sample identification number) with a recent travel history to areas where ZIKV transmission was known to occur in 2015 and 2016. Due to an unclear virus exposure time and the various travel periods of these subjects, we calculated the post-median travel time (PMT) points for samples from asymptomatic individuals from the median day of the travel period to the sample collection date to estimate their potential postexposure time points. For symptomatic subjects, the sample collection time post-symptom onset (PSO; based on the subject's memory of any suspected symptoms during travel) was used to indicate the potential postexposure time. Many subjects frequently visited or stayed in areas where DENV is endemic and that were also affected by the recent ZIKV outbreak. Therefore, some of them may have had previous exposure to 1 or more DENVs and may have recently been infected with ZIKV or another type of DENV (designated secondary flaviviral infections for these cases). On the basis of the original algorithm for serology diagnosis of ZIKV using IgM antibody-capture ELISA and PRNT for antibodies against DENV1, DENV2, and ZIKV in the 33 ZIKV-positive serum samples, 6 samples were identified to have primary flaviviral infections, while 22 samples were identified to have secondary flaviviral infections and the individuals could not be conclusively diagnosed as having a ZIKV or DENV infection. Five specimens negative for any flaviviral infection were included as controls.

MAC-ELISA. The CDC ZIKV IgM antibody-capture ELISA (MAC-ELISA) received the Food and Drug Administration's Emergency Use Authorization on 26 February 2016 (<https://www.cdc.gov/zika/pdfs/zika-mac-elisa-protocol-eua-english.pdf>). All ELISA reagents were provided by CDC's Arboviral Diseases Branch diagnostic laboratory. Briefly, the inner 60 wells of 96-well high-affinity Immulon 2HB microtiter plates (Thermo Scientific, Rochester, NY) were coated with goat anti-human IgM antibody (SeraCare Life Sciences, Milford, MA) diluted at 1:2,000 in carbonate-bicarbonate buffer (50 mM sodium carbonate, 50 mM sodium bicarbonate, pH 9.6) and the plates were incubated overnight at 4°C. After the plates were washed five times in phosphate-buffered saline (PBS)–0.05% Tween wash buffer, nonspecific binding sites were blocked with 5% milk–0.5% Tween 20 in PBS and the plates were incubated at room temperature for 30 min. Controls and patient serum samples were diluted 1:400 in wash buffer and incubated on the plates for 1 h at 37°C. After the plates were washed, ZIKV Vero E6 antigen (β -propionolactone-inactivated ZIKV strain PRVABC59 from Vero E6 cells) or normal Vero E6 control antigen diluted 1:160 in wash buffer was added and the plates were incubated overnight at 4°C. Detector anti-flavivirus monoclonal antibody (MAb) 6B6C-1 conjugated to horseradish peroxidase was diluted 1:5,000 in wash buffer and added to prewashed plates, and the plates were incubated for 1 h at 37°C. Enhanced K-Blue tetramethylbenzidine substrate (Neogen, Lansing, MI) was added to each well of the plates, and the plates were incubated in the dark at room temperature for 10 min before the reaction was stopped by the addition of 1 N H₂SO₄. The optical density at 450 nm was read on an automatic plate

reader. The results were expressed as a positive-to-negative (P/N) ratio of the observed A_{450} readings and interpreted as follows: positive, A_{450} reading of ≥ 3.0 ; equivocal, A_{450} reading between 2.0 and 3.0; negative, A_{450} reading of < 2.0 . The DENV MAC-ELISA was performed in essentially the same way, except for the substitution of a cocktail of virus-like particle (VLP) antigen containing all four DENV VLPs made from COS-1 cells or normal COS-1 antigen diluted 1:120 in wash buffer (35).

Antibody-capture ELISA for detection of ZIKV- or DENV-specific human IgG. The inner 60 wells of 96-well high-affinity Immulon 2HB microtiter plates (Thermo Scientific, Rochester, NY) were coated with flavivirus cross-reactive 4G2 mouse MAb diluted 1:4,000 in carbonate-bicarbonate buffer and the plates were incubated overnight at 4°C. After the plates were washed, nonspecific binding sites were blocked as described above. ZIKV Vero E6 or normal antigen diluted 1:160 or a cocktail of DENV VLP COS-1 antigen or normal COS-1 antigen diluted 1:80 in wash buffer was added, and the plates were incubated overnight at 4°C. After the plates were washed, controls and patient serum samples were diluted 1:400 in wash buffer and incubated on the plates for 1 h at 37°C. After the plates were washed, goat anti-human IgG conjugated to alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA) diluted 1:4,000 in blocking buffer was added and the plates were incubated for 1 h at 37°C. The plates were washed, and alkaline phosphatase substrate (Sigma-Aldrich, St. Louis, MO) was incubated on the plates for 30 min at room temperature in the dark before the addition of 1 N NaOH stopping solution. The optical density at 405 nm was read. The results were expressed as described above for the MAC-ELISA.

