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Evaluation of a Rapid Immunochromatographic Assay and Two Enzyme-Linked Immunosorbent Assays for Detection of IgM-Class Antibodies to Zika Virus

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ABSTRACT There are currently five serologic assays available for detection of anti-Zika virus (ZIKV) IgM-class antibodies with U.S. Food and Drug Administration emergency use authorization. Among these are the Chembio DPP Zika IgM system (DPP Zika ICA; Chembio, Medford, NY), a rapid immunochromatographic assay (ICA), and the InBios ZIKV Detect 2.0 IgM antibody capture enzyme-linked immunosorbent assay (ZIKV 2.0 MAC-ELISA; InBios international, Inc., Seattle, WA), which has replaced the original InBios ZIKV Detect MAC-ELISA. We evaluated performance of these three serologic assays using 72 specimens characterized by plaque reduction neutralization testing (PRNT) for the presence or absence of neutralizing antibodies (NAbs) to ZIKV, dengue virus (DENV), and West Nile virus (WNV). The InBios ZIKV 2.0 MAC-ELISA was "presumptive Zika positive" in all 15 PRNT-confirmed ZIKV samples, while the Chembio DPP Zika ICA was nonreactive in three (20%) and the InBios ZIKV MAC-ELISA was negative in four (27%). The Chembio DPP Zika ICA and InBios ZIKV 2.0 MAC-ELISA showed >95% specificity in 22 ZIKV/DENV-seronegative specimens and in 13 samples positive for NAbs to non-ZIKV flaviviruses. Comparatively, the InBios ZIKV MAC-ELISA was "presumptive" or "possible Zika positive" in 8 of 12 WNV or DENV PRNT-positive samples and in 12 of 22 PRNT-seronegative sera. Our findings suggest that replacement of the InBios ZIKV MAC-ELISA with the InBios ZIKV 2.0 MAC-ELISA will lead to fewer samples requiring PRNT, minimizing unnecessary anxiety among patients ultimately determined to be seronegative for ZIKV and DENV by PRNT and alleviating some of the testing burden on laboratories performing PRNT.

KEYWORDS IgM antibodies, immunochromatographic assay, MAC-ELISA, Zika virus

During the 2016 Zika virus (ZIKV) outbreak, over 5,000 cases of symptomatic ZIKV infections were reported to the Centers for Disease Control and Prevention (CDC) in the United States, with the majority occurring in travelers returning from areas where ZIKV is endemic (1). Since then, the U.S. case counts have dropped dramatically, with 108 ZIKV disease cases reported to the CDC as of August 2018. The risk of both autochthonous and travel-associated infection with ZIKV remains, however, due to ongoing circulation of the virus throughout the majority of Latin America, much of central Africa, the Indian subcontinent, Southeast Asia, Indonesia, and the Philippines. ZIKV, a member of the *Flavivirus* genus, which includes dengue virus (DENV) and West Nile virus (WNV) among others, is most frequently transmitted through the bite of infected *Aedes* species mosquitos and is the first arbovirus effectively transmitted through sexual contact and vertically from mother to fetus (2–4). Although the majority of ZIKV infections are subclinical, symptomatic patients frequently develop fever alongside a maculopapular rash, arthralgia, and/or conjunctivitis. ZIKV sequelae are most severe in cases of congenital disease, where infection has been associated with the

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development of significant physical abnormalities and neurologic deficits, in some cases leading to fetal demise (4–6).

Currently, the CDC recommends diagnostic testing for ZIKV in patients meeting both clinical and epidemiologic criteria. This includes any pregnant woman (symptomatic or asymptomatic) or symptomatic individual with possible or ongoing exposure to ZIKV through either travel or unprotected sexual contact with a traveler returning from or resident of a ZIKV region of endemicity (7). While different algorithmic testing approaches are recommended depending on the aforementioned patient variables (e.g., pregnancy status, exposure history, presence/absence of symptoms, duration of symptoms, etc.), the routinely available diagnostic tests for ZIKV include reverse transcriptase real-time PCR (rRT-PCR) and serologic tests for detection of IgM-class antibodies to the virus (8). Due to the nature of the outbreak and the significant sequelae associated with ZIKV infection, the U.S. Food and Drug Association (FDA) requires that all commercially available ZIKV assays receive FDA emergency use authorization (EUA) prior to being released for clinical use; currently, 14 rRT-PCR and five anti-ZIKV IgM serologic assays have FDA EUA (9). Among the serologic assays, (i) two are colorimetric IgM antibody capture enzyme-linked immunosorbent assays (MAC-ELISAs) based on detection of IgM to ZIKV envelope proteins (i.e., the CDC Zika MAC-ELISA and the InBios ZIKV Detect MAC-ELISA [InBios International, Inc., Seattle, WA]), (ii) two are chemiluminescent, microparticle IgM capture assays using the ZIKV nonstructural protein 1 (NS1; i.e., the Liaison XL Zika Capture IgM assay [DiaSorin, Inc., Stillwater, MN] and the ADVIA Centaur Zika test [Siemens Healthcare Diagnostics, Inc., Tarrytown, NY]), and (iii) one is an immunochromatographic assay (ICA), also targeting IgM-class antibodies to the ZIKV NS1 protein (DPP Zika IgM system [Chembio Diagnostic Systems, Inc., Medford, NY]). In May 2018, InBios received FDA EUA for the ZIKV Detect 2.0 MAC-ELISA (InBios ZIKV 2.0 MAC-ELISA) and discontinued their prior version (InBios ZIKV MAC-ELISA). Importantly, samples reactive by either of these commercial assays require confirmatory testing by a plaque reduction neutralization test (PRNT), unless the patient is also positive by rRT-PCR for ZIKV (8). PRNT is performed concurrently for ZIKV and DENV due to cocirculation of these antigenically similar viruses and is available through either the CDC or select public health laboratories (10).

