



# Comparison of Four Serological Methods and Two Reverse Transcription-PCR Assays for Diagnosis and Surveillance of Zika Virus Infection

Angel Balmaseda,<sup>a,b</sup> José Victor Zambrana,<sup>b</sup> Damaris Collado,<sup>b</sup> Nadezna García,<sup>a,b</sup> Saira Saborío,<sup>a,b</sup> Douglas Elizondo,<sup>b</sup> Juan Carlos Mercado,<sup>a,b</sup> Karla Gonzalez,<sup>a,b</sup> Cristhiam Cerpas,<sup>a,b</sup> Andrea Nuñez,<sup>a,b</sup> Davide Corti,<sup>c</sup> Jesse J. Waggoner,<sup>d</sup> Guillermina Kuan,<sup>b,e</sup> Raquel Burger-Calderon,<sup>b,f</sup> Eva Harris<sup>b,f</sup>

<sup>a</sup>Laboratorio Nacional de Virología, Centro Nacional de Diagnóstico y Referencia, Ministry of Health, Managua, Nicaragua

<sup>b</sup>Sustainable Sciences Institute, Managua, Nicaragua

<sup>c</sup>Humabs Biomed SA, subsidiary of Vir Biotechnology, Inc., Bellinzona, Switzerland

<sup>d</sup>Department of Medicine, Division of Infectious Diseases, Emory University, Atlanta, Georgia, USA

<sup>e</sup>Health Center Sócrates Flores Vivas, Ministry of Health, Managua, Nicaragua

<sup>f</sup>Division of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley, Berkeley, California, USA

**ABSTRACT** Zika virus (ZIKV) is a mosquito-borne flavivirus that is responsible for recent explosive epidemics in the Americas. Notably, ZIKV infection during pregnancy has been found to cause congenital birth defects, including microcephaly, and ZIKV has been associated with Guillain-Barré syndrome in adults. Diagnosis and surveillance of Zika in the Americas have been challenging due to similar clinical manifestations and extensive antibody cross-reactivity with endemic flaviviral diseases, such as dengue. We evaluated four serological and two reverse transcription-PCR (RT-PCR) methods in acute-phase (mean day, 1.8), early-convalescent-phase (mean day, 16.7), and late-convalescent-phase (mean, ~7 months) samples from the same individuals in a long-term pediatric cohort study in Nicaragua. Well-characterized samples from 301 cases of Zika, dengue, or non-Zika, nondengue febrile illnesses were tested. Compared to a composite reference, an in-house IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) and the NIAID-Biodefense and Emerging Infections (BEI) MAC-ELISA measuring IgM yielded sensitivities of 94.5% and 70.1% and specificities of 85.6% and 82.8%, respectively. The NS1 blockade-of-binding ELISA measuring anti-ZIKV NS1 antibody levels yielded sensitivities of 85.0% and 96.5% and specificities of 91.4% and 92.6% at early and late convalescence, respectively. An inhibition ELISA detecting total anti-ZIKV antibodies had sensitivity and specificity values of 68.3% and 58.3% for diagnosis and 94.0% and 98.6% for measuring annual infection incidence. Finally, the ZCD and Triplex real-time RT-PCR assays detecting Zika, chikungunya, and dengue viruses both yielded a sensitivity of 96.1% and specificity of 100%. Together, these assays resolve the urgent need for diagnostic and surveillance tools for countries affected by Zika virus infections.

**KEYWORDS** ELISA, RT-PCR, Zika virus, dengue virus, diagnosis, serological assay, surveillance

Zika virus (ZIKV) is an emerging arbovirus belonging to the *Flavivirus* genus of the *Flaviviridae* family. ZIKV produces mild disease characterized by rash, moderate fever, conjunctivitis, and arthralgia; however, ZIKV has also been found to cause congenital birth defects, including microcephaly, as well as Guillain-Barré syndrome (1–7). The Zika epidemic in the Americas began in 2015 in Brazil and spread rapidly

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Address correspondence to Eva Harris, eharris@berkeley.edu.

throughout the hemisphere (8, 9). The first laboratory-confirmed case in Nicaragua was detected in January 2016, with an explosive epidemic occurring from late June to mid-September.

