Use of a Blockade-of-Binding ELISA and Microneutralization Assay to Evaluate Zika Virus Serostatus in Dengue-Endemic Areas

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Abstract. Zika virus (ZIKV) serological diagnostics are compromised in areas where dengue viruses (DENV) co-circulate because of their high levels of protein sequence homology. Here, we describe the characterization of a Zika blockade-of-binding ELISA (Zika BOB) and a Zika microneutralization assay (Zika MN) for the detection of ZIKV non-structural protein 1 (NS1)-specific antibodies and ZIKV neutralizing antibodies, respectively. Zika BOB and Zika MN cutoffs were established as 10 and 100 endpoint titers, respectively, using samples collected pre- and post-virologically confirmed ZIKV infection from subjects living in DENV-endemic areas. Specificity of the assays was equally high, whereas sensitivity of Zika BOB was lower than that of Zika MN, especially in samples collected > 6 months post-infection. Immunosurveillance analysis, using combined results from both Zika BOB and Zika MN, carried out also in DENV-endemic regions in Colombia, Honduras, Mexico, and Puerto Rico before (2013–2014) and after (2017–2018) ZIKV introduction in the Americas suggests unapparent ZIKV seroprevalence rates ranged from 25% to 80% over the specified period of time in the regions investigated.

INTRODUCTION

Whereas Zika virus (ZIKV) was initially detected in 2013 in the Americas,1 its first apparent outbreak with widespread clinical manifestation was detected in Brazil in early 2015. Since then, ZIKV rapidly disseminated with high attack rates2 throughout South and Central America and the Caribbean, especially in areas where the seroprevalence of dengue viruses (DENV) is high.3–6 Those outbreaks were linked with neurological disorders in adults7,8 and devastating neurological consequences in the children of mothers infected during pregnancy.9 Both DENV and ZIKV are members of the genus Flavivirus, which are transmitted by the same mosquito vector (predominantly Aedes aegypti) and share a high degree of protein identity.10 The factors contributing to the severity of clinical outcomes following ZIKV infection are not fully understood, although a potential role of DENV (and other arboviruses) co-transmission has been proposed.11 Several reports have suggested that antibodies directed to the DENV envelope protein (E) may enhance the infection of phagocytes by ZIKV in vitro,12,13 although such an association has not been confirmed either clinically or epidemiologically.14,15 In addition, the high homology in the primary amino acid sequence between DENV and ZIKV poses a significant challenge in defining virus serostatus in areas of endemic transmission where these viruses co-circulate.13,16,17 As serum antibodies from DENV-immune subjects cross-react with ZIKV antigens and vice versa,18–22 Neutralizing antibodies titers assessed by plaque-reduction neutralization test (PRNT) have successfully been used to differentiate ZIKV from DENV infections, especially at the late convalescent stage of infection, despite a persistent degree of cross-reactivity after recent exposure to both viruses.23 In addition, low-throughput, longer turnaround time, and the need for experienced and highly trained personnel make PRNT a challenge for high-demand clinical testing.24 On the other hand, most of the in-house and commercially available immunoassays are based on detection of antibodies to the two major targets of immune responses following ZIKV infection, the E and non-structural 1 (NS1) proteins, and have limited specificity.13,18,21,22 with the exception of a ZIKV NS1 IgG3 ELISA2 and Zika NS1 blockade-of-binding (BOB) ELISA.19,25 The BOB ELISA is a competitive ligand-binding assay and relies on the ability of serum antibodies to block the binding of a highly specific monoclonal antibody (mAb) to an antigen adsorbed on a microtiter ELISA plate.26 This approach has been used with high specificity to detect antibodies against many viruses, including Crimean–Congo hemorrhagic fever virus,27 foot-and-mouth disease virus,28 bluetongue virus,29 and West Nile virus (WNV).30 We carried out a proof-of-concept study to determine Zika serostatus using a Zika NS1 BOB ELISA (Zika BOB) and a high-throughput colorimetric Zika microneutralization assay (Zika MN) in samples collected in DENV-endemic areas affected by ZIKV outbreaks in 2016. In addition, the combination of results of both methods was used to estimate unapparent ZIKV exposure (recent and remote) in Colombia, Honduras, Mexico, and Puerto Rico before (2013–2014) and after (2017–2018) the introduction of ZIKV to the Americas.