The Chembio DDP Zika IgM system (Chembio DPP Zika ICA) is unique among the other serologic assays since it is a rapid (~20 min), immunochromatographic test that can be performed on multiple sources (i.e., serum, plasma, and venous or fingerstick whole blood) and uses a portable DPP Micro Reader to determine the presence or absence of test and control bands on the reagent strip. Here, we evaluated performance of the Chembio DPP Zika ICA and the new InBios ZIKV 2.0 MAC-ELISA for the detection of anti-ZIKV IgM-class antibodies in serum or plasma specimens characterized by PRNT for the presence or absence of neutralizing antibodies (NAbs) to ZIKV, DENV, and WNV.

MATERIALS AND METHODS

Study samples. A total of 72 samples were included in this evaluation, 63 of which were archived, including clinical residual serum specimens with PRNT results indicative of the presence or absence of NAbs to ZIKV, DENV, WNV, St. Louis encephalitis virus (SLEV), Jamestown Canyon virus (JCV), and/or Powassan virus (POWV). All samples were stored at −70°C prior to testing in a blinded fashion. These 63 sera were collected between September 2016 and September 2018 and can be divided into two groups: (i) those originally submitted for anti-ZIKV IgM ($n = 53$) testing to Mayo Clinic Laboratories (MCL; Rochester, MN) and (ii) those submitted to MCL for other, non-ZIKV arbovirus serologic testing ($n = 10$) (see Fig. S1 in the supplemental material). Patient samples submitted for ZIKV serologic testing were required to meet current CDC testing criteria, which was determined based on provider responses (yes or no) to "ask at order entry" questions regarding pregnancy status, travel history, and the presence of ZIKV-related symptoms as outlined previously (8). Detailed clinical information (e.g., duration of symptoms, route of exposure, etc.) was not available due to receipt of these samples through our MCL reference laboratory practice. Sera from patients meeting these criteria were screened in our laboratory by either the CDC Zika MAC-ELISA ($n = 11$; used September to November 2016), the InBios ZIKV MAC-ELISA ($n = 49$; used November 2016 to July 2018), or the InBios ZIKV 2.0 MAC-ELISA ($n = 3$; used July 2018 to the present). Multiple anti-ZIKV IgM ELISAs were used during this period due to discontinuation of the commercial production of reagents for the CDC Zika MAC-ELISA in November 2016 and discontinuation of the InBios ZIKV MAC-ELISA in mid-2018. Per CDC ZIKV testing guidelines, reactive

samples by any anti-ZIKV IgM screening assay require additional, supplemental testing by PRNT for ZIKV and DENV antibodies. Among the 53 anti-ZIKV IgM screen reactive samples, PRNT showed evidence of infection with ZIKV in six specimens and evidence of DENV infection in three samples, and 22 sera showed no evidence of infection with either virus. NAbs to both ZIKV and DENV were present in 22 samples, resulting in a PRNT interpretation of "evidence of infection with a flavivirus, specific virus unable to be identified." To supplement the number of ZIKV PRNT positive samples from our laboratory, the AccuSet Zika performance panel (plasma, $n = 9$; catalogue no. 0845 to 0142, batch 10245884) collected from patients between February and August 2016 was purchased from SeraCare (Milford, MA).

The second group of samples included residual sera ($n = 10$) collected between September 2016 and October 2017 from Minnesota patients, which were submitted to our laboratory for non-ZIKV arbovirus serologic testing as part of routine clinical care. In adherence to the original provider test request, samples were tested for IgM- and IgG-class antibodies to WNV using the WNV IgM Capture and WNV IgG DxSelect test kits (Quest Diagnostics, Cypress, CA) and/or for IgM- and IgG-class antibodies to SLEV, California encephalitis virus (CEV), Eastern equine encephalitis virus (EEEV), and Western equine encephalitis virus (WEEV) using the arbovirus IgM and IgG immunofluorescence test kits (Quest Diagnostics). Per Minnesota state requirements, anti-WNV IgM reactive samples and those positive for IgM and/or IgG to the other arboviruses were submitted for additional testing to the Minnesota Department of Health, which forwards samples as necessary to the CDC for supplemental arbovirus PRNT; the results were returned to our laboratory (Fig. S1). Briefly, five positive and two equivocal anti-WNV IgM samples and two anti-SLEV IgG (titers 1:320 to 1:1,280)-positive samples from our laboratory were evaluated at the CDC by PRNT for WNV, SLEV, and/or POWV, which confirmed infection with WNV in eight cases and with POWV in one sample. One additional sample, originally positive in our laboratory for IgG antibodies to SLEV (titer 1:640) and CEV (titer 1:80), was tested by PRNT for WNV, JCV, EEEV, and La Crosse virus and showed evidence of infection with both WNV (PRNT titer 1:2,560) and JCV (PRNT titer >1:2,560).