Serum IgG depletion. IgG was depleted from the serum samples using protein G Sepharose 4 Fast Flow beads (GE Healthcare, Chicago, IL), provided at a concentration of 2 mg protein G/ml. Specimens were diluted 1:5 in BA1 diluent (Hanks M-199 salts, 0.05 M Tris, pH 7.6, 1% bovine serum albumin, 0.35 g/liter sodium bicarbonate, 100 U/ml penicillin, 100 mg/liter streptomycin, 1 mg/liter amphotericin B [Fungizone]) and heat inactivated at 56°C for 30 min. Initially, IgG was depleted from three serum specimens using the protocol previously described with some modifications (36). Briefly, approximately 13.3 μ g of protein G was added to each well of a 96-well filter plate (Millipore Sigma, Burlington, MA), and the plate was washed twice with PBS before incubation with 100 μ l/well of diluted, heat-inactivated serum. Six replicates were processed for each diluted sample (a total of 0.08 mg protein G for each 600 μ l of diluted sample). Subsequently, the method was further optimized using a larger amount of protein G. Diluted serum samples (600 μ l each) were either incubated twice consecutively with 0.2 mg of protein G beads for 1 h at room temperature or incubated with 0.2 mg protein G a single time at 4°C overnight with constant mixing in a microcentrifuge tube. Samples incubated twice for 1 h at room temperature were centrifuged after the first incubation, and the supernatant was transferred to newly washed protein G Sepharose beads. Percent IgG depletion was calculated by testing serum samples before and after IgG depletion using a quantitative human IgG antigen ELISA kit (Innovative Research Inc., Novi, MI) following the manufacturer's protocol.

PRNT. The plaque reduction neutralization test (PRNT) was performed in six-well plates of Vero cells as described previously (31, 37). Twofold serial dilutions of heat-inactivated human serum specimens were made in BA1 diluent to a final volume of 60 μ l/dilution. The PRNTs were performed with the addition of a standard amount of exogenous complement (naive human serum collected, pooled, and standardized for the CDC diagnostic PRNT). Briefly, each chimeric virus was diluted in BA1 diluent containing a final volume of 8% naive human serum to 2 times the desired virus input titer for the assay. Sixty microliters of $2\times$ input virus was added to each serially diluted serum sample to achieve a target of 80 to 100 PFU/ml of input virus. After incubation at 4°C overnight, 100 μ l/well of the virus-serum mixture was added to confluent Vero cells. Back titration of the input virus was conducted by 2-fold serial dilution (6 replicates each) of the $2\times$ virus preparation in each assay. The plates were adsorbed and overlaid with agarose medium and incubated at 37°C with 5% CO₂. After 48 h, the cells were stained with a secondary agarose overlay medium containing neutral red. Virus plaques were counted on day 3 postinfection, and PRNT endpoint titers were expressed as the reciprocal of the serum dilution titer yielding at least a 90% reduction of the input number of PFU (PRNT₉₀ titer). A sample was considered ZIKV or DENV positive when the PRNT₉₀ titer was at least 20 and the ratio of the PRNT₉₀ titer of antibody to one virus was at least 4-fold greater than the titer of antibody to the other viruses tested.

RESULTS

Optimization of IgG depletion. IgG is the most common antibody isotype found in humans, making up approximately 80% of serum antibodies. IgG antibody concentrations can range from 5.5 to 16 mg/ml in healthy adults. In order to determine the optimal protocol to deplete IgG from the diagnostic specimens, two different methods and concentrations of protein G were tested. First, three samples were depleted using the protocol for IgG depletion of diagnostic specimens for testing in a microsphere immunoassay designed to detect anti-West Nile virus and anti-St. Louis encephalitis virus IgM antibodies (36). We determined that only $31.5\% \pm 9.89\%$ (standard error [SE]) of the IgG was depleted from three different serum specimens, with IgG levels ranging from 6.5 to 19 mg/ml before depletion, based on the quantitative IgG ELISA results (Fig. 2). Since the binding capacity of protein G is approximately 10 mg of human IgG/mg beads (based on the manufacturer's product specification), this protocol, which utilizes a total of 0.08 mg of protein G, had the capacity to bind only about 0.8 mg of human

