Standardization and Evaluation of an Anti-ZIKV IgM ELISA Assay for the Serological Diagnosis of Zika Virus Infection

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Abstract. Here, we describe the development of the in-house anti-Zika virus (ZIKV) IgM antibody capture ELISA (in-house ZIKV IgM ELISA) for the detection and diagnosis of acute ZIKV infections. We compared the in-house ZIKV IgM ELISA assay performance against two commercial kits, Euroimmun ZIKV IgM and InBios 2.0 ZIKV IgM ELISA. We tested the assays’ ability to detect anti-ZIKV IgM using a well-defined serum sample panel. This panel included 80 ZIKV negative samples (20 negative, 20 found to be primary dengue virus [DENV] infections, 20 secondary DENV infections, and 20 Japanese encephalitis virus [JEV] infections) and 67 ZIKV reverse transcriptase–polymerase chain reaction–positive acute serum samples. The OD values were calculated to enzyme immunoassay (EIA) units by comparing them to weak positive controls. The results demonstrated the high sensitivity (88.06%) and specificity (90.00%) of our in-house ZIKV IgM ELISA and its 89.12% overall percentage agreement. The kappa values were deemed to be within excellent range and comparable to the InBios ZIKV IgM ELISA. Some cross-reactivity was observed among secondary DENV and JEV samples, and to a much lower extent, among primary DENV samples. These data indicate that our in-house ZIKV IgM ELISA is a reliable assay for the detection of anti-ZIKV IgM antibodies in serum.

INTRODUCTION

Zika virus (ZIKV) is a mosquito-borne pathogen, belonging to the Flaviviridae family, in the Spondweni sero-complex group.1 ZIKV virus was first isolated from a sentinel Rhesus monkey (Macaca mulatta) during a yellow fever study in the Zika forest near Entebbe, Uganda, in 1947.2 In 1948, the virus was isolated from pooled Aedes africanus circulating in the same forest.3 ZIKV virus has also been isolated from many other species of Aedes mosquitoes,4,5 including Aedes aegypti mosquitoes,6 the most significant vector of ZIKV transmission. Human-to-human transmission can also occur through blood transfusions, sexual contact, and vertically from mother to fetus.7

The first ZIKV infection in humans was reported in Nigeria in 1954.8 ZIKV virus infections usually manifest as asymptomatic or mild disease, most commonly accompanied by mild fever, arthralgia in small joints of the hands and feet, myalgia, headache, retro-orbital pain, conjunctivitis, and cutaneous maculopapular rash. Clinical diagnosis is often difficult because symptoms are shared by infections with other arboviruses, like dengue virus (DENV) and chikungunya virus (CHIKV). Abdominal pain, diarrhea, and arthritis can also appear in some cases of ZIKV infections.9 In 2007, a large outbreak of ZIKV disease took place in Yap state, Micronesia, infecting approximately 70% of the population.10 Since then, ZIKV has spread throughout various regions of the world,11–13 becoming a significant public health threat due to its association with significant neurological disorders in infants.14–16 Given the persistent circulation of ZIKV in some areas of the world, including Thailand,17 improvements in early detection and responses, vector control programs, effective therapeutics and vaccines are needed to control infection and transmission.18

The laboratory diagnosis of ZIKV mostly relies on the detection of viral RNA in whole blood (also serum and plasma), cerebrospinal fluid, saliva, urine, and semen.19,20 ZIKV virus viremia can be detected for up to 5 days after symptoms onset, peaking when clinical signs appear.10 Some evidence shows longer detectable periods of ZIKV viremia in urine and semen than in whole blood or saliva.21,22 Serological tests like ELISAs can provide a wider window for diagnosis because they are capable of detecting ZIKV antibodies’ response early during an acute event and through convalescence. Zika virus IgM typically develops around 5 days after symptom onset and remains detectable for at least 12 weeks, whereas ZIKV IgG can be detected a few days later and remains detectable for at least 1 year.20 Despite its high cross-reactivity to other flaviviruses,23 detection of circulating ZIKV IgG antibodies continues to be widely used to identify prior ZIKV exposures in individuals.24 We seek to test the less cross-reactive ZIKV IgM antibodies as a tool to distinguish acute ZIKV infections from other flaviviruses and extend the diagnostic window provided by reverse transcriptase–polymerase chain reaction (RT-PCR). Here, we standardized and characterized the performance of the in-house ZIKV IgM ELISA using patient sera and compared it with two commercial ELISA kits including Euroimmun ZIKV IgM ELISA (Euroimmun ELISA; Euroimmun AG, Lübeck, Germany) and InBios ZIKV detect 2.0 IgM capture ELISA (InBios MAC-ELISA; InBios international, Inc., Seattle, WA).

