AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Clinical Microbiology®

VIROLOGY

Serologic Testing for Zika Virus: Comparison of Three Zika Virus IgM-Screening Enzyme-Linked Immunosorbent Assays and Initial Laboratory Experiences

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ABSTRACT Serologic evaluation for Zika virus (ZIKV) infection currently includes an initial screen using an anti-ZIKV IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) followed by supplemental testing of specimens with non-negative results by a plaque reduction neutralization test (PRNT). We compared the performance characteristics of three ELISAs for the detection of IgM class antibodies to ZIKV, including the Centers for Disease Control and Prevention (CDC) Zika MAC-ELISA, the InBios ZIKV *Detect* MAC-ELISA, and the Euroimmun anti-Zika Virus IgM ELISA. Additionally, we present our initial experiences with ZIKV serologic testing from a national reference laboratory perspective. Using both retrospectively and prospectively collected specimens from patients with possible ZIKV infection, we show that the CDC and InBios MAC-ELISAs perform comparably to each other, with positive agreement, negative agreement, and interrater kappa values ranging from 87.5% to 93.1%, 95.7% to 98.5%, and 0.52 to 0.83, respectively. In contrast, comparison of the Euroimmun ZIKV ELISA to either the CDC or InBios MAC-ELISAs resulted in positive agreement, negative agreement, and interrater kappa values ranging from 17.9% to 42.9%, 91.7% to 98.6%, and 0.10 to 0.39, respectively. Among the 19 prospective samples submitted for PRNT, nine were negative, eight specimens had neutralizing antibodies to a flavivirus (unable to be identified), and one sample each was confirmed for ZIKV or dengue virus infection. This study highlights the ongoing challenges associated with serologic diagnosis of ZIKV infection. Although the availability of a commercial serologic test for ZIKV has greatly expanded the national capacity for such testing, the need to further characterize and improve these assays, particularly with regard to specificity, remains.

KEYWORDS Zika virus, serology

Zika virus (ZIKV) emerged from obscurity in early 2015 following its detection in Bahia, Brazil (1). Over the next year, ZIKV spread rapidly throughout Latin America, the Caribbean, and into the southern United States, resulting in a major and still ongoing international outbreak (2). Currently, over 1 million cases of suspected or confirmed ZIKV infection have been documented in the Americas by the Pan American Health Organization (3). ZIKV, a single-stranded RNA virus and member of the *Flavivirus* genus, is primarily transmitted through infected *Aedes* species mosquitoes, which are also primary vectors for dengue (DENV) and chikungunya (CHIKV) viruses, both of which cocirculate in many regions where ZIKV is now considered endemic (4). ZIKV transmis-

Received 6 April 2017 **Returned for modification** 19 April 2017 **Accepted** 20 April 2017

Accepted manuscript posted online 26 April 2017

Citation Granger D, Hilgart H, Misner L, Christensen J, Bistodeau S, Palm J, Strain AK, Konstantinovski M, Liu D, Tran A, Theel ES. 2017. Serologic testing for Zika virus: comparison of three Zika virus IgM-screening enzyme-linked immunosorbent assays and initial laboratory experiences. J Clin Microbiol 55:2127–2136. https://doi.org/10.1128/JCM.00580-17.

Editor Alexander J. McAdam, Boston Children's Hospital

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sion can also occur through sexual contact, vertically, through blood transfusion, and possibly via secondary, nonsexual contact (5–9).

While the majority (~80%) of ZIKV infections are unapparent, symptomatic patients often present with a pruritic maculopapular rash, arthralgia, fever, and/or nonpurulent conjunctivitis (10). Although ZIKV has been linked to development of Guillain-Barré syndrome, the most devastating consequence of ZIKV infection occurs in fetuses infected *in utero* (11, 12). A causal relationship between ZIKV and congenital disease has been established, and due to these severe sequelae, the World Health Organization declared the ongoing ZIKV outbreak a Public Health Emergency of International Concern in February 2016, its fourth such declaration in history, which lasted until November 2016 (13).

Recommended diagnostic testing algorithms for ZIKV have been issued through the Centers for Disease Control and Prevention (CDC) and the World Health Organization (14, 15). Although understanding of the serologic response to ZIKV remains limited, prior studies indicate that IgM class antibodies to the virus are detectable as early as 4 to 5 days post illness onset, peak 2 weeks following infection, and typically become undetectable by 12 to 14 weeks (16). Based on this, for patients with less than 2 weeks of symptoms and any pregnant woman who presents with or without symptoms within 2 weeks of the last possible exposure, the CDC recommends that real-time reverse transcription PCR (rRT-PCR) for detection of ZIKV RNA in blood and urine be performed. For symptomatic patients and pregnant women beyond this time point, the CDC recommends serologic evaluation for IgM class antibodies to ZIKV via an IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) (17). Due to the possibility of antibody cross-reactivity between closely related flaviviruses, supplemental testing by a plaque reduction neutralization test (PRNT) is required for all specimens reactive by a ZIKV MAC-ELISA.