FIG 2 Percent IgG depletion under various conditions. The mean percent IgG depletion $\pm$ SE was calculated for three serum samples. An aliquot of 600 μ l of a 1:5-diluted serum sample was used for each method for comparison. 1X 30 min, serum incubated with 0.08 mg protein G once for 30 min at room temperature; 1X 15 min, serum incubated with 0.2 mg protein G once for 15 min at room temperature; 2X 30 min, serum incubated with 0.2 mg protein G twice for 30 min each time at room temperature; 1X 60 min, serum incubated with 0.2 mg protein G once for 60 min at room temperature; 1X O/N, serum incubated with 0.2 mg protein G once overnight at 4°C; 2X 60 min, serum incubated with 0.2 mg protein G twice for 60 min each time at room temperature. Statistically significant differences were calculated using Tukey's method of multiple comparisons with an overall confidence level of 95%. *, a significantly lower percentage of IgG depletion was obtained using 0.08 mg protein G with one 30-min incubation compared to that obtained using all other methods; #, a significantly lower percentage of IgG depletion was obtained using 0.2 mg protein G with one 15-min incubation compared to that obtained using the overnight incubation or incubation with 0.2 mg protein G 2 times for 60 min each time.

IgG, which was insufficient to bind the majority of IgG in the 600 μ l of a 1:5-diluted sample. In order to deplete the maximum quantity of IgG from the samples, the amount of protein G was increased to approximately 0.2 mg for each diluted serum sample and the sample was incubated with constant mixing under various conditions. When the samples were incubated with 0.2 mg of protein G for 15 min at room temperature, approximately 76.7% $\pm$ 2.31% (SE) of total IgG was depleted from the samples. When the incubation time was increased so that the samples were incubated for two 30-min incubations with 0.2 mg of fresh beads or one 60-min incubation with 0.2 mg of beads, the percent IgG depletion was 87.3% $\pm$ 0.88% (SE) and 95.3% $\pm$ 4.15% (SE), respectively. When the samples were incubated for either two 60-min incubations with fresh beads at room temperature or once overnight at 4°C, the percent IgG depletion increased to 99.97% $\pm$ 0.03% (SE) and 99.8% $\pm$ 0.2% (SE), respectively (Fig. 2). Using Tukey's method of multiple comparisons with a confidence coefficient of 0.95, the percent IgG depletion obtained by the method using 0.08 mg of protein G with one 30-min incubation was significantly lower than that obtained by all the other methods using 0.2 to 0.4 mg of total protein G (indicated by an asterisk in Fig. 2). Similarly, the IgG depletion obtained using 0.2 mg of protein G with one 15-min incubation was also significantly lower than that obtained using the overnight incubation or the method with 2 60-min incubations (indicated by a hash tag in Fig. 2). No significant difference was found among the other 4 methods, and the most optimal depletion methods, either the two 60-min incubations at room temperature or the one overnight incubation at 4°C, were used to deplete IgG from all specimens for the study.

To test whether depleting the sample of IgG would have an effect on the neutralizing antibody endpoint titers, the same samples were tested in the assay for the PRNT₉₀ titer before and after the use of two different depletion methods, either 30 min with 0.08 mg protein G (indicated 30 min/0.08 mg in Table 1 and below) or overnight at 4°C with 0.2 mg of protein G (indicated overnight/0.2 mg in Table 1 and below). A clear difference in the neutralizing endpoint titers of antibodies to ZIKV, DENV1, and DENV2 was displayed after the use of the different IgG depletion methods (Table 1).

TABLE 1 PRNT₉₀ titers of selected samples under various IgG depletion conditions

Serum sample identifier	PRNT ₉₀ titer								
	Before depletion			30 min/0.08 mg ^a			Overnight/0.2 mg ^b		
	ZIKV	DENV1	DENV2	ZIKV	DENV1	DENV2	ZIKV	DENV1	DENV2
17	2,560	<10	<10	1,280	<10	<10	640	<10	<10
20A	1,280	2,560	2,560	640	640	1,280	40	10	<10
23	5,120	10,240	10,240	2,560	5,120	5,120	1,280	80	1,280

^aHeat-inactivated serum samples were incubated with 0.08 mg of protein G for 30 min at room temperature before PRNT was performed.

^bHeat-inactivated serum samples were incubated with 0.2 mg of protein G overnight at 4°C before PRNT was performed.