MATERIALS AND METHODS

Serum specimens. The human serum samples used in this study (Table 1) were obtained from Thai patients and confirmed by RT-PCR under a non-human subject research study.
approved by Walter Reed Army Institute of Research and local Institutional Review Boards. The deidentified samples consisted of a total of 147 pairs of acute and convalescent samples, including 67 ZIKV RT-PCR–positive samples and 80 ZIKV RT-PCR–negative samples. The RT-PCR–negative samples were composed of samples with no evidence of flavivirus infection \((N = 20)\), primary DENV infections \((N = 20)\), or secondary DENV infections \((N = 20)\) and were Japanese encephalitis virus (JEV) IgM positive \((N = 20)\).

**Viral RNA detection by RT-PCR.** Viral RNA was extracted using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instruction. Samples were tested by nested RT-PCR for detection of DENV and JEV\(^{25}\) and real-time RT-PCR for detection of ZIKV. For ZIKV realtime RT-PCR, the method was modified from Lanciotti et al.\(^{20}\) by using the two primer/probe sets. The first set includes ZIKV 1086 forward primers, ZIKV 1162c reverse primers, and a ZIKV 1107-FAM probe.\(^{26}\) The second set includes ZIKV 4434 forward primers, ZIKV 4524c reverse primers, and a ZIKV 4479c-FAM probe.\(^{27}\) All real-time assays were performed by using the SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen, Waltham, MA) with amplification using the Applied Biosystems 7500 Fast Real-Time PCR systems (Life Technologies, Carlsbad, CA) following the manufacturer’s protocol.

**Anti-DENV/JEV IgM and IgG ELISA.** Anti-DENV/JEV IgM and IgG ELISAs were performed in three independent experiments following procedure described elsewhere.\(^{28,29}\) Briefly, flat-bottom microplates were coated with 100 \(\mu\)L/well of 1:1,600 dilution of goat anti-human IgM or IgG (KPL, Gaithersburg, MD) in 0.018 M carbonate buffer (pH 9.0). After overnight incubation at 4°C, the plates were washed with PBS (pH 7.4) containing 0.5% Tween 20 (PBS-T). Next, 50 \(\mu\)L/well of 1:100 dilution of test serum, NC, WPC and SPC in PBS were added and incubated overnight at 4°C. After washing with PBS-T, 50 \(\mu\)L/well of 0.2 M sulfuric acid. The absorbance at OD450 was measured and calculated for EIA units as previously described.\(^{28,29}\) The WPC for anti-ZIKA IgM ELISA was obtained from the serum of a rhesus monkey (Macaca mulatta) 15 days after inoculation with 5 \(\times\) 10\(^6\) PFU of ZIKV (MR766; Uganda, 1947).

**Euroimmun anti-Zika virus IgM ELISA.** The Euroimmun ZIKA IgM ELISA test kit was designed to detect specific IgM against recombinant ZIKV NS1 coated on the plate. The Euroimmun ZIKA Virus ELISA kits (Cat. No. E21668-9601M) were produced by Euroimmun AG (Lübeck, Germany). The assays were carried out according to the manufacturer’s instructions.\(^{30}\)

**InBios ZIKV detect\(^\text{TM}\) 2.0 IgM capture ELISA (InBios ZIKV-IgM).** The InBios ZIKV IgM ELISA detects IgM antibodies targeting the recombinant ZIKV envelope glycoproteins (Cat. No. ZKM2-1). The InBios ZIKV-IgM ELISA kit was produced by the InBios International, Inc. (Seattle, WA) and performed according to the manufacturer’s instructions.\(^{31}\)

**Data analysis.** Prism-GraphPad (GraphPad software Inc., La Jolla, CA) was used to create the receiver operating characteristic (ROC) curve to identify an appropriate cut-off value for anti-ZIKV IgM ELISA. The assay performance comparison to commercial kits (assay agreement) was evaluated by Kappa values, indicating excellent agreement if > 0.75, fair agreement if 0.40–0.75, and poor agreement if < 0.40.\(^{32}\)