In total, 72 PRNT-characterized samples were included in this evaluation and were tested by the Chembio DPP Zika ICA, the InBios ZIKV MAC-ELISA, and the InBios ZIKV 2.0 MAC-ELISA, with results compared to each other and to PRNT (Fig. S1). This study was approved by the Mayo Clinic Institutional Review Board.

Chembio DPP Zika IgM system. The DPP Zika IgM assay system (Chembio Diagnostic Systems, Inc., Medford, NY) is a single-use ICA, which received FDA EUA in September 2017 for use on serum, plasma, finger stick whole-blood, and venous whole-blood samples. A detailed description of the format of this assay has been provided previously (9). Serum and plasma samples were tested in this study according to the manufacturer's instructions for use. The Chembio DPP Zika ICA utilizes a dual path platform (DPP) technology. The device is designed in a cartridge format, which has two perpendicular membrane pathways for separate delivery of specimen and detection conjugate to a reagent strip containing immobilized ZIKV NS1 antigen (test band) and protein A (control band). Briefly, 10 μl of diluted patient sera or plasma is added to well 1 on the reagent membrane and allowed to migrate via capillary flow during a 5-min incubation at room temperature. If present in the patient sample, anti-ZIKV IgM antibodies will bind to the ZIKV NS1 antigens on the test band, while nonspecific antibodies will bind to the protein A control band. Next, five drops of sample buffer are added to well 2 on the cartridge, hydrating the antibody-binding, colored conjugate, which will migrate and bind to any bound anti-ZIKV IgM antibodies and nonspecific antibodies on the test and control bands, respectively, during a 10- to 15-min room temperature incubation. The presence or absence of test and control antibody bands is determined using a portable, battery- or USB-powered Chembio DPP Micro Reader, which utilizes assay-specific cutoff values to report qualitative results of reactive ($\geq$20 index), nonreactive ($<$20 index), or invalid. Reactive and nonreactive results are interpreted as "presumptive Zika IgM positive" and "negative", respectively.

InBios ZIKV Detect IgM Capture ELISAs. The InBios ZIKV Detect IgM Capture ELISA (InBios MAC-ELISA) was granted FDA EUA in August 2017 and was performed in our laboratory in accordance with manufacturer instructions and as described previously (9, 11). InBios discontinued this assay after acquiring FDA EUA in May 2018 for a new version of this test, the InBios ZIKV Detect 2.0 IgM Capture ELISA (InBios ZIKV 2.0 MAC-ELISA) (12). The InBios ZIKV 2.0 MAC-ELISA was performed according to the manufacturer's instructions and is similar to the InBios ZIKV MAC-ELISA, including use of the same ZIKV envelope glycoproteins for the target antigens. There are a number of key differences between the InBios ZIKV and the InBios ZIKV 2.0 MAC-ELISAs. First, detection of the captured anti-ZIKV IgM antibodies was changed from a one-step to a two-step process for the InBios ZIKV 2.0 assay. The InBios ZIKV MAC-ELISA detects captured anti-ZIKV IgM antibodies during a single 60-min incubation with horseradish peroxidase (HRP)-conjugated monoclonal anti-flavivirus antigen antibodies, whereas this step has been split into two 30-min incubations for the InBios ZIKV 2.0 MAC-ELISA: the first with ready-to-use reagent containing mouse antibodies against flavivirus antigens and the second with HRP-conjugated anti-mouse antibody. The second change includes extension of the incubation time for the 3,3′,5,5′-tetramethylbenzidine (TMB) chromogenic substrate from 10 min in the InBios ZIKV MAC-ELISA to 20 min for the InBios ZIKV 2.0 MAC-ELISA. Finally, several changes to the interpretive guide were made for the InBios ZIKV 2.0 MAC-ELISA, including the addition of an initial optical density (OD) threshold analysis, which was not present in the InBios ZIKV MAC-ELISA. For this threshold analysis, the OD value from the patient sample incubated with ZIKV antigen is compared to a calculated threshold value (i.e., the average OD value of the negative control plus 0.130). If the patient sample OD value is greater than or equal to the threshold value, a ZIKV immune status ratio (ISR) is calculated. The ISR is the OD value from the patient sample incubated with ZIKV antigen divided by the cross-reactive control antigen (CCA) OD value. Patient ISR values of greater than or equal to 1.70 are interpreted as "presumptive Zika positive," while values of

<1.70 are further evaluated by comparison of the CCA OD to the normal cell antigen (NCA) OD (CCA/NCA) ratio of the patient sample. CCA/NCA ratios of ≥5.00 or <5.00 are interpreted as "presumptive other flavivirus positive (non-Zika)" or "negative", respectively. Initial ZIKV ISR results of 1.50 to 1.90 are retested in duplicate, and the mean ISR value is evaluated using a 1.70 cutoff value. If the original patient OD value is less than the threshold value, only the CCA/NCA ratio is determined, and results are interpreted as described above. This interpretive scheme is notably different than that for the InBios ZIKV MAC-ELISA, which does not include a threshold comparator value, has an ISR retest range of 1.60 to 1.80, utilizes a CCA/NCA ratio cutoff ≥1.70, and includes a ZIKV antigen/NCA ratio analysis for the additional interpretive category of "possible Zika positive."