The cocirculation of several arboviruses in the Americas that cause similar symptoms and display antibody cross-reactivity poses a challenge for diagnosis and surveillance (10–14). In particular, detection and differentiation of the antigenically related flaviviruses dengue virus (DENV) and ZIKV have relied to date mostly upon molecular methods (15–17). However, ZIKV viral loads are low, viremia is short-lived, and serological methods are cheaper and require less sample volume. Serological dengue and Zika assays are designed to detect antibodies to the envelope (E) and/or prM/M proteins on the virion surface or to nonstructural protein 1 (NS1) that is secreted from infected cells (18, 19). However, the standardization of serological methods that are sufficiently sensitive and that discriminate between anti-DENV and anti-ZIKV antibodies has been challenging.

Lack of access to well-characterized samples has been the fundamental problem for evaluation and standardization of new assays. In Nicaragua, a cohort of ~3,600 children has been followed since 2004; each febrile episode of suspected dengue, chikungunya, or Zika or of undifferentiated fever is well-documented and accompanied by blood samples drawn during the acute and early convalescent phases (20, 21). Additionally, an annual healthy blood sample is drawn from all participants during the dry season, when arboviral transmission is minimal. This study serves as a source of samples from DENV-immune individuals where the serotype and immune status are known and from ZIKV-infected participants, stratified by prior DENV exposure.

Here we evaluated the sensitivity and specificity of 2 real-time reverse transcription-PCR (rRT-PCR) assays, one designed by the CDC (15) and the other by Waggoner et al. (16), and of 4 serological methods (2 detecting IgM and 2 detecting total antibodies) using well-characterized samples from the cohort study. Two in-house serological assays, namely, an IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) and an inhibition ELISA method (IEM) detecting total anti-ZIKV antibodies, are new and were developed at the Centro Nacional de Diagnóstico y Referencia (CNDR) of the Nicaraguan Ministry of Health. Additionally, two existing assays were evaluated: an IgM capture technique developed by the CDC and supplied by the Biodefense and Emerging Infections (BEI) Research Resources Repository (NIAID-BEI MAC-ELISA) and a competition ELISA detecting anti-ZIKV NS1 antibodies (NS1 blockade-of-binding ELISA [BOB ELISA]) (22, 23).

## MATERIALS AND METHODS

**Ethics statement.** The Pediatric Dengue Cohort Study (PDCS) was approved by the Institutional Review Boards of the Nicaraguan Ministry of Health and the University of California, Berkeley. Parents or legal guardians of all subjects provided written informed consent, and subjects who were  $\geq 6$  years old provided assent.

**Study population and sample selection.** The patient population of the PDCS is a community-based prospective cohort of children 2 to 14 years of age; the study has been ongoing since August 2004 in Managua, Nicaragua (20). In 2014, chikungunya was added to the PDCS, and in August 2015, ZIKV screening was initiated for participants meeting the clinical definition of dengue and/or chikungunya or presenting with undifferentiated fever. In February 2016, the case definition was expanded to include those presenting with signs and symptoms of Zika but without fever. Participants present at the first sign of illness to the Health Center Sócrates Flores Vivas, and study physicians use standardized forms to collect clinical data. Blood samples are collected during the acute phase and again during early convalescence for dengue, chikungunya, and Zika diagnostic testing. In addition, a healthy blood sample is collected annually from participants to determine inapparent infections (20).