**RESULTS**

**Receiver operating characteristic curve of anti-ZIKV IgM ELISA.** Table 1 shows the composition and characteristics of the serum panel used. All of the samples were tested by RT-PCR and DENV/JEV IgM/IgG ELISA. The ROC curve (Figure 1) was used to determine an optimal EIA

| Group | Label | Cases (N = 147) | RT-PCR | DENV/JEV MAC-ELISA |
|-------|-------|----------------|--------|---------------------|
| 1     | Negative | 20 0 0 0 0 0 0 | 0 0 0 0 |
| 2     | 1° DENV | 20 20 0 0 0 0 0 | 20 4 0 0 |
| 3     | 2° DENV | 20 20 0 0 0 0 0 | 20 0 0 0 |
| 4     | JEV    | 20 0 0 0 0 0 0 | 20 0 0 0 |
| 5     | ZIKV   | 67 0 0 0 0 0 0 | 16 8 0 0 |

\(\text{DENV} = \text{dengue virus}; \text{JEV} = \text{Japanese encephalitis virus}; \text{MAC-ELISA} = \text{IgM antibody capture enzyme-linked immunosorbent assay}; \text{ND} = \text{not determined}; \text{RT-PCR} = \text{reverse transcriptase-polymerase chain reaction}; \text{ZIKV} = \text{Zika virus.}\)
The detection of ZIKV IgM by anti-ZIKV IgM ELISAs. The performance of the in-house ZIKV IgM ELISA was compared with two commercial ELISA kits: Euroimmun ZIKV IgM ELISA and InBios ZIKV IgM ELISA. Table 2 shows the serum panel results done by all three ZIKV IgM ELISA tests. The negative ZIKV samples were found to be 90% (72/80) negative when tested by the in-house ZIKV IgM ELISA. Fifty-nine of 67 (88.06%) ZIKV RT-PCR–positive samples were found to be positive when tested by the in-house ZIKV IgM ELISA. The Euroimmun ZIKV IgM ELISA showed 98.75% (79/80) agreement with the ZIKV RT-PCR–negative samples but only 13.43% (9/67) agreement with the ZIKV RT-PCR–positive samples, with an additional 4.48% (3/67) labeled as borderline. The InBios ZIKV IgM ELISA had a 94.03% (63/67) agreement with the ZIKV RT-PCR–positive samples with an additional one sample (1.5%) identified as “other flavivirus.” The InBios ZIKV IgM ELISA also had a 91.25% (73/80) agreement ZIKV RT-PCR–negative samples, which also includes the correct assessment of 47/60 (78.33%) as other flaviviruses.

Overall sensitivity and specificity. Table 3 shows the case distribution and comparison of sensitivity, specificity, overall agreement, and Kappa assessment values (95% CI) of the in-house ZIKV IgM ELISA assay to two commercial ELISA kits (Euroimmun ZIKV IgM ELISA and InBios ZIKV IgM ELISA). By using ZIKV RT-PCR and DENV/JE IgM ELISA as reference standard methods, the sensitivities of the in-house ZIKV-IgM ELISA, Euroimmun ZIKV IgM ELISA, and InBios ZIKV IgM ELISA were 88.06% (59/67), 10.45% (7/67), and 94.03% (63/67), respectively. The assay specificities were 90.00% (72/80), 98.75% (79/80), and 83.75% (67/80) for in-house ZIKV-IgM ELISA, Euroimmun ZIKV IgM ELISA, and InBios ZIKV IgM ELISA, respectively. The percentage overall agreement to the ZIKV RT-PCR and DENV/JE EIA of the in-house ZIKV IgM ELISA, Euroimmun ZIKV IgM ELISA, and InBios ZIKV IgM ELISA were 89.12% (131/147), 58.5% (86/147), and 88.4% (130/147), respectively. Kappa assessment values define the in-house ZIKV-IgM ELISA (Kappa value: 0.83) and InBios ZIKV IgM ELISA (Kappa value: 0.81) as excellent, whereas the Euroimmun ZIKV IgM (Kappa value: 0.45) was classified as fair.

DISCUSSION

Serological diagnosis of ZIKV infection is challenging, often leading to misinterpretation due to the cross-reactive nature of antibodies elicited by infection to other flaviviruses bearing common antigenic determinants or by vaccination. Reverse transcriptase–polymerase chain reaction is the most reliable assay today for ZIKV detection and diagnosis but is limited by the short-lived presence of viral RNA in acute serum, often lasting only 3–5 days after symptoms. Validated serological assays of high specificity and sensitivity, capable of detection of ZIKV-specific antibodies circulating during the acute and early convalescence phases, would expand the window of detection in support of ZIKV diagnosis and treatment.