Sample 17, which was shown to be from a patient with a primary ZIKV infection with only neutralizing antibody titers to this virus before IgG depletion, retained ZIKV-specific neutralizing antibody titers within a 2- to 4-fold difference of the titer for unabsorbed serum after IgG depletion (Table 1). When sample 20A (from a person who had experienced multiple flavivirus exposures) was tested in the assay for the PRNT₉₀ titer before depletion, no distinct difference in the titers of antibodies to the three viruses (within a 2-fold difference) was seen. When the sample was tested with the incomplete depletion method (30 min/0.08 mg), the endpoint titers of antibodies to the three viruses remained indistinguishable, with only a 2- to 4-fold drop in the PRNT₉₀ titers of antibodies against each virus being detected; however, after complete depletion by the overnight/0.2 mg method, the neutralization titers of antibodies to DENV1 and DENV2 dropped more than 256-fold from the PRNT₉₀ titers for non-IgG-depleted samples. The PRNT₉₀ titers of antibodies to ZIKV after complete IgG depletion, on the other hand, decreased just 32-fold and resulted in a 4-fold higher titer of antibodies to ZIKV than antibodies to DENV1 and DENV2, suggesting a possible recent ZIKV infection with at least a previous DENV infection (Table 1). Like sample 20A, sample 23 was from a secondary flavivirus infection case, and the test for the PRNT₉₀ titer before IgG depletion did not provide a diagnosis distinguishable between a recent ZIKV or DENV infection when the diagnosis was based on at least a 4-fold difference of the endpoint titers. When sample 23 was tested after the 30-min/0.08 mg depletion method, the titers of antibodies to all three viruses remained high, with only a 2-fold drop in the titers of antibodies to each of the three viruses; however, after complete IgG depletion (overnight/0.2 mg), the endpoint titers of antibodies to DENV1 decreased 128-fold, those of antibodies to DENV2 decreased 8-fold, and those of antibodies to ZIKV decreased only 4-fold, indicating that the majority of the cross-reactive neutralizing antibodies in samples 20A and 23 were likely cross-reactive flavivirus IgG boosted from a previous DENV infection. However, unlike sample 20A, the IgM-PRNT₉₀ titer did not result in at least a 4-fold higher ZIKV antibody titer than DENV antibody titer for sample 23. It is interesting to note that sample 20A was collected from a person 65 days PMT to an area where DENV is endemic and experiencing a ZIKV outbreak, while sample 23 was collected from a person with illness 19 days PSO. These differences suggest that the timing of sample collection postexposure may affect the usefulness of IgG depletion in differentiating neutralizing antibody endpoint titers for diagnosis of secondary flavivirus infections.

Sera from primary infections remain specific for the infecting virus in the IgM-PRNT. To determine whether IgG depletion would significantly decrease the sensitivity of PRNT among primary infection cases, six serum samples from individuals with primary flaviviral infections collected between 1 and 7 days PSO were tested before and after IgG depletion. Five of the six samples determined to be from patients with primary ZIKV infections before depletion were shown to maintain their neutralizing antibody titers within 2- to 8-fold of the titer of the unabsorbed serum sample after IgG depletion (Table 2). Among these 5 samples, sample 16 was positive in both the ZIKV and DENV MAC-ELISAs, while samples 57, 19, 18, and 17 were positive only in the ZIKV MAC-ELISA. None of the samples, except sample 57, had detectable IgG by ELISA.

TABLE 2 Serology assay results for samples from persons with a primary flaviviral infection

Serum sample identifier	Day after symptom onset	MAC-ELISA result ^a		IgG ELISA result ^a		PRNT ₉₀ titer			IgM-PRNT ₉₀ titer		
		ZIKV	DENV	ZIKV	DENV	ZIKV	DENV1	DENV2	ZIKV	DENV1	DENV2
16	1	+	+	—	—	80	<10	<10	40	<10	<10
57	2	+	—	+	e	1,280	<10	10	160	<10	<10
19	6	+	e	—	—	640	<10	<10	320	<10	<10
18	7	+	—	—	—	640	<10	<10	320	<10	<10
17	7	+	—	—	—	2,560	<10	<10	640	<10	<10
25	7	+	+	—	—	<10	<10	320	<10	<10	160

^aMAC ELISA and IgG ELISA results are shown as positive (+), negative (—), or equivocal (e).

Sample 57, which was positive for both anti-ZIKV IgM and IgG, had the most significant (8-fold) drop of the anti-ZIKV PRNT₉₀ titer after IgG depletion. One specimen, sample 25, was positive for anti-ZIKV and anti-DENV IgM antibodies by ELISA, but it had detectable titers of neutralizing antibodies only against DENV2 before (titer, 320) and after (titer, 160) IgG depletion (Table 2), suggesting a possible recent DENV primary infection. These results confirmed that a significant portion of the virus-neutralizing antibody response in these recent primary infections was contributed by virus-specific IgM within 7 days PSO and remained detectable by PRNT after complete IgG depletion. No anti-ZIKV- or anti-DENV-neutralizing antibody was detected in the five negative controls before or after IgG depletion (data not shown).