RESULTS

Patient demographics and summary of PRNT results. In total, 63 patient serum samples received through MCL were included in this evaluation (Fig. S1). Among the 53 samples submitted for anti-ZIKV IgM testing, all but one patient (a pregnant female) reported travel to a ZIKV region of endemicity (specific locations not provided). A total of 32 (60%) samples were from pregnant females (age range, 17 to 43 years), while 11 (21%) and 9 (17%) were from symptomatic males (age range, 29 to 69 years) and nonpregnant, symptomatic females (age range, 21 to 78 years), respectively. One sample was collected from a 1-day-old infant born to a symptomatic mother with travel to an area where ZIKV is endemic. All samples were reactive in our laboratory by the anti-ZIKV IgM MAC-ELISA used at the time, including either the CDC-ZIKV MAC-ELISA ($n = 11$), the InBios ZIKV MAC-ELISA ($n = 49$), or the InBios ZIKV 2.0 MAC-ELISA ($n = 3$). Subsequent PRNT for NAbs to ZIKV and DENV on each of these screen-reactive samples showed evidence of ZIKV infection in six patients, DENV infection in three patients, and no evidence of infection with either virus in 22 cases. The remaining 22 sera had evidence of NAbs to both ZIKV and DENV, resulting in an interpretation of "evidence of infection with a flavivirus, specific virus unable to be identified." The additional nine plasma samples positive by PRNT for NAbs to ZIKV were purchased from SeraCare. These samples were from two male and seven female patients ranging in age from 23 to 49 years; pregnancy status, travel history, and clinical symptoms were not available for these samples. Finally, an additional 10 samples included in this evaluation were confirmed by PRNT for evidence of infection with WNV in nine patients, one of whom had a coinfection with JCV, and evidence of POWV infection in one sample. These 10 individuals ranged in age from 42 to 51 years, and the majority were male ($n = 6$). Disease duration, symptoms, and exposure history were not available for these patients.

Performance of the Chembio DPP Zika ICA, the InBios ZIKV MAC-ELISA, and the InBios ZIKV 2.0 MAC-ELISA in PRNT-characterized samples. Among the 15 samples with evidence of ZIKV infection by PRNT, the InBios ZIKV 2.0 MAC-ELISA yielded results of "presumptive Zika positive" in all 15 (100%) (Table 1). The Chembio DPP Zika ICA and InBios ZIKV MAC-ELISA were reactive or "presumptive ZIKV positive" in 12 (80%) and 11 (73.3%) samples, respectively (Table 1). Both of these assays were nonreactive in three ZIKV PRNT-positive sera, with ZIKV titers ranging from >1:80 to >1:1,280 (DENV PRNT titers <1:10) (Table 2). In addition, the InBios ZIKV MAC-ELISA was negative in one of the SeraCare ZIKV positive plasma samples, which had a ZIKV PRNT titer of ≥1:320 (Table 2).

Of the 13 samples determined to be positive by PRNT for evidence of infection with DENV ($n = 3$), WNV ($n = 9$), or POWV ($n = 1$) and for the 22 samples without evidence of ZIKV or DENV infection, the Chembio DPP Zika ICA was negative in all (100%) cases. In comparison, the InBios ZIKV 2.0 MAC-ELISA yielded a result of "other flavivirus positive" in eight (61.5%) of the 13 samples positive for NAbs to WNV, DENV, or POWV and was negative in the remaining five samples, whereas the InBios ZIKV MAC-ELISA resulted as "presumptive Zika positive" for a PRNT confirmed DENV positive sample, "possible Zika positive" for seven of the sera, and "other flavivirus positive" in four specimens (Tables 1 and 2). Among the 22 samples negative for NAbs to ZIKV and DENV by PRNT, the InBios ZIKV 2.0 MAC-ELISA result was "presumptive Zika positive" in one (4.5%) sample, while the InBios ZIKV MAC-ELISA was nonnegative in 13 (59%) (Tables 1 and 2).

TABLE 1 Summary of the performance characteristics for the Chembio DPP Zika IgM ICA, the InBios ZIKV Detect MAC-ELISA, and the InBios ZIKV Detect 2.0 MAC-ELISA in 72 arbovirus PRNT-characterized specimens[a]

Test	Finding	PRNT result interpretation, no. of samples (%)			
		Evidence of infection with:			
		ZIKV ($n = 15$)	WNV, DENV, or POWV ($n = 13$)[b]	Flavivirus, unspecified (ZIKV and/or DENV) ($n = 22$)[c]	No evidence of infection with ZIKV or DENV ($n = 22$)
InBios ZIKV MAC-ELISA	Presumptive ZIKV positive	11 (73.3)	1 (7.7)	15 (68.2)	5 (22.7)
	Possible ZIKV positive	0 (0)	7 (53.8)	4 (18.2)	7 (31.8)
	Other flavivirus positive	0 (0)	4 (30.8)	1 (4.5)	1 (4.5)
	Negative	4 (26.7)	1 (7.7)	2 (9.1)	9 (40.9)
InBios ZIKV 2.0 MAC-ELISA	Presumptive ZIKV positive	15 (100)	0 (0)	13 (59.1)	1 (4.5)
	Other flavivirus positive	0 (0)	8 (61.5)	2 (9.1)	0 (0)
	Negative	0 (0)	5 (38.5)	7 (31.8)	21 (95.5)
Chembio DPP Zika ICA	Reactive	12 (80.0)	0 (0)	9 (40.9)	0 (0)
	Nonreactive	3 (20.0)	13 (100)	13 (59.1)	22 (100)

[a]Abbreviations: ICA, immunochromatographic assay; WNV, West Nile virus; DENV, dengue virus; POWV, Powassan virus; ZIKV, Zika virus; MAC-ELISA, IgM antibody capture ELISA.
[b]PRNT showed evidence of neutralizing antibodies to WNV ($n = 9$), DENV ($n = 3$), or POWV ($n = 1$).
[c]PRNT unable to differentiate between infection with ZIKV and/or DENV.