MATERIALS AND METHODS

Ethics statement. The trial protocols were approved by all relevant ethics review boards, and parents or guardians provided written informed consent and older children provided written informed assent before participation in the study, in accordance with local regulations. All data were anonymized such that no patient identifiers were present in the data files received for analysis.

Recombinant proteins. Recombinant NS1 proteins from the following flaviviruses were obtained commercially (Native Antigen Company, Oxfordshire, United Kingdom): Zika virus (ZIKV, strain Suriname, which circulated in the Americas in 2015); DENV serotypes 1 (strain Nauru/Western Pacific/1974), 2 (strain Thailand/16681/84), 3 (strain Sri Lanka D3/H/IMTSSASRI/2000/1266), and 4 (strain Sri Lanka D3/H/IMTSSA-SRI/2000/1266); yellow fever virus (YFV; strain 17D); Japanese encephalitis virus (JEV; strain SA-14); tick-borne
encephalitis virus (TBEV; strain Neudoerfl); West Nile virus (WNV; strain NY99); and Usutu virus (USUV; strain Vienna 2001).

**Unrelated proteins.** Non-recombinant Bordetella pertussis toxin (Mardy L’Etoile, France) and Clostridium difficile toxin B (Swiftwater) were manufactured by Sanofi Pasteur and were used as unrelated antigens in specificity (competition) experiments.

**Zika NS1 BOB ELISA procedure.** Zika NS1 BOB ELISA measures the levels of serum antibodies that block the binding of a highly specific mAb to Zika NS1 as described as follows: Thermo Immulon 2HB (Thermo Scientific, Waltham, MA) 96-well flat-bottom microtiter plates were coated with Zika NS1 in carbonate/bicarbonate buffer (pH 9.6 ± 0.1) overnight at 4°C. The plates were washed with 0.1 M phosphate-buffered saline (PBS) with 0.05% Tween 20 (PBS-T; HyClone Laboratories, Logan, UT) and blocked with PBS-T supplemented with 1% (v/v) goat normal serum (1% GNS; Gibco Laboratories, Gaithersburg, MD) for 45 ± 5 minutes at 21°C. The plates were washed with PBS-T, then 2-fold serially diluted human samples and internal quality controls (IOC; human samples obtained commercially from ZikaVirus-exposed individuals in Colombia [ABO Pharmaceuticals, San Diego, CA]) in 1% GNS were supplemented with a pool of DENV NS1 from all four DENV serotypes (at 1:1:1:1 ratio) in carbonate/bicarbonate buffer (pH 9.6 ± 0.1) overnight at 4°C. The plates were washed with PBS-T and incubated for 60 ± 5 minutes at 21°C to reduce cross-reactivity by DENV-specific antibodies to Zika NS1–coated plates. A solution containing Zika NS1–specific mouse mAb, clone 1F11.B7.A2.F9 (Native Antigen Company; see Supplemental Table 1 and Figure 1A for binding specificity analysis), at 0.5 μg/mL prepared in 1% GNS was immediately pipetted on top of the human samples, mixed, and incubated for 10 ± 5 minutes at 21°C. The plates were washed with PBS-T and incubated for 60 ± 5 minutes at 21°C with peroxidase-conjugated Fab(ab′)2 goat anti-mouse IgG Fcγ fragment (Jackson ImmunoResearch Laboratories, West Grove, PA) prepared in 1% GNS. The plates were washed with PBS-T and developed with SureBlue Reserve tetramethylbenzidine (TMB) microwell peroxidase substrate (SeraCare, Milford, MA) for 30 ± 2 minutes at 21°C. The reaction was stopped with 2N sulfuric acid (Machinery, France). The plates were washed with PBS-T and incubated with horseradish-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) with 80% cells of a freshly prepared suspension of Vero cells (CCL-81, ATCC) was added to each well of the plates. The plates were incubated for 4 days at 37°C with 5% CO2 and humidity, then washed and fixed with 80% acetone (Thermo Fisher Scientific), followed by blocking with 5% nonfat milk (Carnation Company, Los Angeles, CA) in PBS-T, and incubated with an anti-pan Flavivirus mAb, HB112-4G2 (Biotem Inc., Apprieu, France). The plates were washed with PBS-T and incubated with horseradish-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) for 1 hour. The plates were washed with PBS-T and developed with the TMB microwell peroxidase substrate system (SeraCare) for 45 minutes at 20°C. The reaction was stopped with 2N sulfuric acid (Machinery, Center Valley, PA) and the plates were read in a SpectraMax 384 (Molecular Devices) microplate reader at 450 nm (650 nm as the reference wavelength) using SoftMax Pro software version 6.5.1 (Molecular Devices). The serum titers were determined by calculating the sample dilution that neutralized 50% of the maximum signal using SoftMax Pro software. The assay acceptance criteria include three IQCs and the TCID_{50} in each assay run for data validity (Supplemental Table 2).