**Study samples and case definitions.** A total of 301 PDCS participants were included in the study, each providing an acute-phase sample (number of days post-onset of symptoms,  $1.8 \pm 0.9$  [mean  $\pm$  standard deviation {SD}]), an early-convalescent-phase sample (mean of  $16.7 \pm 2.9$  days), and paired annual samples before and after the infection in question, although a few samples could not be tested by all methods due to insufficient volume (see Table S1 in the supplemental material). The first annual sample served as a baseline, and the second served as a late-convalescent-phase sample ( $\sim 7$  months  $\pm 29$  days). The ZIKV-positive controls consisted of 127 children who tested positive for ZIKV infection by both rRT-PCR and at least one serological method (Zika MAC-ELISA, NS1 BOB ELISA, or IEM) (Table 1). Serologically positive cases were defined as (i) those with ZIKV seroconversion detected between acute-phase samples and either early or late convalescent-phase samples or between consecutive annual

**TABLE 1** Characteristics of study population

Characteristic	<i>n</i> of patients	% of patients <sup>a</sup>
Total	301	
Sex		
Female	162	53.8
Age		
2–4 yrs	37	12.3
5–10 yrs	146	48.5
11–14 yrs	118	39.2
ZIKV positive	127	42.2 <sup>a</sup>
DENV naïve	62	48.8 <sup>b</sup>
DENV immune	65	51.2 <sup>b</sup>
DENV positive	97	32.2 <sup>a</sup>
DENV1 positive	24	24.7 <sup>c</sup>
Primary	12	50.0 <sup>d</sup>
Secondary	12	50.0 <sup>d</sup>
DENV2 positive	34	35.1 <sup>c</sup>
Primary	17	50.0 <sup>e</sup>
Secondary	17	50.0 <sup>e</sup>
DENV3 positive	39	40.2 <sup>c</sup>
Primary	19	48.7 <sup>f</sup>
Secondary	20	51.3 <sup>f</sup>
Negative	77	25.6 <sup>a</sup>

<sup>a</sup>The population data for the subgroups denoted by roman superscript letters a through f add up to 100%.

samples or (ii) those for which the titer at early convalescence was  $\geq 4$ -fold higher than the acute-phase titer or for which a  $\geq 4$ -fold increase in titer was observed between consecutive annual samples (IEM). Of the ZIKV-infected patients, 65 (51.2%) had been previously exposed to DENV (DENV-immune patients) and 62 (48.8%) were naïve (DENV-naïve patients). Confirmed ZIKV-positive cases were classified as DENV-naïve cases if they entered the cohort study with no detectable anti-DENV antibodies (as measured by DENV IEM) and had no documented DENV infections (symptomatic or inapparent) during their time in the cohort. Confirmed ZIKV-positive cases were classified as DENV-immune cases if they either entered the cohort study with detectable anti-DENV IEM antibodies or entered the cohort study with no detectable anti-DENV antibodies and had one or more documented DENV infections during their time in the cohort. A total of 97 children with previous RT-PCR-confirmed DENV serotype 1 (DENV1), DENV2, or DENV3 infections from 2007 to 2013, prior to the introduction of ZIKV in 2016, served as DENV-positive ZIKV-negative controls. In addition, 77 individuals who had never experienced ZIKV or DENV infection served as negative controls.

**Composite reference.** As there is no gold standard for Zika diagnosis, a composite reference standard was defined for the calculation of assay sensitivity and specificity (24, 25). To be considered Zika positive by the composite reference, the acute-phase serum sample of a case had to test positive by at least one molecular method and the patient had to seroconvert on the basis of at least one serological method. Sensitivity for each assay was defined as the percentage of cases that tested positive among the composite reference-positive cases. Specificity for each assay was defined as the percentage of cases that tested negative among the composite reference-negative cases.