Effect of IgM-PRNT in differentiation of secondary flaviviral infections. Before IgG depletion was performed, only 22.7% (95% confidence interval [CI], 10.1 to 43.4%) of the samples, or 5 out of 22 samples (samples 44, 51, 33A, 24A, and 22A), from secondary flavivirus infection cases had PRNT₉₀ titers able to differentiate (by use of at least a 4-fold difference) the three tested viruses (Tables 3 and 4); however, one of these samples (sample 44), which was taken 1 day PSO and which was confirmed to be positive for ZIKV by reverse transcription-PCR, had an 8-fold higher PRNT₉₀ titer of antibodies against DENV1 than antibodies against ZIKV. This sample demonstrated an anamnestic antibody response to a possible previous DENV infection and supports the notion that the 4-fold differential algorithm with traditional PRNT₉₀ titers is not reliable for differentiation between recent ZIKV and DENV infections. Interestingly, the anti-ZIKV PRNT₉₀ titer remained the same with or without IgG depletion for sample 44, while IgG depletion decreased the PRNT₉₀ titers of antibodies against both DENVs 4-fold (Table 3). Although the IgG depletion did not result in 4-fold higher neutralizing titers of antibodies against ZIKV than antibodies against DENV1 for this case, it is obvious that

TABLE 3 Serology assay results for samples from persons with a secondary flaviviral infection with blood collected before 21 days after possible exposure^a

Serum sample identifier	Days PMT ^b	Day PSO	MAC-ELISA result ^c		IgG ELISA result ^c		PRNT ₉₀ titer			IgM-PRNT ₉₀ titer		
			ZIKV	DENV	ZIKV	DENV	ZIKV	DENV1	DENV2	ZIKV	DENV1	DENV2
44		1	+	e	+	+	320	2,560	320	320	640	80
56		7	+	—	+	+	>20,480	>20,480	>20,480	2,560	5,120	320
50		9	+	+	+	e	1,280	1,280	1,280	640	80	40
39A ^d	13 ^e		+	— ^f	+	+	1,280	1,280	640	10	10	<10
51		14	+	+	e	—	5,120	1,280	<10	640	320	<10
23		19	+	+	+	+	5,120	5,120	10,240	1,280	80	1,280
33A	20 ^e		+	—	+	+	20,480	5,120	5,120	20	20	<10

^aPRNT₉₀ titer values in boldface indicate that the titer of one virus was at least 4-fold higher than that of the others (regardless of IgG depletion). The shading indicates that the titer of one virus was at least 4-fold higher than that of the others in the IgM-PRNT₉₀.

^bThe median day of travel and sample collection date were used to calculate the days post-median travel time.

^cMAC-ELISA and IgG ELISA results are shown as positive (+), negative (—), or equivocal (e).

^dA, asymptomatic pregnant traveler.

^eOnly one travel date was recorded on the intake form, and it is unknown if it was from the beginning or the end of travel.

^f—, the serum sample displayed a high background with the negative-control antigen. The P/N ratio could not be determined.

TABLE 4 Serology assay results for samples from persons with a secondary flaviviral infection with blood collected after 21 days after possible exposure^a

Serum sample identifier	Day PMT ^b	Day PSO	MAC-ELISA result ^c		IgG ELISA result ^c		PRNT ₉₀ titer			IgM-PRNT ₉₀ titer		
			ZIKV	DENV	ZIKV	DENV	ZIKV	DENV1	DENV2	ZIKV	DENV1	DENV2
21		24	+	e	+	+	10,240	5,120	640	2,560	320	<10
38A ^d	27		+	—	+	+	640	1,280	1,280	80	20	<10
49		40	+	—	+	+	5,120	5,120	5,120	1,280	320	320
47A	43		+	—	+	+	1,280	2,560	1,280	10	40	10
48A	44		+	—	+	+	2,560	5,120	40	40	160	<10
53A	46 ^f		+	—	+	+	1,280	2,560	1,280	160	160	40
24A	52		+	+	+	+	20	2,560	320	<10	640	80
55A	54 ^e		+	—	—	e	1,280	2,560	320	160	40	<10
54A	58		e	—	+	+	320	1,280	1,280	10	<10	10
52A	60 ^e		e	—	+	+	80	80	160	<10	10	<10
22A	64		+	—	+	e	2,560	320	80	320	<10	<10
20A	65		e	—	+	+	1,280	2,560	2,560	40	10	<10
32A	74 ^f		+	—	+	+	2,560	640	2,560	40	10	10
45A		87	+	e	+	+	2,560	1,280	2,560	160	80	40
30	89		+	e	+	+	160	1,280	640	20	10	<10

^aPRNT₉₀ titer values in boldface indicate that the titer of one virus was at least 4-fold higher than that of the others (regardless of IgG depletion). Shading indicates that the titer of one virus was at least 4-fold higher than that of the others in IgM-PRNT₉₀.