Since recognition of ZIKV as a public health threat, multiple molecular and serologic assays have become commercially available, and many have received emergency use authorization (EUA) through the Food and Drug Administration (FDA) (18). The goal of this study was to compare three ZIKV IgM screening ELISAs using retrospectively collected specimens from symptomatic individuals presenting to a hospital in Colombia and from U.S. residents with recent exposure or travel to a region with ongoing ZIKV transmission. The ELISAs included in this comparison are the CDC Zika MAC-ELISA (CDC MAC-ELISA), the InBios ZIKV *Detect* IgM capture ELISA (InBios MAC-ELISA; InBios International, Inc., Seattle, WA), and the Euroimmun anti-Zika Virus IgM ELISA (Euroimmun ELISA; Euroimmun AG, Lübeck, Germany).

RESULTS

Patient demographics. The median ages of patients included in the retrospective and prospective arms were 43 years and 29 years, respectively (Table 1). The majority of specimens were collected from females (68.5% and 80.1% in the retrospective and prospective arms, respectively), of whom 18.9% were pregnant in the retrospective arm compared to 65.2% pregnant in the prospective arm. All 54 samples in the retrospective arm were collected from individuals who resided in a region where Zika is endemic (i.e., Colombia), whereas 91.4% of patients in the prospective arm reported travel to a region with autochthonous ZIKV transmission. Nearly all patients in the retrospective arm (92.6%) reported at least one symptom consistent with ZIKV infection compared to 48.3% of patients in the prospective arm. Data regarding specific patient symptoms were available for the retrospective sample set and showed that the majority of patients presented with fever (90%) and/or rash (84%) (Table 1); detailed clinical data were available for select patients in the prospective arm and are discussed separately.

Comparison of the ZIKV IgM screening ELISAs. Using specimens in the retrospective arm, the InBios MAC-ELISA showed positive, negative, and overall percent agreements of 93.1% (27/29), 95.7% (22/23), and 90.7% (49/54), respectively, compared to the CDC MAC-ELISA, whereas these same performance measures for the Euroimmun ELISA were 20.7% (6/29), 95.7% (22/23), and 51.9% (28/54), respectively (Table 2). The

TABLE 1 Summary of patient demographics

Demographic	Retrospective samples	Prospective samples[c]
No. of patients	54	151
Median age (range, yrs)	43[a] (6–88)	29 (1 days to 79)
No. of female patients (% of total samples)	37 (68.5)	121 (80.1)
No. of pregnant patients (% of females)	7 (18.9)	79 (65.2)
Reported travel to or residence in area with local Zika virus transmission (% of total samples)	54 (100)	138 (91.4)
No. of symptomatic patients (% of total samples)[b]	50 (92.6)	73 (48.3)
No. of patients with Fever (%)	45 (90)	NA[d]
No. of patients with Rash (%)	42 (84)	NA
No. of patients with Conjunctivitis (%)	28 (56)	NA
No. of patients with Arthralgia (%)	30 (60)	NA
No. of patients with GBS[e] (%)	7 (14)	NA

[a]Age was not documented for 1 patient.
[b]Symptomatic defined as presenting with at least one of the following symptoms: fever, conjunctivitis, rash, arthralgia, Guillain-Barré syndrome.
[c]Patient travel and symptom data acquired from ask at order entry questions answered by physician upon order entry. Detailed information regarding travel and specific symptoms was not provided.
[d]NA, not available.
[e]GBS, Guillain-Barré syndrome.

interrater agreement kappa value for the InBios MAC-ELISA was excellent (0.83), while the Euroimmun assay was considered poor (0.15). Compared to the InBios MAC-ELISA, the Euroimmun ELISA showed positive, negative, and overall agreements of 17.9% (5/28), 91.7% (22/24), and 50% (27/54), respectively, with a kappa value of 0.10 (poor) (Table 3). Among these 54 retrospective samples, five (9.3%) were reactive for IgM antibodies to DENV. The CDC and InBios ZIKV MAC-ELISAs resulted as presumptive positive in four of five of these specimens compared to the results of the Euroimmun ZIKV ELISA, which were presumptive positive in only one of these samples (data not shown).