**Dengue NS1 IgG ELISA procedure.** Dengue NS1 IgG ELISA is an immunoassay to quantify IgG to DENV NS1 and is described elsewhere. Briefly, 96-well flat-bottom microtiter plates were coated with pooled DENV NS1 from all four DENV serotypes (at 1:1:1:1 ratio) in carbonate/bicarbonate buffer (pH 9.6) and left overnight at 4°C. The plates were washed with PBS-T and then blocked with PBS-T supplemented with 1% GNS for 45 minutes at 21°C. After washing, 2-fold serially diluted human samples and IQCs (described earlier) in 1% GNS were added and incubated for 60 minutes at 37°C. The plates were washed with PBS-T and incubated for 60 minutes at 37°C with an anti-human IgG detection antibody, developed with a TMB substrate, and read as described earlier.

**Zika immunoassay characterization parameters.** Assay performance evaluation of both Zika NS1 BOB ELISA and Zika MN was carried out based on the International Conference for Harmonization Harmonized Tripartite Guideline and is shown in the Supplemental Material.

**Virologically confirmed Zika (VCZ) and dengue (VCD) samples.** Longitudinal Zika antibody-positive human serum samples were obtained from febrile (≥ 38°C for at least 2 days) subjects who had a VCZ by real-time reverse transcriptase-polymerase chain reaction (qRT-PCR; ARUP Laboratories, Salt Lake City, UT). Samples available before and after the qRT-PCR diagnosis were used for evaluation of specificity and sensitivity, respectively, of Zika BOB and Zika MN. Subjects had participated in the CYD15 Phase III efficacy clinical...
The selected subjects (n = 97) were on average (min, max) 12 (9, 17) years old, 55% female (n = 53), who had a detectable ZIKV infection by PCR (VCZ) in 2016. The majority of these individuals (74 of 97) had positive dengue NS1 IgG titers in samples collected before ZIKV infection (pre-Zika). These samples were considered immune to DENV before ZIKV exposure (Table 1).

Zika microneutralization assay and Zika BOB were performed on the pre- (n = 78) and post- (n = 202) ZIKV infection samples, and the kinetics of antibody production are shown in Figure 1A and B, respectively. The specificity of Zika MN and Zika BOB was 71% and 97%, respectively, and the sensitivity of Zika MN and Zika BOB was 99% and 78%, respectively, when a cutoff titer of 10 was applied to both methods (Table 2). However, the specificity of Zika MN increased to 100% while maintaining a very high sensitivity of 98% when a cutoff titer of 100 was used (Table 2). Of note, multiple samples following ZIKV infection were used from the same subject in this analysis.

Unlike the Zika MN, the sensitivity of the Zika BOB was influenced by the timing of sample collection post-infection as it reached 86% on samples collected at a median of 130.5 days (1st/2nd bleed; mostly from a single sample per subject) and declined to 74% and 65% in samples collected at 220 days (3rd bleed) and 298 days (≥4th bleed), respectively (Table 3). A similar trend was also observed in relation to Zika BOB geometric mean titers (Table 3).