^bThe median day of travel and the sample collection date were used to calculate the days post-median travel time.

^cMAC-ELISA and IgG ELISA results are shown as positive (+), negative (—), or equivocal (e).

^dA, asymptomatic pregnant traveler.

^eThe individual was a recent immigrant to the United States from a country affected by the 2016 ZIKV epidemic. Ninety days before and the time of arrival in the United States were used to calculate the median.

^fOnly one travel date was recorded on the intake form, and it is unknown if it was from the beginning or the end of travel.

IgG depletion significantly decreased the level of the anamnestic cross-reactive flavivirus antibodies.

Since our data showed that IgG depletion prior to PRNT can significantly reduce cross-reactive neutralizing antibody titers, we decided to use the 4-fold differential algorithm to gauge the usefulness of the IgM-PRNT₉₀ in confirming and differentiating the diagnosis of the secondary flavivirus infection cases. Overall, among all of the samples from secondary infection cases tested, 50.0% (95% CI, 30.7 to 69.3%) of these samples (11/22) had an IgM-PRNT₉₀ titer able to differentiate between ZIKV and DENV. Among these 11 samples differentiated after IgG depletion, 8 were from individuals with a recent ZIKV infection, while 3 were from individuals with a recent DENV infection (Tables 3 and 4).

When the time of sample collection was taken into account, the IgM-PRNT successfully differentiated only one (sample 50) of the seven samples collected before 21 days PSO or PMT (Table 3). IgG depletion significantly decreased the anti-DENV PRNT₉₀ titers of this sample (at least a 16-fold drop), but the titers remained within a 2-fold range after depletion of IgG for ZIKV. Two samples (samples 51 and 33A) had PRNT₉₀ titers 4-fold higher for ZIKV antibody before IgG depletion; however, after IgG depletion, no significant difference in virus-neutralizing antibody titers was detected (Table 3). Sample 33A, collected at 20 days PMT, had anti-ZIKV IgM and IgG and anti-DENV IgG but no anti-DENV IgM measured by ELISA, suggesting a possible recent ZIKV infection. The IgM-PRNT₉₀ titers for this sample dropped significantly (more than a 256-fold drop to a titer of ≤20) for all three viruses, suggesting that almost all of the PRNT₉₀ titers for the sample were from highly cross-reactive anamnestic IgG and that the sample had very low levels of IgM neutralizing antibodies. For this case, complete IgG depletion resulted in very low IgM-PRNT₉₀ titers of antibodies to all three viruses and was not useful for differential diagnosis.

Among the samples collected between 21 and 74 days PSO or PMT, the percentage of discernible cases increased from 15.4% (95% CI, 4.3 to 42.2%), or 2/13 samples, before IgG depletion to 76.9% (95% CI, 49.7 to 91.8%), or 10/13 samples, after IgG depletion (Table 4). Seven samples (samples 21, 38A, 49, 55A, 22A, 20A, and 32A) that were positive/equivocal for anti-ZIKV IgM and negative/equivocal for anti-DENV IgM by

ELISA were found to have discernible anti-ZIKV IgM-PRNT₉₀ titers, but only one of them (sample 22A) had discernible anti-ZIKV PRNT₉₀ titers before IgG depletion. After IgG depletion, only ZIKV-specific neutralizing antibody titers were detected from sample 22A, indicating that the depletion successfully removed all of the detectable cross-reactive neutralizing antibody in this case. Interestingly, two samples (samples 47A and 48A) that were also positive for anti-ZIKV IgM and negative for anti-DENV IgM by ELISA were found to have discernible DENV1 IgM-PRNT₉₀ titers. We confirmed that the majority of the DENV1-neutralizing antibody was due to IgM, since the percent IgG depletion for samples 47A and 48A was determined to be 99.5 and 99.3%, respectively. The discrepancy in the results between the negative DENV MAC-ELISA results and the positive DENV1 IgM-PRNT results may be explained by the difference in serum dilutions used for the assays. Serum samples were tested at a single 1:400 dilution in the MAC-ELISA, while the highest IgM-PRNT₉₀ endpoint titer for DENV1 was 1:40 and 1:160 for samples 47A and 48A, respectively. Based on these results, we believe that the quantitative IgM-PRNT starting from a lower dilution level likely provides a more sensitive outcome than the MAC-ELISA performed with a single dilution.