In the prospective arm, the InBios MAC-ELISA had positive, negative, and overall percent agreements of 87.5% (7/8), 98.5% (130/132), and 91.4% (138/151), respectively, versus the CDC MAC-ELISA. Comparatively, the Euroimmun ELISA showed positive, negative, and overall percent agreement values of 37.5% (3/8), 98.5% (130/132), and 88.1% (133/151) (Table 4). Kappa values for the InBios and Euroimmun assays were 0.52 (fair) and 0.11 (poor), respectively. Compared to the InBios MAC-ELISA, the Euroimmun assay showed positive, negative, and overall percent agreements of 42.9% (3/7), 98.6% (139/141), and 94.0% (142/151), respectively, with a kappa value of 0.39 (fair) (Table 5). Among the prospective specimens, one was positive for IgM antibodies to DENV and both the CDC and InBios ZIKV MAC-ELISAs resulted as presumptive positive for this specimen; the Euroimmun ZIKV ELISA was negative (data not shown).

Among the 151 prospective specimens, 19 had nonnegative results (10 inconclusive, 1 equivocal, and 8 presumptive ZIKV positive) by the screening ZIKV MAC-ELISA and

TABLE 2 Comparison of the InBios and Euroimmun Zika IgM ELISAs to the CDC Zika MAC-ELISA using retrospective clinical specimens

ELISA	Result	CDC Zika MAC-ELISA			Positive % agreement (95% CI)	Negative % agreement (95% CI)	Overall % agreement (95% CI)	Kappa (95% CI), assessment
		No. presumptive positive	No. negative	No. other[b]				
InBios Zika MAC	Positive[a]	27	0	1	93.1 (77–99.2)	95.7 (77.3–100)	90.7 (79.7–96.4)	0.83 (0.69–0.96), excellent
	Negative	1	22	1				
	Other[b]	1	1	0				
Euroimmun Zika	Positive	6	0	0	20.7 (9.5–38.8)	95.7 (77.3–100)	51.9 (38.9–64.6)	0.15 (0.02–0.29), poor
	Negative	23	22	2				
	Other[b]	0	1	0				

[a]The positive category for the InBios Zika MAC-ELISA includes both presumptive Zika positive and possible Zika positive results.
[b]Other indicates result of inconclusive for the CDC Zika MAC-ELISA, borderline for the Euroimmun Zika IgM ELISA, and other flavivirus positive for the InBios Zika MAC-ELISA.

TABLE 3 Comparison of the InBios and Euroimmun Zika IgM ELISAs using retrospective clinical specimens

ELISA	Result	InBios Zika MAC-ELISA			Positive % agreement (95% CI)	Negative % agreement (95% CI)	Overall % agreement (95% CI)	Kappa (95% CI), assessment
		No. positive[a]	No. negative	No. other[b]				
Euroimmun Zika	Positive[a]	5	1	0	17.9 (7.4–36.1)	91.7 (73.0–98.9)	50 (37.1–62.9)	0.10 (−0.05–0.25), poor
	Negative	23	22	2				
	Other[b]	0	1	0				

[a]The positive category for the InBios Zika MAC-ELISA includes both presumptive Zika positive and possible Zika positive results.
[b]Other indicates a result of inconclusive or equivocal for the CDC Zika MAC-ELISA, borderline for the Euroimmun Zika IgM ELISA, and other flavivirus positive for the InBios Zika MAC-ELISA.

were submitted to the CDC or a CDC-designated laboratory for supplemental testing by PRNT as part of routine clinical care (Table 6). All 10 specimens with inconclusive results by the CDC MAC-ELISA were negative by both the InBios and Euroimmun assays, and 8 of these were also negative by PRNT. Plaque reduction neutralization testing demonstrated evidence of infection with a flavivirus but was unable to differentiate between ZIKV and DENV for two of the 10 CDC MAC-ELISA inconclusive specimens. The one specimen that resulted as equivocal by the CDC MAC-ELISA was resulted as "other flavivirus positive" by the InBios MAC-ELISA, negative by the Euroimmun ELISA, and showed evidence of infection with an unspecified flavivirus by PRNT. Three of the eight presumptive positive ZIKV specimens by the CDC MAC-ELISA were also presumptively positive by both the InBios and Euroimmun assays; PRNT confirmed evidence of ZIKV infection in one of these specimens (Table 6, patient number 1). For the other two patients, PRNT indicated evidence of infection with an unspecified flavivirus, though notably, one of these patients (Table 6, patient number 3) was positive for ZIKV by rRT-PCR in urine. Four of the 19 specimens were presumptively positive by only the CDC and InBios MAC-ELISAs, among which PRNT confirmed a DENV infection in one sample (Table 6, patient number 2) and evidence of an unspecified flavivirus infection in the other three specimens. Finally, 1 of 19 specimens that was presumptive ZIKV positive by the CDC MAC-ELISA was negative by all other assays.