In this study, we developed a specific in-house ZIKV IgM ELISA and measured its specificity and sensitivity using a well-defined serum panel consisting of ZIKV RT-PCR–positive serum samples and ZIKV RT-PCR–negative serum samples of other or unknown etiologies. Receiver operating
characteristic curve analysis using the differences between true positive rate (sensitivity) and false positive rate (1 – specificity) showed that 40 EIA units represents the optimal cut-off point. Using this cut-off point, the AUC approximates 1 (AUC = 0.941) and indicates excellent diagnostic capabilities and accuracy of our in-house ZIKV IgM ELISA, with 88.06% sensitivity and 90.00% specificity.

We used the in-house DENV/JEV IgM/IgG ELISA as a model to develop the in-house ZIKV IgM ELISA. Evidence of ZIKV infection was classified when the ratio of ZIKV IgM/DENV IgM and ZIKV IgM/JEV IgM was ≥ 1.0. Using this criteria, we found ease in distinguishing between negative/primary DENV and ZIKV samples. However, we found low levels of cross-reactivity to ZIKV IgM when testing secondary DENV samples. Even though ZIKV cross-reactivity has been reported, we were unable to search the medical literature on the possible cause of the high anti-JEV IgM levels, which are cross-reactive and interfering with other flavivirus serological tests. Ultimately, there is a risk of false positives when ZIKV IgM ELISAs are used in areas where ZIKV co-circulates with various flaviviruses. Nonetheless, our data show that the in-house ZIKV IgM ELISA is highly specific, even when testing samples from patients with primary flavivirus infections.

Evaluations of various Zika ELISA assays have been published elsewhere. In general, envelop-based Zika IgM ELISA assays increase detection rates significantly over NS-1-based assays and provide a larger diagnostic window. These observations are reflected in the performance described here for the Euroimmun ZIKV IgM ELISA, a NS1-based assay with moderate results. In contrast, ZIKV IgM ELISA (using suckling mouse brain extracted crude antigen) and the InBios ZIKV IgM ELISA (recombinant envelope protein) performed substantially better. Euroimmun has recently recommended the use of the combined ZIKV IgA/IgM to improve its sensitivity.

In conclusion, our study demonstrates that the in-house ZIKV IgM ELISA can be an important tool for detecting ZIKV infections in humans. The assay is an affordable and reliable option for diagnosis of ZIKV patients, especially in flavivirus-endemic countries. A limitation in the use of the in-house ZIKV IgM ELISA is that its interpretation requires the use of

| ELISAs          | Results     | ZIKV (N = 67) | Other flavivirus (N = 147) | Negative (N = 20) | Total (N = 147) |
|-----------------|-------------|---------------|---------------------------|-------------------|-----------------|
| In-house ZIKV IgM | ZIKV        | 59            | 2                         | 4                 | 0               | 147             |
|                 | Negative    | 8             | 18                        | 18                | 16              | 20              |
| Euroimmun ZIKV IgM | ZIKV        | 9             | 0                         | 1                 | 0               | 147             |
|                 | Borderline  | 3             | 0                         | 0                 | 0               | 20              |
|                 | Negative    | 55            | 20                        | 19                | 20              | 20              |
| InBios 2.0 ZIKV IgM | ZIKV        | 63            | 0                         | 6                 | 0               | 147             |
|                 | Other flavivirus | 1       | 20                        | 9                 | 0               | 20              |
|                 | Negative    | 3             | 0                         | 1                 | 5               | 20              |

IgM = immunoglobulin M; ZIKV = Zika virus.

* Other includes a) 1°, 2° dengue virus, and Japanese encephalitis virus positive of validation; b) borderline for Euroimmun; and c) other flavivirus positive for InBios ZIKV IgM ELISAs.
DENV and JEV antibodies ELISAs, which may limit its usability in some laboratories.

Received February 9, 2021. Accepted for publication May 24, 2021. Published online August 2, 2021.

Financial support: The research was funded by a grant from the Armed Forces Health Surveillance Branch (AFHSB) and its Global Emerging Infectious Surveillance (GEIS) Section, United States, under grant number P0108_19_AF_07 for fiscal year 2019.

Disclaimer: This material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting true views of the Department of the Army or the Department of Defense.

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