Sample 24A had discernible PRNT₉₀ titers of antibodies to DENV1 that were highest both before and after IgG depletion (Table 4). The IgG depletion resulted in a nondetectable anti-ZIKV PRNT₉₀ titer for this sample, indicating a recent DENV secondary infection. Three samples (samples 52A, 53A, and 54A) collected between 21 and 76 days after possible exposure still did not have IgM-PRNT₉₀ titers able to discern between ZIKV and DENVs. The IgM-PRNT₉₀ titers for samples 52A and 54A decreased to ≤ 10 for all three viruses, which was not surprising, considering that both of the samples had equivocal or nondetectable IgM results for ZIKV and DENV by ELISA (Table 4). As the IgM level was low in these samples, it is understandable that the IgM-PRNT may not be very useful in these cases.

Only two samples collected more than 12 weeks postexposure were available to us at the time of the study. Sample 45A (collected 87 days PMT) and sample 30 (collected 89 days PSO) had undiscernible PRNT₉₀ titers for antibodies to the viruses before and after IgG depletion (Table 4). Although we had only two samples from time points beyond 12 weeks PSO or PMT, the results suggest that the IgM-PRNT may not be very efficient in providing a differential diagnosis for samples collected this long after exposure.

DISCUSSION

This report describes the ability of IgG depletion to reduce the cross-reactive neutralizing antibodies in diagnostic serum samples resulting from a secondary flaviviral infection. Our results show that the timing of collection of convalescent-phase specimens may affect the usefulness of IgG depletion to reduce cross-reactivity. Among the 13 samples with recent secondary flavivirus (ZIKV or DENV) infections collected between 21 and 74 days PSO or PMT, IgG depletion increased the percentage of samples with discernible PRNT₉₀ titers to 76.9%, while IgG depletion prior to PRNT helped to differentiate only 11.1% of samples collected outside that time frame. These results highlight the dynamic antibody response elicited during secondary flavivirus infections. In addition to evaluating the utility of the IgM-PRNT in the differential diagnosis of ZIKV and DENV infection, this study also demonstrates the potential usefulness of the assay to advance knowledge of the ant flavivirus IgM and IgG neutralizing antibody response over time in primary and secondary flavivirus infections.

It is well-known that ant flavivirus IgG is highly cross-reactive, and the presence of IgG is often indicative of long-term acquired immunity from a previous flaviviral infection (38). For this reason, ELISAs measuring ant flavivirus IgG are often nonspecific and usually require PRNT for confirmatory diagnosis; however, PRNT is also highly cross-reactive among flaviviruses in secondary infection cases. During the analysis of the ZIKV outbreak in Yap State, Federated States of Micronesia, it was determined that the majority of presumed secondary flaviviral infections showed measurable anti-DENV neutralizing antibody titers in PRNT (13, 39). This is due to the anamnestic cross-reactive

flavivirus antibody response, which can sometimes yield higher titers of neutralizing antibodies to a different flavivirus causing the previous infection, leading to an uninterpretable diagnosis on the basis of the results of ELISA and PRNT (13, 40). Historically, the differential diagnosis of flavivirus infection using PRNT from a single specimen was determined using a 4-fold difference in the antibody titers; however, during the 2015–2016 ZIKV epidemic in the Western Hemisphere, the CDC guidelines changed so that only samples with positive anti-ZIKV PRNT₉₀ titers without detectable anti-DENV PRNT₉₀ titers were considered ZIKV positive. This change was made to prevent the misdiagnosis of ZIKV in vulnerable populations with probable past flaviviral infections that may have affected the accuracy of the serological testing results. On the basis of the results of MAC-ELISA, IgG ELISA, and traditional PRNT for ZIKV and two DENVs, we confirmed that the traditional PRNT algorithm could not always reliably confirm or facilitate the differential diagnosis between ZIKV and DENVs in suspected secondary flavivirus infection cases.