**NIAID-BEI MAC-ELISA.** A MAC-ELISA, developed on the basis of the initial CDC Emergency Use Authorization (March 2016), was manufactured and supplied by the NIAID-NIH BEI Resources Repository (catalog no. NR-50449) for the assessment of human IgM antibody to ZIKV in human serum (HS) by enzyme immunoassay (23). Briefly, 75  $\mu$ l anti-human IgM antibody was coated on 8-well polystyrene Maxisorp strips (Nunc Thermo Scientific) and incubated overnight, followed by one wash with phosphate-buffered saline with 0.05% Tween 20 (PBS-T) and blocking with PBS-T containing 5% nonfat dry milk. After a 30-min incubation at room temperature, 50  $\mu$ l of serum samples and negative control diluted 1:400 in PBS-T and positive control (anti-ZIKV human IgM positive control from the kit) diluted 1:125 in PBS-T were added to individual wells. After 1 h of incubation at 37°C, 50  $\mu$ l of ZIKV antigen and negative-control antigen was added, and the reaction mixture was incubated overnight, followed by addition of 50  $\mu$ l horseradish peroxidase (HRP)-conjugated monoclonal antibody (anti-flavivirus MAb clone 6B6C-1, which binds to a conserved domain of flavivirus E protein) diluted 1:1,000 in blocking solution (5% nonfat dry milk), and 75  $\mu$ l of 3,3',-5,5' tetramethylbenzidine (TMB; Sigma). The reaction was terminated with 50  $\mu$ l 12.5% sulfuric acid. After each incubation period, wells were washed 4 times with PBS-T and 5 times after the addition of conjugated antibody. Optical absorbance was measured using a Microplate Photometer (Multiskan FC Thermo Scientific) at 450 nm. All samples with absorbance values of  $\geq 0.2$  were considered positive. A paired sample was considered positive when the acute-phase sample was negative and the convalescent-phase sample was positive (seroconversion).

**CNDR MAC-ELISA.** ZIKV-specific IgM antibodies were detected by an in-house IgM-capture ELISA (CNDR MAC-ELISA) similar to that described for the detection of anti-DENV and anti-chikungunya virus (anti-CHIKV) IgM antibodies (26, 27). The assay was adapted to the detection of anti-ZIKV IgM antibodies using the strongly neutralizing ZIKV-specific monoclonal antibody (MAb) ZKA64 (Humabs Biomed SA) directed to ZIKV E domain III (14). Briefly, strips of polystyrene microwells were coated with 6.6  $\mu\text{g}/\text{ml}$  goat anti-human IgM (Life Technologies) in sodium carbonate-bicarbonate buffer (pH 9.5) and incubated overnight at room temperature, followed by blocking in 150  $\mu\text{l}$  of 1% bovine serum albumin (BSA) fraction V (Fisher) diluted in PBS-T. Each serum specimen was diluted 1:20 in PBS-T, and 50  $\mu\text{l}$  was added to the wells and incubated for 1 h at 37°C; all samples were processed in duplicate. ZIKV antigen was produced from strain MR766 (obtained from Michael S. Diamond, Washington University in St. Louis) in suckling mouse brain (BALB/c strain) using the sucrose-acetone extraction method (28). A 50- $\mu\text{l}$  volume of antigen diluted 1:20 in PBS-T was added to the wells, and the reaction mixture was incubated for 1 h at 37°C, followed by addition of 50  $\mu\text{l}$  of MAb ZKA64 conjugated to horseradish peroxidase (HRP) diluted 1:1,000 in PBS-T with 2.5% normal human serum (NHS). After 1 h at 37°C, 50  $\mu\text{l}$  of TMB substrate was added, and the strips were held at room temperature for 10 min until the reaction was terminated by the addition of 50  $\mu\text{l}$  of 12.5% sulfuric acid. Optical absorbance was measured using a Microplate Photometer at 450/630 nm. After each incubation period, the wells were washed 4 times with PBS-T and 5 times after the addition of conjugated antibody. The cutoff value was determined so as to maximize specificity on the basis of testing negative controls and known DENV-positive samples collected during the period before the introduction of Zika and as such was defined as 6.6 times the average of the negative controls. All samples with absorbance values above the cutoff value were considered positive. As described above, seroconversion in paired samples indicated a positive result for the case.