Twenty-two samples had NAbs to both ZIKV and DENV by PRNT and were classified as "evidence of infection with a flavivirus, specific virus unable to be identified." Among these, the Chembio DPP Zika ICA, the InBios ZIKV 2.0 MAC-ELISA, and the InBios ZIKV MAC-ELISA results were "presumptive Zika positive" in 9 (40.9%), 13 (59.1%), and 15 (68.2%) samples, respectively. The InBios ZIKV 2.0 and InBios ZIKV MAC-ELISAs yielded results of "other flavivirus positive" in 2 and 1 of the 22 samples, respectively, and the InBios ZIKV MAC-ELISA had an additional four samples reported as "possible Zika positive" (Tables 1 and 2). ZIKV rRT-PCR (RealStar Zika Virus RT-PCR; Altona Diagnostics, Hamburg, Germany) was concurrently ordered from urine samples from four patients and from either serum only or serum and urine samples for two patients (Table 2). One urine sample was determined to be positive by the ZIKV rRT-PCR, for which all three serologic assays were also positive (Table 2, specimen 24). For the remaining five ZIKV rRT-PCR negative patients, the InBios ZIKV MAC-ELISA, the InBios ZIKV 2.0 MAC-ELISA, and the Chembio DPP Zika ICA yielded "presumptive Zika positive" results in four, three, and two of the samples, respectively.

DISCUSSION

We present the first independent evaluation of the Chembio DPP Zika ICA and the new InBios ZIKV 2.0 MAC-ELISA in comparison to results from the original InBios ZIKV MAC-ELISA (now discontinued) and to PRNT, considered the reference method for detection of antibodies to arboviruses. Our findings suggest that the InBios ZIKV 2.0 MAC-ELISA has enhanced sensitivity for detection of IgM antibodies to ZIKV compared to the Chembio DPP ICA and the InBios ZIKV MAC-ELISA, which were negative in 3 and 4 of 15 PRNT-confirmed ZIKV-positive samples, respectively. In addition, our data indicate that the InBios ZIKV 2.0 MAC-ELISA and Chembio DPP Zika ICA have improved specificity over the InBios ZIKV MAC-ELISA. This was particularly apparent among 13 samples with PRNT evidence of infection with either DENV, WNV, or POWV, for which the InBios ZIKV 2.0 MAC-ELISA and the Chembio DPP Zika ICA were either nonreactive or "other flavivirus positive," and among the 22 specimens determined by PRNT to be negative for NAbs to ZIKV and DENV, where only one sample resulted as "presumptive Zika positive" by the InBios ZIKV 2.0 MAC-ELISA. In comparison, the InBios ZIKV MAC-ELISA resulted as "presumptive" or "possible Zika positive" in 8 (66.7%) of the 12 WNV or DENV PRNT-positive samples and in 12 (54.5%) of the 22 sera determined to be negative for NAbs to ZIKV and DENV by PRNT.

This study included 22 samples for which PRNT detected NAbs to both ZIKV and

TABLE 2 Comparison of the Chembio DPP ZIKV IgM ICA, the InBios ZIKV 2.0 MAC-ELISA, and the InBios ZIKV MAC-ELISA using 72 arbovirus PRNT-characterized specimens[a]