DISCUSSION

This study compared three screening ELISAs designed to detect IgM class antibodies to ZIKV in serum, including the CDC MAC-ELISA, the InBios MAC-ELISA, and the Euroimmun IgM ELISA. Our findings suggest that the CDC and InBios MAC-ELISAs perform similarly, with overall percent agreements above 90% and kappa values in the fair to excellent range. In contrast, regardless of whether the Euroimmun Zika IgM ELISA was compared to the CDC or InBios MAC-ELISA, it demonstrated low positive agreement, ranging from 18% to 43%, and consistently low interrater agreement (kappa <

TABLE 4 Comparison of the InBios and Euroimmun Zika IgM ELISAs to the CDC Zika MAC-ELISA using prospective clinical specimens

ELISA	Result	CDC Zika MAC-ELISA			Positive % agreement (95% CI)	Negative % agreement (95% CI)	Overall % agreement (95% CI)	Kappa (95% CI), assessment
		No. presumptive positive	No. negative	No. other[b]				
InBios Zika MAC	Positive[a]	7	0	0	87.5 (50.8–99.9)	98.5 (94.3–99.9)	91.4 (85.7–95.0)	0.52 (0.30–0.75), fair
	Negative	1	130	10				
	Other[b]	0	2[c]	1				
Euroimmun Zika	Positive	3	1[d]	0	37.5 (13.5–69.6)	98.5 (94.3–99.9)	88.1 (81.9–92.4)	0.11 (0–0.45), poor
	Negative	5	130	11				
	Other[b]	0	1[d]	0				

[a]The positive category for the InBios Zika MAC-ELISA includes both presumptive Zika positive and possible Zika positive results.
[b]Other indicates result of inconclusive or equivocal for the CDC Zika MAC-ELISA, borderline for the Euroimmun Zika IgM ELISA, and other flavivirus positive for the InBios Zika MAC-ELISA.
[c]Both samples resulted as other flavivirus positive by the InBios Zika MAC-ELISA. PRNT was not performed on these samples. Both samples were negative by the Euroimmun Zika IgM ELISA and were negative for dengue virus IgM antibodies.
[d]PRNT was not performed on these samples. Samples were negative by the InBios Zika MAC-ELISA and were negative for dengue virus IgM antibodies.

TABLE 5 Comparison of the InBios and Euroimmun Zika IgM ELISAs using prospective clinical specimens

ELISA	Result	InBios Zika MAC-ELISA			Positive % agreement (95% CI)	Negative % agreement (95% CI)	Overall % agreement (95% CI)	Kappa (95% CI), assessment
		No. positive[a]	No. negative	No. other[b]				
Euroimmun Zika	Positive[a]	3	1	0	42.9 (15.8–75.0)	98.6 (94.7–99.9)	94.0 (88.9–97.0)	0.39 (0.06–0.69), fair
	Negative	4	139	3				
	Other[b]	0	1	0				

[a]The positive category for the InBios Zika MAC-ELISA includes both presumptive Zika positive and possible Zika positive results.
[b]Other indicates result of inconclusive or equivocal for the CDC Zika MAC-ELISA, borderline for the Euroimmun Zika IgM ELISA, and other flavivirus positive for the InBios Zika MAC-ELISA.

0.40). Similar to prior studies, our findings reinforce the limited capability of PRNT to discriminate between infections with closely related flaviviruses (19). In our experience, among the 10 specimens with neutralizing antibodies to flaviviruses, PRNT provided definitive identification for only two specimens.

The significantly lower positive agreement observed for the Euroimmun ELISA compared to those of the two MAC-ELISAs may indicate either decreased sensitivity or alternatively increased specificity for IgM antibodies to ZIKV. Due to the limited number of confirmed ZIKV infections in our prospective study set ($n = 2$), a detailed discussion regarding sensitivity of the Euroimmun assay, which was presumptive positive in both cases, is not possible. Interestingly, however, the Euroimmun ELISA was negative in serum from a 1-day-old infant born, with severe microcephaly and arthrogryposis, to a mother who resided in a region where ZIKV is endemic and had experienced Zika-like symptoms during her first trimester (Table 6, patient number 7). Both the CDC and InBios ZIKV MAC-ELISAs were presumptively positive in this same serum sample, and despite negative ZIKV rRT-PCR results on multiple sources from the baby and a lack of definitive PRNT findings, the infant was clinically diagnosed with congenital ZIKV infection. The negative result by the Euroimmun ZIKV ELISA in this patient was unexpected.