**NS1 blockade-of-binding ELISA (NS1 BOB).** ZIKV-specific anti-NS1 antibodies in samples were detected as described by Balmaseda et al. (22). Briefly, polystyrene Maxisorp plates were coated overnight with 1  $\mu\text{g}/\text{ml}$  of ZIKV NS1 (MR766 strain; Native Antigen Company, Inc.) in coating buffer (PBS) at 4°C. Plates were blocked for 1 h with PBS containing 1% BSA and then washed two times with PBS-T. Serum (1:10 dilution in PBS–1% BSA) was added to NS1-coated ELISA plates. After 1 h, an equal volume of biotinylated anti-NS1 ZKA35 (Humabs Biomed) (20 ng/ml) was added to samples, and the mixture was incubated at room temperature for 15 min (final dilution of the plasma in the mixture, 1:20). Plates were washed with PBS-T four times, and alkaline phosphatase-conjugated streptavidin (Jackson Immuno Research) was added for 1 h. Plates were washed with PBS-T five times, and the substrate *para*-nitrophenyl phosphate was added for 20 min. Optical absorbance was then measured using a microplate photometer at 405 nm. BOB values were calculated as follows:  $\{1 - [(OD \text{ sample} - OD \text{ minimum value}) / \Delta \text{value}]\} \times 100$ , where OD represents optical density. A BOB value of  $\geq 50$  was considered positive, and a BOB value of  $< 50$  was considered negative. As described above, seroconversion in paired samples indicated a positive result for the case.

**Inhibition ELISA method (IEM).** An in-house inhibition ELISA similar to that described by Galo et al. (26) was established and used to detect and determine the titer of total ZIKV-specific antibodies in serum. Briefly, 96-well polystyrene Maxisorp plates were coated with MAb ZKA64 (5  $\mu\text{g}/\text{ml}$ ). After three washes with PBS-T, 150  $\mu\text{l}$  of 1% BSA diluted in PBS-T was added, and the reaction mixture was incubated for 30 min at 37°C. ZIKV antigen (100  $\mu\text{l}$ ) produced in infected suckling mouse brains (see above) and diluted 1:20 in PBS-T was added to each well, and plates were incubated for 1 h at 37°C. After four washes with PBS-T, 100  $\mu\text{l}$  of samples, positive control (serially diluted in PBS-T–0.5% BSA from 1:10 to 1:100,000), and negative control (at a dilution of 1:10) were added, and the reaction mixture was incubated for 2 h at 37°C. After four washes with PBS-T, 100  $\mu\text{l}$  of HRP-conjugated MAb ZKA64 diluted 1:1,000 in PBS–2.5% NHS was added to each well. The plates were incubated for 30 min at 37°C and washed five times with PBS-T, and then 50  $\mu\text{l}$  of substrate TMB was added to each well. The plates were held at room temperature for 30 min, the reaction was stopped with 50  $\mu\text{l}$  of 12.5% sulfuric acid, and the optical density was measured using a Microplate Photometer at 450 nm and 630 nm. The percentage of inhibition was calculated using the following formula:

$$\text{inhibition percent (\%I)} = [1 - (\text{absorbance of sample} / \text{average absorbance of negative controls})] \times 100 \quad (1)$$

The sample titer was then calculated using the Reed and Muench method [29] as follows:

$$10^{\wedge}[\log_{10}(T_{\geq 50\%}) + (D_{=50\%} - 50) / (D_{\geq 50\%} - D_{<50\%})] \quad (2)$$

where  $D_{\geq 50\%}$  is percent inhibition of the last serum dilution with  $\geq 50\%$  inhibition,  $T_{\geq 50\%}$  is the last serum dilution at which  $\geq 50\%$  inhibition was observed, and  $D_{<50\%}$  is percent inhibition of the first serum dilution with  $< 50\%$  inhibition. The cutoff value was determined based on previously published DENV and CHIKV IEM assays (18, 26, 30).

**Molecular testing.** Viral RNA was extracted using a QIAamp viral RNA minikit (Qiagen) with 140  $\mu\text{l}$  of serum and a 60- $\mu\text{l}$  elution volume. All rRT-PCRs were performed on an ABI 7500 Fast instrument (Applied Biosystems) using 25- $\mu\text{l}$  reaction mixtures, a SuperScript III Platinum One-Step qRT-PCR kit (Life Technologies), and 5  $\mu\text{l}$  of RNA template.