PRNT result interpretation	Specimen no.	PRNT titer[b]						InBios ZIKV MAC-ELISA[c]	InBios ZIKV 2.0 MAC-ELISA[d]	Chembio DPP Zika ICA
		ZIKV	DENV1	DENV2	WNV	SLEV	POWV			
Evidence of infection with ZIKV ($n = 15$)	1	>1:1,280	<1:10	<1:10	NT	NT	NT	Positive	Positive	Reactive
	2	1:1,280	<1:10	<1:10	NT	NT	NT	Positive	Positive	Reactive
	3	>1:80	<1:10	<1:10	NT	NT	NT	Positive	Positive	Reactive
	4	>1:80	<1:10	<1:10	NT	NT	NT	Negative	Positive	Negative
	5	>1:80	<1:10	<1:10	NT	NT	NT	Negative	Positive	Negative
	6	>1:80	<1:10	<1:10	NT	NT	NT	Negative	Positive	Negative
	7[e]	≥1:320	NA	NA	NT	NT	NT	Positive	Positive	Reactive
	8[e]	≥1:1,280	NA	NA	NT	NT	NT	Positive	Positive	Reactive
	9[e]	≥1:320	NA	NA	NT	NT	NT	Positive	Positive	Reactive
	10[e]	≥1:40	NA	NA	NT	NT	NT	Positive	Positive	Reactive
	11[e]	≥1:320	NA	NA	NT	NT	NT	Positive	Positive	Reactive
	12[e]	≥1:80	NA	NA	NT	NT	NT	Positive	Positive	Reactive
	13[e]	≥1:320	NA	NA	NT	NT	NT	Positive	Positive	Reactive
	14[e]	≥1:320	NA	NA	NT	NT	NT	Positive	Positive	Reactive
	15[e]	≥1:320	NA	NA	NT	NT	NT	Negative	Positive	Reactive
Evidence of infection with DENV ($n = 3$)	16	<1:10	1:320	1:10	NT	NT	NT	Possible	Negative	Nonreactive
	17	<1:10	1:10	<1:10	NT	NT	NT	Positive	Negative	Nonreactive
	18[f]	NA	NA	NA	NT	NT	NT	Possible	Other	Nonreactive
Evidence of infection with a *Flavivirus*, specific virus unable to be identified ($n = 22$)	19[g]	>1:1,280	>1:1,280	>1:1,280	NT	NT	NT	Positive	Negative	Nonreactive
	20	>1:1,280	>1:1,280	>1:1,280	NT	NT	NT	Positive	Positive	Reactive
	21[g]	>1:1,280	>1:1,280	>1:1280	NT	NT	NT	Positive	Positive	Reactive
	22[h]	>1:1,280	>1:1,280	≥1,280	NT	NT	NT	Other	Other	Reactive
	23	>1:1,280	>1:80	>1:80	NT	NT	NT	Positive	Positive	Reactive
	24	>1:1,280	>1:80	>1:20	NT	NT	NT	Positive	Positive	Nonreactive
	25	>1:1,280	>1:80	>1:20	NT	NT	NT	Positive	Positive	Reactive
	26	>1:1,280	>1:20	1:20	NT	NT	NT	Positive	Positive	Reactive
	27[i]	>1:1,280	>1:20	1:10	NT	NT	NT	Positive	Positive	Reactive
	28[j]	>1:1,280	1:10	<1:10	NT	NT	NT	Positive	Positive	Nonreactive
	29	1:640	1:1,280	1:160	NT	NT	NT	Positive	Positive	Nonreactive
	30	>1:320	>1:320	>1:320	NT	NT	NT	Positive	Negative	Nonreactive
	31	>1:80	>1:1,280	1:1,280	NT	NT	NT	Possible	Negative	Nonreactive
	32	>1:1,280	>1:80	>1:80	NT	NT	NT	Positive	Positive	Reactive
	33	>1:80	>1:80	>1:80	NT	NT	NT	Possible	Negative	Nonreactive
	34	>1:80	>1:80	>1:20	NT	NT	NT	Possible	Negative	Nonreactive
	35	>1:80	>1:20	<1:10	NT	NT	NT	Positive	Positive	Nonreactive
	36[g]	1:320	1:40	<1:10	NT	NT	NT	Positive	Positive	Nonreactive
	37	>1:20	>1:1,280	>1:1,280	NT	NT	NT	Positive	Other	Reactive
	38	>1:1,280	>1:20	<1:10	NT	NT	NT	Possible	Positive	Nonreactive
	39[k]	1:20	>1:80	1:20	NT	NT	NT	Negative	Negative	Nonreactive
	40[k]	1:10	1:20	1:80	NT	NT	NT	Negative	Negative	Nonreactive
No evidence of infection with ZIKV or DENV ($n = 22$)	41[k]	<1:10	<1:10	<1:10	NT	NT	NT	Negative	Negative	Nonreactive
	42[k]	<1:10	<1:10	<1:10	NT	NT	NT	Negative	Negative	Nonreactive
	43[k]	<1:10	<1:10	<1:10	NT	NT	NT	Negative	Negative	Nonreactive
	44[k]	<1:10	<1:10	<1:10	NT	NT	NT	Negative	Negative	Nonreactive
	45[k]	<1:10	<1:10	<1:10	NT	NT	NT	Negative	Positive	Nonreactive
	46[k]	<1:10	<1:10	<1:10	NT	NT	NT	Negative	Negative	Nonreactive
	47[k]	<1:10	<1:10	<1:10	NT	NT	NT	Negative	Negative	Nonreactive
	48	<1:10	<1:10	<1:10	NT	NT	NT	Possible	Negative	Nonreactive
	49	<1:10	<1:10	<1:10	NT	NT	NT	Other	Negative	Nonreactive
	50	<1:10	<1:10	<1:10	NT	NT	NT	Possible	Negative	Nonreactive
	51[k]	<1:10	<1:10	<1:10	NT	NT	NT	Negative	Negative	Nonreactive
	52[k]	<1:10	<1:10	<1:10	NT	NT	NT	Negative	Negative	Nonreactive
	53	<1:10	<1:10	<1:10	NT	NT	NT	Positive	Negative	Nonreactive
	54	<1:10	<1:10	<1:10	NT	NT	NT	Positive	Negative	Nonreactive
	55	<1:10	<1:10	<1:10	NT	NT	NT	Possible	Negative	Nonreactive
	56	<1:10	<1:10	<1:10	NT	NT	NT	Possible	Negative	Nonreactive

(Continued on next page)

TABLE 2 (Continued)

