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Ability To Serologically Confirm Recent Zika Virus Infection in Areas with Varying Past Incidence of Dengue Virus Infection in the United States and U.S. Territories in 2016

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ABSTRACT Cross-reactivity within flavivirus antibody assays, produced by shared epitopes in the envelope proteins, can complicate the serological diagnosis of Zika virus (ZIKAV) infection. We assessed the utility of the plaque reduction neutralization test (PRNT) to confirm recent ZIKAV infections and rule out misleading positive immunoglobulin M (IgM) results in areas with various levels of past dengue virus (DENV) infection incidence. We reviewed PRNT results of sera collected for diagnosis of ZIKAV infection from 1 January through 31 August 2016 with positive ZIKAV IgM results, and ZIKAV and DENV PRNTs were performed. PRNT result interpretations included ZIKAV, unspecified flavivirus, DENV infection, or negative. For this analysis, ZIKAV IgM was considered false positive for samples interpreted as a DENV infection or negative. In U.S. states, 208 (27%) of 759 IgM-positive results were confirmed to be ZIKAV compared to 11 (21%) of 52 in the U.S. Virgin Islands (USVI), 15 (15%) of 103 in American Samoa, and 13 (11%) of 123 in Puerto Rico. In American Samoa and Puerto Rico, more than 80% of IgM-positive results were unspecified flavivirus infections. The false-positivity rate was 27% in U.S. states, 18% in the USVI, 2% in American Samoa, and 6% in Puerto Rico. In U.S. states, the PRNT provided a virus-specific diagnosis or ruled out infection in the majority of IgM-positive samples. Almost a third of ZIKAV IgM-positive results were not confirmed; therefore, providers and patients must understand that IgM results are preliminary. In territories with historically higher rates of DENV transmission, the PRNT usually could not differentiate between ZIKAV and DENV infections.

KEYWORDS confirmatory, serologic, Zika virus

Zika virus (ZIKAV) is a flavivirus related to yellow fever, dengue, West Nile, and Japanese encephalitis viruses (1, 2). Among flaviviruses, ZIKAV and dengue virus (DENV) in particular share similar symptoms of infection, transmission cycles, and

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geographic distribution (3). Laboratory diagnosis of recent ZIKAV infection can be made by molecular testing, particularly if samples are collected shortly after disease onset, or by serological methods.

ZIKAV-specific immunoglobulin M (IgM) antibodies typically develop during the first week of infection (4). Envelope protein-based enzyme-linked immunosorbent assays (ELISAs) are highly sensitive and can be used to screen for anti-ZIKAV IgM antibodies in serum or cerebrospinal fluid; however, these assays can provide false-positive results for the purpose of definitively diagnosing ZIKAV infection because of cross-reactivity with envelope proteins of related flaviviruses or nonspecific reactivity (5, 6). Cross-reactive results in sera occur more frequently among patients with previous flavivirus infection or vaccination against a related flavivirus (4, 7). This cross-reactivity is particularly extensive between ZIKAV and DENV (4), which is important because the results of ZIKAV and DENV testing are essential for guiding clinical management of patients and public health prevention efforts. As a result of these cross-reactivity and nonspecific reactivity issues, testing algorithms have generally recommended that all positive and equivocal ZIKAV IgM results be confirmed with the more specific plaque reduction neutralization test (PRNT) (8).

The PRNT can measure virus-specific neutralizing antibody titers against ZIKAV, DENV, and other flaviviruses to which the person might have been exposed (9). In some situations, the PRNT can resolve nonspecific reactivity and discriminate between cross-reacting antibodies because neutralizing antibodies bind with virus-specific antigens and, unlike ELISA, the PRNT is a quantitative assay in which titers are compared. However, in persons previously infected with or vaccinated against a flavivirus, discriminating between cross-reactive antibodies might still be difficult. A person previously infected with a flavivirus, when exposed to another flavivirus, can have a rapid rise in neutralizing antibodies against multiple flaviviruses because their immune system recognizes and reacts to shared epitopes among these viruses (10). It can be impossible to determine which flavivirus is causing the patient's illness, resulting in the diagnosis of an unspecified flavivirus infection.