The ZCD (Zika virus, chikungunya virus, and dengue virus) rRT-PCR assay detects and differentiates RNA from ZIKV, CHIKV, and DENV during the acute phase of illness, using target sequences in ZIKV NS4B (16). The assay was performed as previously described (16). Each run included a no-template control and positive controls for ZIKV, CHIKV, and DENV. A result was interpreted as positive if the reaction generated an exponential curve that crossed a threshold that was set manually, as described previously (31). The Trioplex real-time RT-PCR assay (Trioplex rRT-PCR) was developed by the CDC for the simultaneous

**TABLE 2** Results of CNDR MAC-ELISA and NIAID-BEI MAC-ELISA in acute-phase and early convalescent-phase samples for diagnosis of Zika cases<sup>a</sup>

Patient group	CNDR MAC-ELISA		NIAID-BEI MAC-ELISA	
	% Sens (95% CI)	% Spec (95% CI)	% Sens (95% CI)	% Spec (95% CI)
Overall	94.5 (89.0–97.8)	85.6 (79.5–90.5)	70.1 (61.3–77.9)	82.8 (76.3–88.1)
ZIKV positive	94.5 (89.0–97.8)	NA	70.1 (61.3–77.9)	NA
DENV naïve	96.8 (88.8–99.6)	NA	69.4 (56.3–80.4)	NA
DENV immune	92.3 (83.0–97.5)	NA	70.8 (58.2–81.4)	NA
DENV positive	NA	77.3 (67.7–85.2)	NA	69.1 (58.9–78.1)
DENV1	NA	66.7 (44.7–84.4)	NA	66.7 (44.7–84.4)
DENV2	NA	82.4 (65.5–93.2)	NA	70.6 (52.5–84.9)
DENV3	NA	79.5 (63.5–90.7)	NA	69.2 (52.4–83)
Primary	NA	70.8 (55.9–83.0)	NA	52.1 (37.2–66.7)
Secondary	NA	83.7 (70.3–92.7)	NA	85.7 (72.8–94.1)
Negative	NA	96.1 (89.0–99.2)	NA	100 (95.3–100.0)

<sup>a</sup>Sens, sensitivity. Sensitivity values were calculated for each assay based on ZIKV-positive samples, stratified by those who had been previously infected with DENV (DENV immune) and those who were DENV naïve. Spec, specificity. Specificity values were calculated for each assay based on two groups of ZIKV-negative samples: (i) ZIKV-negative DENV-positive samples, stratified by DENV subtype (DENV1 to DENV3) and primary and secondary infection, and (ii) ZIKV-negative DENV-negative controls. 95% CI, 95% confidence interval. NA, not applicable: data from ZIKV-positive cases served only for determination of sensitivity and were not used for the specificity calculation. Similarly, data from ZIKV-negative cases served only for determination of specificity and were not used for the sensitivity calculation.