PRNT result interpretation	Specimen no.	PRNT titer[b]						InBios ZIKV MAC-ELISA[c]	InBios ZIKV 2.0 MAC-ELISA[d]	Chembio DPP Zika ICA
		ZIKV	DENV1	DENV2	WNV	SLEV	POWV			
	57	<1:10	<1:10	<1:10	NT	NT	NT	Possible	Negative	Nonreactive
	58	<1:10	<1:10	<1:10	NT	NT	NT	Positive	Negative	Nonreactive
	59	<1:10	<1:10	<1:10	NT	NT	NT	Positive	Negative	Nonreactive
	60	<1:10	<1:10	<1:10	NT	NT	NT	Possible	Negative	Nonreactive
	61	<1:10	<1:10	<1:10	NT	NT	NT	Positive	Negative	Nonreactive
	62	<1:10	<1:10	<1:10	NT	NT	NT	Possible	Negative	Nonreactive
Evidence of infection with WNV ($n = 9$)	63[l]	NT	NT	NT	1:2,560	1:80	NT	Possible	Other	Nonreactive
	64	NT	NT	NT	1:1,280	<1:10	NT	Other	Other	Nonreactive
	65	NT	NT	NT	1:640	1:40	<1:10	Possible	Other	Nonreactive
	66	NT	NT	NT	1:640	<1:10	<1:10	Possible	Other	Nonreactive
	67	NT	NT	NT	1:640	1:20	NT	Other	Negative	Nonreactive
	68	NT	NT	NT	1:320	1:10	NT	Possible	Other	Nonreactive
	69	NT	NT	NT	1:320	<1:10	NT	Possible	Other	Nonreactive
	70	NT	NT	NT	1:80	1:20	NT	Other	Other	Nonreactive
	71	NT	NT	NT	1:80	<1:10	NT	Other	Negative	Nonreactive
Evidence of infection with POWV ($n = 1$)	72	NT	NT	NT	<1:10	<1:10	1:80	Negative	Negative	Nonreactive

[a]Abbreviations: NT, not tested; ICA, immunochromatographic assay; NA, data not available; DENV, dengue virus; DENV1, dengue virus serotype 1; DENV2, dengue virus serotype 2; POWV, Powassan virus; WNV, West Nile virus; SLEV, St. Louis encephalitis virus.
[b]PRNT titers of <1:10 and ≥1:10 are considered negative and positive, respectively, for the viruses indicated.
[c]InBios ZIKV IgM Capture ELISA interpretive guide: positive, presumptive Zika positive; possible, possible Zika positive; other, presumptive other flavivirus positive.
[d]InBios ZIKV 2.0 IgM Capture ELISA interpretive guide: positive, presumptive Zika positive; other, presumptive other flavivirus positive (non-Zika).
[e]Samples are plasma from the SeraCare AccuSet Zika Performance Panel (catalogue no. 0845 to 0142). DENV1 and DENV2 PRNT titers are not provided by the manufacturer.
[f]PRNT was performed at a CDC-designated public health laboratory, and only a qualitative result was reported.
[g]ZIKV rRT-PCR was performed on urine with a result of "not detected." A concurrent serum sample was not submitted.
[h]ZIKV rRT-PCR was performed on serum and urine with results of "not detected."
[i]ZIKV rRT-PCR was performed on urine with a result of "detected." Concurrent serum sample not submitted.
[j]ZIKV rRT-PCR was performed on serum with a result of "not detected."
[k]Samples were originally submitted for PRNT testing due to reactivity by the CDC ZIKV MAC-ELISA (inconclusive [$n = 10$] or presumptive Zika positive [$n = 1$]) used in our laboratory between September and November 2016.
[l]Sample was also PRNT positive for Jamestown Canyon virus (>1:2,560). According to the CDC PRNT interpretive comment, this sample was determined to be positive for coinfection with WNV and Jamestown Canyon virus.

DENV, which precluded distinction between infection with either one or both of these viruses. Among these sera, the InBios ZIKV 2.0 and InBios ZIKV MAC-ELISAs were negative or "other flavivirus positive" for 9 (40.9%) and 3 (13.6%) samples, respectively, compared to the Chembio DPP Zika ICA, which was negative in more than half ($n = 13$ [59%]) of the samples. Admittedly, the interpretation of these findings is challenging due to the absence of complete clinical and laboratory records for these 22 patients. Extrapolating from the aforementioned findings showing strong positive and negative agreement of the InBios ZIKV 2.0 MAC-ELISA in well-characterized ZIKV or DENV PRNT-positive samples and in PRNT-negative sera, the results from these 22 samples may allude to a somewhat improved ability of this assay to discriminate between ZIKV and non-ZIKV IgM-class antibodies. Additional independent evaluation of this assay is necessary, however, both in patients with primary ZIKV infection and in patients with ZIKV/DENV coinfection or secondary infection, in order to better elucidate the performance of the InBios ZIKV 2.0 MAC-ELISA in this subset of individuals. Conversely, given the three false-negative results of the Chembio DPP Zika ICA in PRNT-confirmed ZIKV positive sera, the lack of reactivity of the ICA in this 22-sample set suggests a decreased sensitivity for this assay, bringing into question the accuracy of the nonreactive results for 13 of these sera. Despite the challenges associated with interpretation of results from the Chembio DPP Zika ICA and the InBios ZIKV 2.0 MAC-ELISA in samples with NAbs to both ZIKV and DENV, it is worth noting that laboratories transitioning from the InBios ZIKV MAC-ELISA to either of these two assays are likely to observe an overall decrease in anti-ZIKV IgM reactivity rates following implementation.