DENV transmission in U.S. states has historically been infrequent and localized, and nearly all dengue cases have occurred in travelers. In the U.S. Virgin Islands (USVI) and American Samoa, there have been periodic outbreaks, most recently in 2012 and 2015, respectively. Because of limited surveillance during nonoutbreak periods, the ongoing level of DENV transmission in these locations is unclear. In Puerto Rico, dengue is endemic, and periodic island-wide outbreaks also occur (11, 12). We assessed the utility of the PRNT to confirm ZIKAV infections and identify false-positive IgM results in U.S. states and selected territories with varying incidences of DENV infection.

MATERIALS AND METHODS

We included data for serum samples submitted for routine diagnosis of recent ZIKAV infection that were collected from symptomatic and asymptomatic individuals in all 50 U.S. states, the USVI, American Samoa, and Puerto Rico from 1 January through 31 August 2016. For most of the included time period, the recommended testing algorithm included antibody testing for anyone with symptom onset at $\geq$4 days before sample collection, and this was the primary testing strategy for asymptomatic pregnant women (13, 14). We included samples that had positive or equivocal IgM results by Zika IgM antibody capture enzyme-linked immunosorbent assay (Zika MAC-ELISA) performed at a Centers for Disease Control and Prevention (CDC) laboratory or at the Hawaii Department of Health State Laboratories (for specimens from American Samoa) and also had $PRNT_{90}$ results for both ZIKAV and DENV.

The U.S. Food and Drug Administration has issued an Emergency Use Authorization for the CDC Zika MAC-ELISA for antibody testing (15). The CDC MAC-ELISA is designed to have high sensitivity to ensure flavivirus antibody-positive samples are not missed; thus, some specificity is lost. The ELISA is routinely used in combination with the more specific PRNT, which yields a quantitative result to aid in differentiating between cross-reacting flavivirus antibodies. Samples with detectable Zika IgM antibody with the MAC-ELISA might subsequently be determined by a PRNT to be ZIKAV, unspecified flavivirus, or DENV infections or to have no evidence of ZIKAV infection. In this analysis, for samples interpreted as positive for DENV infection or with no evidence of infection, the IgM result was described as a false-positive result.

The PRNT was performed based on the methods described by Lindsey et al. (16). DENV PRNT results were based on assays using DENV-2 and/or DENV-1. The $PRNT_{90}$ titer is the reciprocal of the endpoint serum dilution that reduces the challenge virus plaque count by 90%. Previously published interpretations of results of neutralizing antibody testing were used, which had been determined to be the most accurate based on preliminary data specific to ZIKAV and earlier flavivirus research (5). If the ZIKAV PRNT

TABLE 1 PRNT interpretations for serum specimens with positive ZIKAV IgM results in U.S. states and three territories from 1 January 2016 to 31 August 2016 (n = 1,037)

	U.S. states (n = 759)		USVI (n = 52)		American Samoa (n = 103)		Puerto Rico (n = 123)	
PRNT interpretation[a]	No.	% (95% CI)	No.	% (95% CI)	No.	% (95% CI)	No.	% (95% CI)
ZIKAV infection	208	27 (21–34)	11	21 (0–48)	15	15 (0–34)	13	11 (0–28)
Unspecified flavivirus infection	344	45 (39–52)	32	62 (40–83)	86	83 (73–94)	103	84 (74–93)
DENV infection[a]	59	8 (1–15)	4	8 (0–35)	1	1 (0–20)	7	6 (0–23)
Negative[a,b]	148	19 (13–26)	5	10 (0–37)	1	1 (0–20)	0	0 (−18–18)

[a]PRNT interpretations of DENV infection or negative were considered ZIKAV false-positive IgM.
[b]No evidence of ZIKAV or DENV infection.