Next, sample classification agreement was evaluated taking into consideration the cutoff titers that yielded optimal specificity and sensitivity for both assays (100 for Zika MN and 10 for Zika BOB). Samples with titers at or above the cutoff were considered positive and titers below the cutoff were considered negative. As shown in Figure 1C, the MN/BOB double-negative group contained almost entirely pre-Zika samples (76/79) and represents individuals naive to ZIKV. Samples that were MN-negative BOB-positive (N = 3) were heterogeneous, comprising both pre- and post-Zika samples and, therefore, classified as undetermined. The MN/BOB double-positive samples consisted entirely (156/156) of post-Zika samples (Figure 1C) collected at an average (95% CI) of 141 (120, 162) days following virus infection (Figure 1D). Samples that were MN-positive BOB-negative also comprised entirely (42/42) post-Zika samples collected at an average (95% CI) of 249 (221, 276) days following virus infection (Figure 1D). Figure 1D shows that MN/BOB double-positive samples were collected at an earlier time point (P < 0.0001 with unpaired Student’s t-test) than the MN-positive BOB-negative

### Table 1

| Variables          | Number of individuals (%) |
|--------------------|---------------------------|
| **Total**          | 97 (100%)                 |
| **Dengue serostatus** |                           |
| Naive              | 9 (9%)                    |
| Immune             | 74 (76%)                  |
| Unknown†           | 14 (14%)                  |
| **Number of bleed(s) per subject** |          |
| 1                  | 16 (16%)                  |
| 2                  | 31 (32%)                  |
| 3                  | 22 (23%)                  |
| ≥ 4                | 28 (29%)                  |

ZIKV = Zika virus.

† Percentage was calculated based on the total number of volunteers analyzed.

Samples were classified as unknown because of lack of samples before ZIKV infection available for dengue nonstructural protein 1 IgG evaluation.
samples, suggesting that Zika BOB likely detects more recent ZIKV exposures. Of note, MN/BOB double-positive samples (75 of 156) were approximately 3-fold (odds ratio 95% CI: 1, 6; \( P = 0.01 \)) more likely to be collected before or at 180 days (6 months) post-ZIKV infection than MN-positive BOB-negative samples (11 of 42).

**False-positive rates of Zika NS1 BOB ELISA in VCD samples.** Samples from repeat DENV exposures have been shown to cause false-positive results on Zika BOB, likely due to cross-reactivity and proximity to the DENV infection. A panel of serum samples from 98 subjects with a VCD infection from the phase III efficacy trials CYD14 and CYD15, collected

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**TABLE 2**

| Assay (cutoff titer) | Pre-Zika samples (n) | Number negative | % (95% CI) | Post-Zika samples (n) | Number positive | % (95% CI) |
|----------------------|----------------------|------------------|------------|-----------------------|----------------|------------|
| Zika BOB (10)        | 78                   | 76               | 97% (91, 100) | 202                   | 157            | 78% (71, 83) |
| Zika MN (10)         | 55                   | 71% (59, 80)     | 71% (59, 80) | 200                   | 99% (97, 100)  | 99% (97, 100) |
| Zika MN (100)        | 78                   | 100% (95, 100)   | 100% (95, 100) | 198                   | 98% (95, 99)   | 98% (95, 99) |

BOB = blockade-of-binding; MN = microneutralization assay.
in 2012 and 2013, at a median (min, max) of 175 (12, 388) days post-infection, was used to determine the extent these DENV-specific antibodies interfere with the Zika BOB (Table 4). However, DENV serology of these VCD cases before infection was not available to determine the history of virus exposure (primary or secondary infections). As shown in Table 4, 24/98 (24%) VCD samples had positive Zika BOB titers indicating cross-reactivity to DENV infection, which included all four serotypes. Interestingly, VCD samples with positive Zika BOB titers had dengue NS1 IgG levels 7-fold higher than those with negative Zika BOB titers (geometric mean concentration of 5,463 EU/mL and 811 EU/mL, respectively, Supplemental Figure 3).