Since the IgM response to virus infection starts earlier than the IgG response, the presence of IgM antibody is typically indicative of a recent infection. MAC-ELISA has been widely used for IgM detection from acute- or early-convalescent-phase specimens from individuals suspected of having a recent flavivirus infection. It has been determined that the detection of IgM by the ZIKV MAC-ELISA can be cross-reactive, leading to 27% of specimens tested in the United States and more than 80% of specimens tested in Puerto Rico, a region where DENVs are endemic, to be identified as being from individuals who have had a recent nonspecific flavivirus infection (41). Furthermore, Lindsey et al. found that nearly a third of ZIKV IgM-positive cases in the United States tested by MAC-ELISA were not confirmed after PRNT, emphasizing the need for a ZIKV-specific confirmatory diagnostic test (41). Although the overall highest anamnestic antibody subtype is IgG, the anamnestic cross-reactive IgM to flaviviruses in some cases still obviously caused significant problems for differential diagnosis when MAC-ELISA results alone were used. Unless paired specimens collected at different time points can be obtained for testing, an individual providing a single specimen with multiple flavivirus-positive MAC-ELISA outcomes can be diagnosed only as having a possible recent flavivirus infection. The current CDC MAC-ELISA used for diagnosis is not a quantitative assay (all specimens are tested at a single 1:400 dilution without endpoint titration). Therefore, quantitative PRNT with ZIKV and DENV is typically used to confirm and provide a potential further differential diagnosis. As the traditional PRNT cannot be reliable for such differentiation between secondary ZIKV and DENV infections, this study was designed to investigate whether IgG depletion prior to PRNT could improve the utility and accuracy of the assay.

While IgG is the most abundant neutralizing antibody isotype elicited during flaviviral infections, IgM antibodies also display virus-neutralizing activity and have been found to be more virus specific or complex specific than IgG (25–29, 42). Other antibody isotypes have been studied, but their contributions to ant flaviviral humoral immunity are less characterized (43). In the present study, we assumed that the majority of the neutralizing antibody detected by PRNT after IgG depletion was dependent on the concentration and virus specificity of ant flaviviral IgM in the serum sample. Exogenous complement has been shown to be an important factor in the efficiency with which IgM neutralizes several viruses (44–48). While the exact mechanism of the association between IgM and complement in many viral infections is not clear, IgM and components of the classical complement pathway have been shown to be associated with influenza viral aggregation and coating of the viral hemagglutinin receptor (49). In our study, we expressly included exogenous human complement in both PRNT protocols with all viruses tested in order to standardize the assays and enable IgM to neutralize virus effectively *in vitro*. Our hypothesis is that depletion of the IgG in the specimen would potentially remove the most abundant anamnestic cross-reactive neutralizing antibodies from samples collected at certain time frames postexposure, thereby improving the virus specificity of the PRNT and allowing a more meaningful 4-fold difference in titer for differential diagnosis. Indeed, using the algorithm with the 4-fold

titer difference to analyze the PRNT results before and after IgG depletion, among all 22 samples from suspected secondary flavivirus infections (Tables 3 and 4), the percentage of samples with discernible titers increased from 18.2% before depletion to 50.0% after depletion. When analyzing the 13 samples collected at about 3 to 12 weeks postexposure, IgG depletion increased the percentage of discernible samples from 15.4% to 76.9%.

Limitations in this study included the lack of definitive molecular testing results for all but one subject. In addition, we were not able to test both acute- and convalescent-phase samples from the same subject to better determine the accurate dynamic effect of IgG depletion on differential PRNT. Furthermore, the time points of sample collections indicated in the sample records that we received, which were analyzed in a blind manner, relied on self-reported travel duration and/or a suspected symptom onset time. Therefore, the collection time points postexposure may not have been very accurate, especially for subjects who stayed at the travel areas for weeks or longer; however, these specimens are examples of the types of samples and accompanying information received by CDC's Arboviral Diseases Branch diagnostic laboratory for confirmatory testing, which makes their inclusion in this study logical. We acknowledge that the sample size was limited, especially for samples collected before 3 weeks or after 12 weeks postexposure. Nevertheless, this study demonstrates the potential of the IgM-PRNT to improve the differential diagnosis in individuals with secondary infections caused by closely related flaviviruses, and the IgM-PRNT warrants further study. Currently, we plan to continue the investigation with specimens collected for several cohort studies at different time points postinfection. Preliminary data for some of these specimens suggest that the IgM-PRNT may have a broader window for use outside the 3- to 12-week-postexposure time point. We also plan to implement a high-throughput, reporter virus-based microneutralization assay utilizing live cell image flow cytometry developed in our laboratory. Along with a modified IgG depletion process for higher throughput using a 96-well filtration system, these modifications will allow analysis of a larger sample size in order to further study the change in specificity of the IgM neutralizing antibody response in the convalescent phase of disease and confirm the usefulness of the IgM-PRNT.

In conclusion, the recent ZIKV epidemic in the Western Hemisphere has highlighted the limitations of current serological assays for the confirmatory diagnosis of infections with flaviviruses, which is especially important in ZIKV infections, due to the complications that may arise for women infected during pregnancy. By utilizing IgG depletion before PRNT, the accuracy of the results may be significantly increased, leading to more defined diagnostic information for patients and health care providers.

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