titer was ≥10 and DENV PRNT titer was <10, the result was interpreted as a ZIKAV infection. If PRNT titers were ≥10 for both ZIKAV and DENV, the result was interpreted as an unspecified flavivirus infection, since this result precludes identification of the specific infecting flavivirus. If the ZIKAV PRNT titer was <10 and DENV PRNT titer was ≥10, the result was interpreted as a DENV infection. Among asymptomatic cases, if PRNT titers were <10 for both ZIKAV and DENV, the result was interpreted as no evidence of ZIKAV or DENV infection (negative). Among symptomatic patients, if PRNT titers were <10 for both ZIKAV and DENV and the sample was collected ≥7 days after illness onset, the result was interpreted as no evidence of ZIKAV or DENV infection. If PRNT titers were <10 for both ZIKAV and DENV and the sample was collected <7 days after illness onset, the negative PRNT titer might reflect collection before development of detectable neutralizing antibodies; reverse transcription-PCR (RT-PCR) is recommended to clarify interpretation (5). For these samples, if RT-PCR was not conducted, samples were excluded from the analysis. If the RT-PCR was negative, the result was interpreted as no evidence of ZIKAV or DENV infection. A positive RT-PCR provided evidence of recent ZIKAV or DENV infection.

We compared the proportions of samples with the defined PRNT result interpretations in U.S. states, the USVI, American Samoa, and Puerto Rico. In addition, we compared PRNT interpretations by the presence of symptoms, age group and sex. The demographic comparisons were limited to symptomatic persons because the asymptomatic persons tested were almost exclusively females of childbearing age, since the testing recommendations for asymptomatic persons were directed to pregnant women.

All testing described here was performed in the course of routine public health practice. Samples were collected by clinicians in the course of clinical care, and they were submitted to the CDC and state laboratories for diagnostic testing. No additional testing was performed for the analysis described here. Because samples were collected and tested in the course of clinical care, these activities were outside the scope of Institutional Review Board requirements.

RESULTS

A total of 1,320 samples met the inclusion criteria for this analysis, including 1,037 samples with ZIKAV IgM-positive results and 283 with IgM-equivocal results. Of the 1,037 IgM-positive samples, 247 (24%) were confirmed by a PRNT to be ZIKAV infections, 565 (54%) were unspecified flavivirus infections, 71 (7%) were DENV infections, and 154 (15%) were negative. For the 225 (22%) samples with the PRNT interpreted as DENV or negative, the Zika IgM result was considered to be a false-positive result.

PRNT result interpretations for ZIKAV IgM-positive samples subdivided by jurisdiction are shown in Table 1. In U.S. states, 27% of the IgM-positive results were confirmed to be ZIKAV compared to 21% in the USVI, 15% in American Samoa, and 11% in Puerto Rico. In American Samoa and Puerto Rico, more than 80% of IgM-positive samples were interpreted as unspecified flavivirus infections. In U.S. states, 27% of IgM-positive results were found to be false-positive results, with the PRNT indicating no evidence of ZIKAV infection compared to 18% in the USVI, 2% in American Samoa, and 6% in Puerto Rico. Of the 225 samples from all jurisdictions with false-positive ZIKAV IgM results, 71 (32%) had results consistent with DENV infection. A larger proportion of results were consistent with DENV infection in the territories than in U.S. states (Table 1).