The noted decrease in reactivity of the Chembio DPP Zika ICA in our study may be

attributable, at least in part, to use of the ZIKV NS1 protein as the target antigen. Structural studies of NS1, a conserved flavivirus protein involved in viral replication and pathogenesis, have revealed virus-specific electrostatic differences at select surface epitopes, which may lead to enhanced antibody specificity and the ability to discriminate between antibodies to different flaviviruses (13, 14). Prior studies evaluating other ZIKV NS1-based serologic assays, including the Liaison XL Zika Capture IgM chemiluminescent assay (CLIA) and the Euroimmun IgM and IgG ZIKV ELISAs (Lübeck, Germany), have shown increased specificity in samples confirmed by PRNT or rRT-PCR for DENV infection compared to MAC-ELISAs based on antibody detection to ZIKV envelope proteins (11, 15, 16). Notably, however, these same reports have also documented decreased sensitivity of NS1-based assays for detection of IgM-class antibodies to ZIKV, ranging from 58 to 85%, in patients with either PRNT or rRT-PCR confirmed ZIKV infection (15–17). Despite the small sample number included in our study, the decreased reactivity of the Chembio DPP Zika ICA in PRNT-confirmed ZIKV samples is consistent with prior evaluations of assays using ZIKV NS1 as the antigenic target. In contrast, the InBios ZIKV 2.0 MAC-ELISA is based on detection of anti-ZIKV IgM to ZIKV envelope proteins, identical to the original InBios ZIKV MAC-ELISA version. Previous studies have shown excellent positive agreement of the InBios ZIKV MAC-ELISA with PRNT, as high as 100%, although specificity of this assay has been variable, ranging from 20 to 74% (16, 18). Interestingly, we show improved specificity of the InBios ZIKV 2.0 MAC-ELISA compared to the original version and suggest that although the assay design is similar between these two InBios MAC-ELISA versions, this increase in specificity may be ascribed to a benefit gained from using separate anti-flavivirus and HRP-conjugated detection antibodies in the updated assay format. This two-step approach likely allowed for greater flexibility in titration and optimization during assay development, which ultimately resulted in lower signal-to-noise ratios and allowed for incorporation of an OD threshold. These design improvements, alongside revised qualitative cutoff values, may collectively lead to a decrease in nonspecific reactivity of the new InBios ZIKV 2.0 MAC-ELISA.

This study is limited by a number of factors, including the limited sample set available, the retrospective selection of samples based on initial anti-ZIKV IgM screen reactivity with supplemental PRNT results, and the absence of detailed clinical information (e.g., duration of symptoms, primary versus secondary infection, etc.), impeding a discussion of sensitivity for either the Chembio DPP Zika ICA or the InBios ZIKV 2.0 MAC-ELISA. We also included 22 samples with NAbs to both ZIKV and DENV, for which interpretation of the Chembio or InBios anti-ZIKV IgM results is particularly challenging given the lack of additional clinical and laboratory findings for these patients. Although definitive conclusions cannot be drawn regarding this subset of specimens, they were included in the study since samples with such PRNT results are among the most commonly received by our laboratory, and we felt it prudent to indicate that laboratories utilizing either the Chembio DPP Zika ICA or the InBios ZIKV 2.0 MAC-ELISA will observe an overall lower reactivity rate than that seen with prior assays. In addition, we supplemented our six ZIKV PRNT confirmed sera with nine commercially available ZIKV PRNT-positive plasma specimens purchased from SeraCare. Notably, these specimens were collected from individuals in regions where ZIKV and DENV are endemic, unlike the majority of samples received through MCL, and PRNT titers for DENV, to confirm the absence of cross-reactivity, dual or secondary infection, were not provided for this sample set. Also, neither of the InBios ZIKV MAC-ELISAs are FDA EUA approved for use on plasma. Finally, while our findings suggest improved specificity of the Chembio DPP Zika ICA and the InBios ZIKV 2.0 MAC-ELISA using the 13 samples confirmed by PRNT for a non-ZIKV flavivirus infection and among the 22 samples negative for antibodies to ZIKV and DENV by PRNT, additional studies using a larger flavivirus cross-reactivity sample set are warranted to confirm our observations.

In summary, we demonstrate that the Chembio DPP Zika ICA and the InBios ZIKV 2.0 MAC-ELISA have >95% specificity in ZIKV/DENV-seronegative specimens and in samples positive for antibodies to other, non-ZIKV flaviviruses, a dramatic improvement

over the specificity documented for the InBios ZIKV MAC-ELISA (43%) in these same samples. In addition, although the sample number is small, we show that the InBios ZIKV 2.0 MAC-ELISA was "presumptive Zika positive" for all 15 PRNT-confirmed ZIKV samples, compared to the Chembio DPP Zika ICA and the InBios ZIKV MAC-ELISA, which were nonreactive in 3 and 4 of these samples, respectively. Overall, our preliminary findings suggest that replacement of the InBios ZIKV MAC-ELISA with the InBios ZIKV 2.0 MAC-ELISA will lead to fewer samples requiring confirmatory PRNT, while maintaining a high level of sensitivity. This will alleviate some of the testing burden on laboratories performing supplemental PRNT, a technically challenging and time-consuming assay, and, more importantly, will minimize unnecessary anxiety among patients ultimately seronegative for antibodies to ZIKV by PRNT.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.01413-18.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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