A prior study evaluating the Euroimmun ZIKV IgM ELISA reported a sensitivity of 58% among 27 patients with confirmed ZIKV infection by rRT-PCR and recommended concurrent anti-ZIKV IgM and IgG testing to improve overall sensitivity for ZIKV infection to 100% (20). Additionally, studies from these authors and one other group suggest that the Euroimmun ZIKV IgM ELISA is highly specific, as reactivity of this assay was rarely observed in patients with confirmed DENV, Japanese encephalitis virus, West Nile virus (WNV) infections, or in individuals previously vaccinated against yellow fever virus (20, 21). From our own experience, it is notable that the Euroimmun ZIKV IgM ELISA was positive in only one of six specimens reactive for IgM antibodies to DENV. Also, the one sample confirmed for DENV by PRNT was negative by the Euroimmun ELISA, supporting the hypothesis of higher specificity for this assay.

The performance differences between these three ELISAs may, in part, be driven by the different antigens and assay formats used. The Euroimmun ELISA is an indirect ELISA based on the ZIKV NS1 antigen, whereas the InBios MAC-ELISA uses a recombinant ZIKV E glycoprotein. Though the precise composition of the ZIKV antigen(s) in the two CDC MAC-ELISA formats is not disclosed, the secondary conjugated monoclonal antibody used in these assays is specific for flaviviruses and was developed against the WNV E glycoprotein (InBios Inc.), suggesting that the ZIKV antigen(s) used in the CDC MAC-ELISAs contains ZIKV E glycoprotein components. While antibodies to the E glycoprotein are highly cross-reactive between ZIKV, DENV, and WNV, antibodies to the NS1 protein appear to be more virus specific, possibly due to unique electrostatic differences in the NS1 surface loop allowing for enhanced antibody specificity (22, 23, 24). While NS1 may indeed provide the needed specificity for ZIKV serologic tests, careful evaluation of the sensitivity of assays using this antigen in large, prospective study sets is warranted.

The CDC and InBios MAC-ELISAs performed comparably in both arms. One specimen with discordant results in the prospective set was considered particularly significant

TABLE 6 PRNT results for prospective specimens with nonnegative results by the CDC ZIKV MAC-ELISA[a]

Patient no.	Age/sex[e]	CDC Zika MAC-ELISA	InBios Zika MAC-ELISA	Euroimmun Zika IgM ELISA	PRNT result—evidence of infection with:	PRNT titer results	Zika rRT-PCR (source)
1	29/F	Presumptive positive	Presumptive positive	Presumptive positive	Zika virus	DENV 1, <10 DENV2, <10 ZIKV, >80	ND[g]
2	66/M	Presumptive positive	Possible positive	Negative	Dengue virus[b]	NA[b,f]	ND
3	29/M	Presumptive positive	Presumptive positive	Presumptive positive	Flavivirus	DENV1, >20 DENV2, 10 ZIKV, >1,280	Positive (urine)
4	69/M	Presumptive positive	Presumptive positive	Presumptive positive	Flavivirus	DENV1, >20 DENV2, 20 ZIKV, >1,280	ND
5	64/M	Presumptive positive	Presumptive positive	Negative	Flavivirus	DENV1, >1,280 DENV2, >1,280 ZIKV, >1,280	ND
6	55/M	Presumptive positive	Presumptive positive	Negative	Flavivirus	DENV1, >1,280 DENV2, >1,280 ZIKV, >1,280	Negative (serum)
7	1 day/F[d]	Presumptive positive	Presumptive positive	Negative	Flavivirus	DENV1, 10 DENV2, <10 ZIKV, >1,280	Negative (serum and urine)
8	28/F	Presumptive positive	Negative	Negative	Negative	DENV1, <10 DENV2, <10 ZIKV, <10	ND
9	72/F	Equivocal	Other flavivirus	Negative	Flavivirus	DENV1, >1,280 DENV2, ≥1280 ZIKV, >1280	Negative (serum and urine)
10	23/F	Inconclusive	Negative	Negative	Flavivirus	DENV1, >80 DENV2, 20 ZIKV, 20	ND
11	29/M	Inconclusive	Negative	Negative	Flavivirus	DENV1, 20 DENV2, 80 ZIKV, 10	ND
12	49/F	Inconclusive	Negative	Negative	Negative	DENV1, <10; DENV2, <10; ZIKV, <10	ND
13	61/F	Inconclusive	Negative	Negative	Negative		Negative (urine)
14	32/M	Inconclusive	Negative	Negative	Negative		ND
15	23/F	Inconclusive	Negative	Negative	Negative		ND
16	17/F[c]	Inconclusive	Negative	Negative	Negative		ND
17	25/F[c]	Inconclusive	Negative	Negative	Negative		Negative (urine)
18	31/F[c]	Inconclusive	Negative	Negative	Negative		ND
19	32/F[c]	Inconclusive	Negative	Negative	Negative		ND