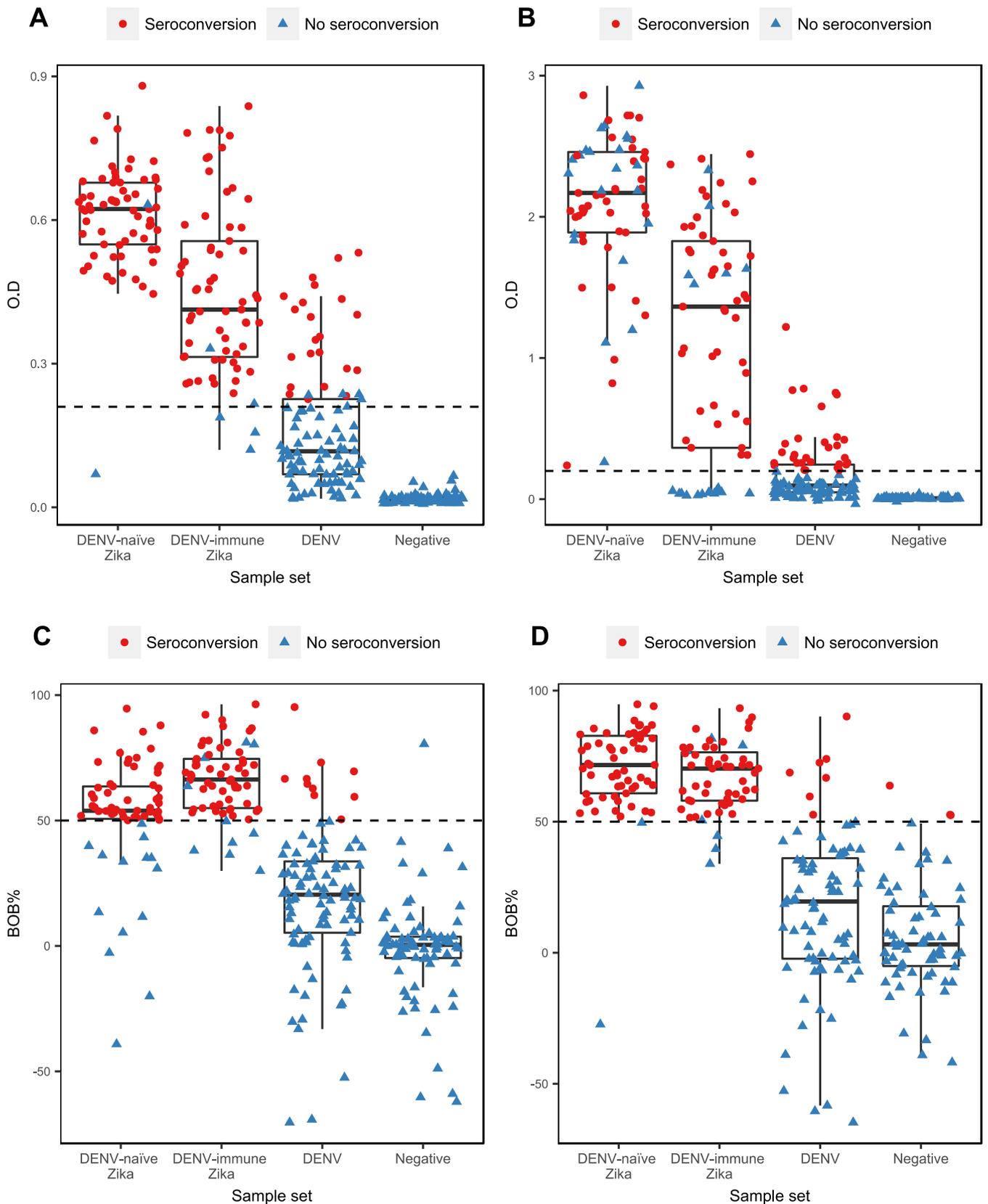
qualitative detection of RNA from ZIKV, DENV, and CHIKV. The ZIKV primers and dually labeled hydrolysis probe target sequences in the E gene. The Triplex assay was performed and interpreted according to the package insert instructions. The ZIKV result was interpreted as positive if the reaction generated an exponential curve that crossed the instrument-defined threshold within  $\leq 38$  cycles (15).

**Statistical analysis.** Sensitivity and specificity values and 95% confidence intervals were calculated using Epi Info version 3.3.2 (CDC). Relative and absolute frequencies are reported for categorical variables, and means and standard deviations are reported for quantitative variables. We used RStudio Inc. software version 1.0.136 with data packet ggplot2 version 2.20 for generating graphs. Fisher's exact test was used to calculate *P* values.

## RESULTS

Four serological and two molecular methods were evaluated for detection of ZIKV infection in 301 participants of the Pediatric Dengue Cohort Study (PDCS). Demographic data are shown in Table 1 (see also Table S1 in the supplemental material). Among the 301 individuals included, 97 had cases of RT-PCR-confirmed DENV infections (half primary and half secondary infections with each of serotypes DENV1, DENV2, and DENV3), 127 represented ZIKV-positive cases (62 DENV-naïve and 65 DENV-immune patients), and 77 were ZIKV negative and DENV negative (Table 1).