Among the 1,037 samples with ZIKAV IgM-positive results from all jurisdictions, 255 were from asymptomatic patients, and 782 were from symptomatic patients. Among the 255 samples from asymptomatic patients, 15 (6%) were confirmed by a PRNT to be ZIKAV infections, 148 (58%) were unspecified flavivirus infections, 21 (8%) were DENV, and 71 (28%) were negative (Table 2). Of the 186 samples from asymptomatic patients from U.S. states, 6 (3%) were confirmed by a PRNT to be ZIKAV infections, 100 (54%) were unspecified flavivirus infections, 14 (8%) were DENV, and 66 (35%) were negative. Of the 69 samples from asymptomatic patients from the three territories, 9 (13%) were

TABLE 2 PRNT interpretations for serum specimens with positive ZIKAV IgM results by presence of symptoms in U.S. states and three territories from 1 January 2016 to 31 August 2016 (n = 1,037)

	Asymptomatic (n = 255)		Symptomatic (n = 782)	
PRNT interpretation	No.	% (95% CI)	No.	% (95% CI)
ZIKAV infection	15	6 (0–18)	232	30 (23–36)
Unspecified flavivirus infection	148	58 (48–68)	417	53 (47–59)
DENV infection	21	8 (0–20)	50	6 (0–13)
Negative[a]	71	28 (16–40)	83	11 (4–18)

[a]No evidence of ZIKAV or DENV infection.

confirmed by a PRNT to be ZIKAV infection, 48 (70%) were unspecified flavivirus infections, 7 (10%) were DENV, and 5 (7%) were negative.

Of the 782 samples with positive ZIKAV IgM results from symptomatic patients from all jurisdictions, 232 (30%) were confirmed by a PRNT to be ZIKAV infections, 417 (53%) were unspecified flavivirus infections, 50 (6%) were DENV, and 83 (11%) were negative (Table 2). Of the 573 samples from symptomatic patients from U.S. states, 202 (35%) were confirmed by a PRNT to be ZIKAV infections, 244 (43%) were unspecified flavivirus infections, 45 (8%) were DENV, and 82 (14%) were negative. Of the 209 samples from the three territories, 30 (14%) were confirmed by a PRNT to be ZIKAV infection, 173 (83%) were unspecified flavivirus infections, 5 (2%) were DENV, and 1 (<1%) was negative.

Among the 782 samples from symptomatic persons, 492 (63%) were from female patients, 244 (31%) were from males, and 46 (6%) had missing sex data. The proportion confirmed as ZIKAV infection was higher for males (35%, 95% confidence interval [CI] = 23 to 47%) than females (28%, 95% CI = 19 to 36%), but the difference was not statistically significant. The percentage of false-positive IgM results was similar for males (18%, 95% CI = 5 to 30%) and females (17%; 95% CI = 9 to 26%). The percentage of IgM-positive results that were confirmed to be ZIKAV infections was highest in the youngest age group (aged <10 years; 57% of IgM-positive samples), followed by those aged 10 to 19 years (39%) (Table 3). Similar demographic trends were seen for U.S. states and territories separately (data not shown).

Of the 283 samples with equivocal ZIKAV IgM results, 17 (6%) were confirmed by a PRNT to be ZIKAV infections, 59 (21%) were unspecified flavivirus infections, 37 (13%) were DENV, and 170 (60%) were negative. Of the 255 samples with equivocal results from U.S. states, 16 (6%) were confirmed by a PRNT to be ZIKAV infections, 49 (19%) were unspecified flavivirus infections, 25 (10%) were DENV, and 165 (65%) were negative. Thus, among samples with equivocal IgM results in U.S. states, a confirmatory PRNT indicated that 75% had no evidence of ZIKAV infection. Of the 28 equivocal IgM results from the three territories, 1 (4%) was confirmed by the PRNT to be a ZIKAV infection, 10 (36%) were unspecified flavivirus infections, 12 (43%) were DENV, and 5 (18%) were negative. In the three U.S. territories, 60% of the samples with equivocal IgM results had no evidence of ZIKAV infection.