Zika immunosurveillance using Zika NS1 BOB ELISA and Zika MN. To evaluate the performance of Zika BOB and ZIKV MN as immunosurveillance tools to determine Zika serostatus in DENV-endemic areas and to differentiate possible unapparent recent and remote ZIKV exposure, we screened paired samples from the same healthy individuals collected from the immunogenicity subset of the CYD15 Phase III efficacy trial in Colombia, Honduras, Mexico, and Puerto Rico before (2013–2014) and after (2017–2018) the introduction of ZIKV in the Americas (Table 5). Zika serostatus was defined using Zika BOB combined with Zika MN as follows: MN-positive BOB-negative and MN/BOB double-positive results were indicators of remote (> 6 months) and recent (≤ 6 months) virus exposures, respectively. Microneutralization assay/blockade-of-binding double-negative results represented Zika-naive status, whereas MN-negative BOB-positive results were of an undetermined status. The results showed that false-positive Zika serostatus classification in samples collected before ZIKV introduction, whether considered remote or recent, ranged from 1% to 3% or 0% to 1%, respectively, depending on the country of origin (Table 5). By contrast, Zika seropositive rates ranged from 25% to 80% in samples collected at the 2017–2018 time point (Table 5). These samples were collected mostly (20–57%) from recent ZIKV infections, whereas remote exposures accounted for 5–23% of the samples evaluated (Table 5).

### DISCUSSION

In this study, we evaluated the performance of two Zika immunoassays, Zika BOB and Zika MN, to detect ZIKV exposures in DENV-endemic areas. The specificity and sensitivity of Zika BOB were 97% and 78% (cutoff titer of 10), respectively, using samples from VCD cases collected before and after ZIKV infection. In addition, as expected, we found that Zika BOB sensitivity was greater and titers were higher in samples collected at earlier time points post-infection. Thus, unlike the Zika BOB previously described using ZKA35 mAb, serum antibodies blocking the binding of the F9 mAb are transiently detected in the Zika BOB (within 6 months from the time of infection) and, in line with DENV NS1- and ZIKV NS1-specific IgG3 assays, may represent an alternative tool to identify recent virus exposure. On the other hand, the specificity and sensitivity of Zika MN were 100% and 98%, respectively, at a cutoff titer of 100. Neutralizing antibodies, which have the ability to target multiple epitopes on the virion, were consistently detected by Zika MN throughout the course of the study and, therefore, cannot distinguish between recent and remote virus exposure. Both assays may be used separately to determine Zika serostatus, even in the background of DENV pre-immunity, although some limitations should be considered: 1) Zika MN is a laborious and relatively low-throughput method that takes 4 days to be completed and requires an infrastructure that may not be widely available in most laboratories, and 2) Zika BOB takes 1 day to be completed, although it needs longitudinal samples collected at least every 6 months, for optimal performance. In addition, Zika BOB results should be confirmed by other
specific methods, especially during active DENV outbreaks. Thus, we propose the combination of the results of both methods not only to define Zika serostatus, but also to qualitatively discern recent ZIKV infections as follows: MN/BOB double negative represents samples from ZIKV-naive individuals, MN/BOB double positive may represent samples collected from individuals recently (e.g., ≤6 months) exposed to ZIKV, whereas MN positive BOB negative likely represents remote (e.g., >6 months) ZIKV exposure. Subjects who are MN negative BOB positive are likely false positive to ZIKV infection due to recent DENV infection. Additional studies are necessary to further define the mean duration of recent infection and false-recency rate of BOB titers and establish the duration it remains detectable as previously shown for DENV NS1 IgG.33