[a]This includes samples with results of presumptive positive, equivocal, or inconclusive by the CDC Zika MAC-ELISA. A total of 19 specimens were used.
[b]Specimen was submitted to a CDC-designated public health laboratory for testing and only a qualitative result was reported.
[c]Patient was pregnant.
[d]Patient presented with severe microcephaly and arthrogryposis consistent with congenital ZIKV infection, born to a mother who immigrated from Guatemala and reported fever and maculopapular rash during first trimester of pregnancy.
[e]F, female; M, male.
[f]NA, not available.
[g]ND, not performed.

due to a result of presumptive positive by the CDC assay and negative results by the other two ELISAs and PRNT, suggesting a false-positive CDC MAC-ELISA result. Recently, the FDA alerted laboratorians and clinicians to a high rate of specimens reported as presumptive Zika positive by the InBios MAC-ELISA that were not confirmed by PRNT (25). Our observations show that out of seven presumptive or possible ZIKV-positive results by the InBios MAC-ELISA, only one was confirmed by PRNT for ZIKV, similar to the performance of the CDC MAC-ELISA. The remaining specimens only showed evidence of a flavivirus infection. Notably, one of the possible results by the InBios MAC-ELISA is "other flavivirus positive," which suggests an ability of this assay to distinguish antibodies to ZIKV from antibodies to other flaviviruses. Interestingly, however, the PRNT-confirmed DENV sample was reported as "possible Zika positive" by the InBios MAC-ELISA. Further studies assessing whether the InBios MAC-ELISA can provide enhanced specificity for antibodies to ZIKV are needed.

A high number of prospective specimens resulted as inconclusive (6.6%) by the CDC MAC-ELISA, indicating high background reactivity; this was not observed in the retrospective study set. Notably, testing of the prospective and retrospective specimens occurred at different laboratories. The difference in inconclusive rates may be explained, in part, by inherent interlaboratory variability associated with the CDC MAC-ELISA, as the assay requires site-specific optimization of the dilutions used for select reagents (e.g., ZIKV antigen, goat anti-human IgM, conjugated secondary antibody, etc.; see Materials and Methods). Similar to the results of the InBios MAC-ELISA, for the majority of specimens with reactive results by the CDC assay, PRNT could not distinguish whether the antiflavivirus antibodies present were specific to ZIKV or DENV. Prior studies have shown that while PRNT is highly specific in cases of primary flavivirus infection, secondary flavivirus infections often stimulate the original antigenic sin phenomenon, leading to significant antibody cross-reactivity between closely related flaviviruses. Because of this and cocirculation of ZIKV and DENV, even a $\geq$4-fold difference between ZIKV and DENV PRNT titers, which was observed in five of our patients, cannot be relied upon to accurately distinguish between flaviviruses (14, 19). Collectively, this underscores the ongoing challenges associated with serologic evaluation for ZIKV.

This study has several limitations. First, PRNT was only performed for reactive specimens screened by a ZIKV MAC-ELISA as part of routine clinical care in the prospective study set. Specimens in this arm that were reactive by only the InBios or Euroimmun ELISAs ($n = 4$) and any reactive retrospective samples were not eligible for evaluation by PRNT as testing was performed for study purposes, not clinical care, and availability of PRNT during the ZIKV outbreak was limited. This inherently biased our study toward the CDC MAC-ELISA and limited our ability to evaluate the accuracy and, more so, the specificity of the ZIKV IgM screening ELISAs to only 19 samples. Second, only two samples were confirmed by either PRNT or rRT-PCR for ZIKV in this study. While this is a significant limitation, this report is the first "real world" account of serologic testing for ZIKV at a commercial reference laboratory and presents that, among all specimens with reactivity by the ZIKV IgM screening ELISAs, there remains a high rate of ultimately ambiguous serologic results by PRNT. Though a result of "antibodies to flavivirus detected" by PRNT provides some information to clinicians, the lack of definitive viral identification remains problematic, particularly for pregnant women. Finally, detailed clinical presentation and exposure histories were not available for all patients in the prospective arm, thus limiting our ability to correlate results with clinical findings.