TABLE 3 PRNT interpretations for serum specimens from symptomatic patients with positive ZIKAV IgM results by age group in U.S. states and three territories from 1 January 2016 to 31 August 2016 (n = 745)[a]

	Age, <10 yrs (n = 30)		Age, 10–19 yrs (n = 59)		Age, 20–39 yrs (n = 386)		Age, 40–59 yrs (n = 191)		Age, ≥60 yrs (n = 79)	
PRNT interpretation	No.	% (95% CI)	No.	% (95% CI)	No.	% (95% CI)	No.	% (95% CI)	No.	% (95% CI)
ZIKAV infection	17	57 (27–86)	23	39 (15–62)	107	28 (18–37)	56	29 (16–43)	20	25 (4–47)
Unspecified flavivirus infection	10	33 (0–67)	29	49 (27–71)	190	49 (41–58)	115	60 (49–72)	47	59 (42–77)
DENV infection	2	7 (0–42)	5	8 (0–34)	26	7 (0–17)	14	7 (0–21)	2	3 (0–25)
Negative[b]	1	3 (0–39)	2	3 (0–29)	63	16 (6–26)	6	3 (0–17)	10	13 (0–35)

[a]Age data are missing for 37 symptomatic patients.
[b]No evidence of ZIKAV or DENV infection.

DISCUSSION

The PRNT plays an important role in confirming ZIKAV infections, as well as in identifying false-positive ZIKAV IgM results to rule out ZIKAV infection. Of samples collected from patients in U.S. states, 27% of ZIKAV IgM-positive and 75% of equivocal results were not confirmed by the PRNT. Therefore, it is crucial that these patients receive pre- and posttest counseling to explain that a positive IgM result is only considered a preliminary presumptive positive result until confirmatory testing by the PRNT has been conducted. In addition, all patients and providers should be aware that most ZIKAV IgM-equivocal results will not be confirmed as ZIKAV infection since many are the result of nonspecific cross-reactivity. This analysis also showed that in areas where DENV circulation has been historically high, the PRNT often could not differentiate between ZIKAV and DENV infections. In such settings, the PRNT might not provide substantial benefit for routine laboratory testing, particularly considering the time-consuming and costly nature of the test. In fact, laboratory guidelines for serological testing in Puerto Rico indicate that PRNT confirmation is no longer routinely recommended (17).

ZIKAV IgM test results were more likely to be falsely positive among asymptomatic patients, a group in which a lower prevalence of infection is expected, compared to symptomatic patients (18). The proportions of ZIKAV IgM-positive results confirmed as ZIKAV infection were similar among males and females. However, an age-stratified analysis suggested a greater likelihood of a confirmed ZIKAV interpretation in patients in younger groups, likely because of less frequent previous DENV exposure.

Because of the importance of appropriate clinical management of DENV infection and the need for careful monitoring of pregnant women infected with ZIKAV, a conservative clinical management approach is recommended for individuals with unspecified flavivirus infections, unless additional testing such as molecular testing can provide a definitive diagnosis (5). Therefore, DENV infection should be considered a possibility in symptomatic patients, and they should receive appropriate management to reduce the risk for hemorrhage and shock (19). Likewise, pregnant women with unspecified flavivirus infections should be evaluated and managed with consideration for possible ZIKAV infection and the potential for adverse pregnancy outcomes (5).

There are some limitations of this analysis. The numbers included in the analysis are small (particularly for the territories), so the likelihood of detecting significant differences in proportions was low. Background levels of DENV infection may differ between different areas within jurisdictions, so results might be different if analyzed in smaller geographical areas. As levels of ZIKAV and DENV transmission change, the utility of the PRNT could also change and would need to be reevaluated. Finally, the IgM results included in this analysis were generated using the CDC-developed ELISA and may not be directly transferable to commercially available ZIKAV IgM assays.

In summary, the PRNT plays an important role in confirming ZIKAV infections and identifying false-positive ZIKAV IgM results, particularly in areas without high historical DENV circulation. In U.S. states, more than half of those tested by the PRNT received a conclusive result either indicating no evidence of infection or confirming ZIKAV or DENV infection. Almost 30% of the positive ZIKAV IgM results were not confirmed by the PRNT, highlighting the importance of physician understanding of the limitations of IgM results so that patients can be properly counseled.

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