Using the Zika serostatus classification shown previously, we also evaluated Zika serostatus in healthy individuals in Colombia, Honduras, Mexico, and Puerto Rico. The subjects were 12 years old on average at the time of enrollment, one of the most susceptible age groups to arbovirus infection in Latin America.35 Paired samples were collected from the same individuals (N = 1,283) at various time points in 2013–2014 and in 2017–2018. Samples collected in 2013–2014 were shown not to hold significant rates of either recent (≤1%) or remote (≤3%) ZIKV exposures, despite the occurrence of well-documented DENV outbreaks around the same time period in those countries.36,37 Conversely, the frequency of Zika seropositive samples increased as expected in all countries investigated in 2017–2018. Zika seroprevalence in Colombia, Honduras, Mexico, and Puerto Rico was 48%, 80%, 39%, and 25%, respectively, and supports the hypothesis that high levels of herd immunity (decreased number of susceptible naive individuals) are observed in some geographical areas where ZIKV circulated,7 impacting disease incidence.38 Because the study participants did not report signs or symptoms consistent with ZIKV infection, seropositivity was likely due to unapparent infections. Haby and colleagues in a systematic review and meta-analysis study showed a heterogeneity in the prevalence of unapparent ZIKV infection in the general population (from 29% to 82%),5 which corroborates with the wide seroprevalence rates observed in our study. Furthermore, most of the seropositive samples were collected from individuals likely exposed to ZIKV within 6 months from sample collection, which is consistent with the reports of ZIKV transmission in the participating countries in 2016 and 2017.39 Moreover, Honduras held the highest rates of remote (>6 months) ZIKV exposure (23%), followed by Colombia (16%), Mexico (7%), and Puerto Rico (5%), which corresponds to the spatiotemporal spread of ZIKV among these countries from 2015 to 2017.40 Factors associated with susceptibility/resistance to ZIKV infection could not be determined in our study because of the lack of longitudinal samples collected shortly before virus exposure. Rodríguez-Barraruer et al.,2 however, demonstrated that preexisting immunity to DENV is protective, whereas recent DENV exposures transiently increases susceptibility to ZIKV infection in a community cohort in Salvador, Brazil. Of note, ever since ZIKV was introduced in the Americas, the number of DENV cases, including disease severity and fatalities, decreased substantially until 201738 and started to reemerge in 2018.41 Recent ZIKV exposures observed in our study in 2016 and 2017 support the hypothesis of a possible transient cross-immunity between these viruses as previously suggested.38

Serological Zika diagnosis is extremely difficult in dengue-endemic areas.13,16,17 Zika PRNT offers satisfactory specificity, especially in late convalescent samples,23 although its low-throughput and interpretation subjectivity become a liability in high-demand testing environments.24 The use of Zika BOB along with Zika MN provides an alternative approach to better determine Zika serostatus even in areas where DENV (and other flaviviruses) circulates. In addition, the proposed strategy also potentially allows the differentiation between recent and remote ZIKV exposures, which could be beneficial for calculation of the prevalence and attack rates for immunosurveillance studies.

Received April 8, 2019. Accepted for publication June 15, 2019.

Note: Supplemental information, table, and figures appear at www.ajtmh.org.

Acknowledgments: Editorial assistance was provided by Phil Jones, inScience Communications. We also thank Melissa Velasco and Evan Yost for technical support and Sandrine Buisson for editorial assistance and manuscript coordination on behalf of Sanofi Pasteur.

Financial support: Sanofi Pasteur partially funded this study. This project has been also funded with Federal funds from the Department of Health and Human Services; Office of the Assistant Secretary for Preparedness and Response; Biomedical Advanced Research and Development Authority, under Contract No. HHSO1002016000039C for Zika NS1 BOB ELISA development and evaluation of its performance.
Disclosure: E. J. M. N., M. I. B., P. L., T. S. V., B. H., J. K. G., G. A., F. N., L. Z., and J. W. H. are employees of Sano Pasteur.

Disclaimer: Parts of the work have been presented at the 6th Pan-American Dengue Research Network Meeting, April 9–12, 2018, Galveston, TX, and at the 67th Annual Meeting of the American Society of Tropical Medicine & Hygiene (ASTMH), October 28–November 1, 2018, New Orleans, LA.

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