In conclusion, we show that among the three ZIKV IgM screening ELISAs, the CDC and InBios MAC-ELISAs performed comparably. In contrast, the Euroimmun ZIKV IgM ELISA appears to be less sensitive, though whether this is due to a higher level of specificity for antibodies to ZIKV versus truly a lack of sensitivity for this analyte requires additional follow up studies using well-characterized specimens. Finally, in our experience, for the majority of reactive samples by the CDC MAC-ELISA, definitive identification of the infecting virus (e.g., ZIKV versus DENV) was not possible. This highlights

the ongoing challenges associated with diagnosis of ZIKV infection and the importance of concurrent testing, when appropriate, using molecular methodologies. Overall, while the availability of commercial serologic tests for antibodies to ZIKV has expanded the national capacity for such testing beyond public health laboratories, the need for improved serologic methods for ZIKV detection, particularly with regard to assay specificity, remains.

MATERIALS AND METHODS

Study design. This study was divided into two arms. The first arm included 54 retrospective serum samples, of which 30 were collected in February 2016 from patients presenting with ZIKV-related symptoms (e.g., fever, maculopapular rash, conjunctivitis, arthralgia, etc.) to a hospital in Medellin, Colombia and 24 were purchased from Access Biologicals LLC (Vista, CA). The 24 Access Biologicals samples were also collected from Colombian patients presenting with ZIKV-related symptoms between December 2015 and February 2016. All retrospective specimens were accompanied by demographic and limited clinical data. These specimens were evaluated by the CDC MAC-ELISA at the Minnesota Department of Health ($n = 8$; MDH, St. Paul, MN) and the New York City Department of Health and Mental Hygiene ($n = 46$; NYC DOHMH, New York City, NY).

The second arm included 157 prospective serum specimens submitted to Mayo Medical Laboratories (MML, Rochester, MN) for ZIKV IgM serologic testing by the CDC MAC-ELISA between 1 September and 17 November 2016. MML transitioned to the InBios MAC-ELISA for clinical testing on 18 November 2016, and all specimens with reactive results by this assay received through 31 December 2016 ($n = 3$) were also included in the prospective arm for a total of 160 specimens. Following routine clinical testing, 151 specimens had sufficient volume remaining for evaluation by all three ZIKV IgM ELISAs and were included in the study. Responses to "ask at order entry" (AOE) questions regarding pregnancy status, ZIKV-related symptoms, and travel history were required at the time of order placement and answers (yes/no responses) were recorded. Patient charts were reviewed for ZIKV rRT-PCR results and clinical presentation as available.

All retrospective and prospective specimens were evaluated for the presence of IgM class antibodies to ZIKV using the CDC MAC-ELISA, the InBios MAC-ELISA, and the Euroimmun ELISA in a blinded manner. Due to the lack of a reference screening method, the CDC MAC-ELISA was used as the reference comparator method. Retrospective samples were stored at ≤ -70°C prior to testing and underwent one to two freeze-thaw cycles between testing by the various assays. Prospective samples were evaluated by all three ZIKV assays within 5 days of storage at 2 to 8°C. Supplemental PRNT was performed at the CDC or a CDC-designated laboratory as part of routine clinical care for samples with nonnegative screen results in the prospective arm only. A case of ZIKV infection was confirmed if PRNT and/or rRT-PCR were positive for ZIKV. All samples were also tested for IgM antibodies to DENV using the InBios DENV *Detect* IgM ELISA per the manufacturer's recommendations. This study was approved by the Mayo Clinic Institutional Review Board.

Serologic assay methods. Instructions for use of the CDC and InBios ZIKV MAC-ELISAs, which have received FDA EUA, were followed meticulously and can be found on the FDA EUA website for ZIKV devices (26, 27). Performance and interpretation of each assay will be briefly described here.

CDC Zika MAC-ELISA. This assay received FDA EUA on 26 February 2016. Reagents for the CDC MAC-ELISA were provided to MDH, NYC DOHMH, and MML by the CDC, and select assay components required site-specific titrations. Prior to implementation of the CDC MAC-ELISA for clinical use, all centers passed a mandatory ZIKV serology panel (provided by the CDC) using the site-specific assay parameters. Briefly, 96-well high-affinity microtiter plates (Immulon 2 HB) were coated with goat anti-human IgM antibody (KPL, Gaithersburg, MD) at site-specific dilutions (1:2,000 at MDH and MML and 1:3,000 at NYC DOHMH) and incubated at 4°C overnight. Negative control (negative serum samples) and patient serum samples were used at a 1:400 dilution at all sites. A positive flavivirus IgM control was used at dilutions of 1:3,200 at MDH, 1:4,500 at NYC DOHMH, and 1:1,000 at MML. Controls and patient serum samples were added to a block of 6 wells and incubated for 1 h at 37°C. ZIKV antigen, either the Zika Vero E6 tissue culture antigen (MDH and NYC DOHMH) or Zika COS-1 recombinant antigen (MML), was added in triplicate to each patient and control block and incubated overnight at 4°C. Normal Vero E6 or COS-1 control antigens were used at the respective dilutions. Following washing, site-specific dilutions of the 6B6C-1 monoclonal antibody (1:2,000 at MDH, 1:1,000 at NYC DOHMH, 1:4,000 at MML), a chimeric monoclonal antibody specific for *Flavivirus* conjugated to horseradish peroxidase (MDH and NYC DOHMH purchased from Hennessey Research Associates, Shawnee, KS; MML purchased from InBios, Inc.), were added to the plates, which were further processed and analyzed per manufacturer recommendations. Results of the CDC MAC-ELISA were determined by calculating the ratio of the mean optical density (OD) of the patient sample (P) divided by the mean OD of the negative control (N), each reacted with the ZIKV antigen. P/N ratios of <2 were considered negative. Specimens with P/N ratios of ≥ 2 that showed background reactivity, evaluated by comparison of patient's OD value reacted with ZIKV antigen versus control antigen, were reported as inconclusive. Specimens with acceptable background reactivity levels and P/N ratios of ≥ 3 or between 2 and <3 were reported as presumptive positive or equivocal, respectively.

InBios ZIKV *Detect* IgM capture ELISA. This assay received FDA EUA on 17 August 2016. The InBios MAC-ELISA was performed and results interpreted per manufacturer recommendation, without deviation; only the manufacturer recommended interpretive criteria are presented here. An immune status ratio (ISR) was determined by dividing the OD of the patient sample reacted with the ZIKV recombinant

envelop (E) glycoprotein by the OD of the patient sample reacted with a cross-reactive control antigen (CCA; not further defined). ISR values of ≤1.60 are considered negative while values of ≥1.80 are presumptive positive for IgM antibodies to ZIKV. Specimens with ISR values between 1.61 and 1.79 were retested, and ISR results of ≥1.70 following repeat were considered presumptive positive. Specimens with ISR values of <1.70 on repeat testing were further analyzed, including determination of the ratio of the patient sample reacted with ZIKV Ag or CCA divided by a normal control Ag (NCA; not further defined). Samples with ZIKV Ag/NCA ratio values of ≥1.70, regardless of the CCA/NCA value, were interpreted as possible ZIKV positive. Specimens with a ZIKV Ag/NCA ratio of <1.70 and CCA/NCA ratio of ≥1.70 or <1.70 were interpreted as other flavivirus positive or negative, respectively.

Euroimmun anti-Zika virus IgM ELISA. The Euroimmun ZIKV IgM assay is an indirect, colorimetric ELISA for detection of IgM antibodies to ZIKV. Microtiter wells, precoated with recombinant ZIKV nonstructural protein 1 (NS1), were incubated with patient serum in singlet for 1 h at 37°C. Following a wash step, peroxidase-conjugated goat anti-human IgM is added and incubated for 30 min at room temperature (RT). Wells were washed and incubated with 3,3′,5,5′-tetramethylbenzidine and hydrogen peroxide in the dark for 15 min at RT, followed by addition of sulfuric acid stop solution. The OD of each well was measured by spectrophotometry at 450 nm with a 620-nm reference wavelength. Ratios were determined by dividing the OD of the patient's sample by the OD of the assay calibrator. Ratio values of <0.8, ≥0.8 to <1.1, and ≥1.1 were interpreted as presumptive negative, borderline, and presumptive positive, respectively, for the presence of IgM antibodies to ZIKV.

InBios DENV *Detect* IgM capture ELISA. The InBios DENV IgM ELISA is FDA cleared and was used according to the manufacturer's recommendations. This is a qualitative, sandwich-format colorimetric ELISA for detection of IgM class antibodies to DENV recombinant antigens (DENRA; not further described by the manufacturer). Patient samples are incubated with DENRA and normal control antigen (NCA), and an ISR is determined by dividing the OD from the DENRA well by the OD from the NCA well. Per manufacturer recommendations, ISR values of ≤1.65, >1.65 to <2.84, and ≥2.84 were considered negative, borderline, and positive, respectively, for the presence of IgM class antibodies to DENV.

Statistics. GraphPad software was used to calculate percent positive, negative, and overall agreement (GraphPad, La Jolla, CA, USA). This software was also used to calculate kappa values and 95% confidence intervals (CIs). Kappa values of >0.75, 0.40 to 0.75, and <0.40 were considered indicative of excellent, fair, and poor interrater agreement, respectively (28).

ACKNOWLEDGMENTS

We thank Luis Jaime Gomez, Jorge Mosquera, and Alejandro Mosquera Molina for their assistance with this study.

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