For detection of IgM seroconversion in acute-phase and early-convalescent-phase samples among Zika patients, the in-house CNDR MAC-ELISA had an overall sensitivity of 94.5% (95% confidence interval, 89.0 to 97.8), whereas the BEI MAC-ELISA yielded a sensitivity of 70.1% (61.3 to 77.9) (Table 2). Statistical analysis indicated that the CNDR MAC-ELISA was significantly more sensitive than the NIAID-BEI MAC-ELISA ( $P = 0.0399$ , Fisher's exact test). The respective sensitivities of the two assays for DENV-naïve and DENV-immune ZIKV infections were comparable. Panels A and B of Fig. 1 present the data obtained with early-convalescent-phase samples, and results from paired acute-phase and convalescent-phase samples are shown in Fig. 2A and B, with both figures indicating which individuals underwent seroconversion. The overall specificities were 85.6% (79.5% to 90.5%) and 82.8% (76.3% to 88.1%) for the CNDR MAC-ELISA and NIAID-BEI MAC-ELISA, respectively. Restricting the analysis to confirmed ZIKV-negative DENV-positive cases, the ZIKV specificity of the CNDR assay was higher (77.3% [67.7% to 85.2%]) than that of the BEI assay (69.1% [58.9% to 78.1%]). Interestingly, DENV1



**FIG 1** Box-and-whisker plots graphing results from convalescent-phase samples obtained using each serological method, stratified by sample set. (A) CNDR MAC-ELISA, early-convalescent-phase samples. (B) NIAID-BEI MAC-ELISA, early-convalescent-phase samples. (C) ZIKV NS1 BOB ELISA, early-convalescent-phase samples. (D) ZIKV NS1 BOB ELISA, late-convalescent-phase samples. Red dots represent results from convalescent-phase samples that underwent seroconversion (Continued on next page)

showed the greatest serological cross-reactivity with ZIKV among the three DENV serotypes.

For the NS1 BOB ELISA, we analyzed acute-phase/early-convalescent-phase seroconversion and acute-phase/late-convalescent-phase seroconversion separately to evaluate its utility for diagnosis as well as for surveillance in samples collected several months postinfection. The NS1 BOB ELISA was significantly more sensitive at the late-convalescent-phase time point, with a sensitivity of 96.5% (91.2 to 99.0) compared to 85.0% (77.6 to 90.7) at the early-convalescent-phase time point ( $P = 0.0015$ ; Table 3). This is clearly depicted in Fig. 1C and D and in plots of paired acute-phase and convalescent-phase samples (Fig. 2C and D), where the individuals who underwent seroconversion are indicated. At early convalescence, the NS1 BOB ELISA displayed higher sensitivity in DENV-immune patients (92.3% [83.0 to 97.5]) than in DENV-naïve patients (77.4% [65.0 to 87.1]); however, this difference was not apparent at late convalescence. High specificity was observed at both the early (91.4% [86.2 to 95.1]) and late (92.6% [87.2 to 96.3]) time points.

With the IEM, a case was considered positive when the titer at early convalescence was  $\geq 4$ -fold higher than the acute-phase titer or when seroconversion was observed. In this diagnostic scenario, the sensitivity was 68.3% (59.4% to 76.3%) and the specificity was 58.8% (51.0% to 66.3%) (Table 4; see also Fig. S1 in the supplemental material). Another application of this method is comparing titers in paired annual samples in cohort studies to determine the incidence of ZIKV infection (20). When paired annual samples were evaluated with the IEM, positivity was defined as seroconversion or a  $\geq 4\times$  increase in titer (Fig. S1), yielding a sensitivity of 94.0% (88.0% to 97.5%) and a specificity of 98.6% (94.9% to 98.8%) (Table 4). The IEM was significantly more sensitive and specific ( $P < 0.0001$  for both) in paired annual samples than in samples from the acute-phase and early-convalescent-phase time points. Comparing the NS1 BOB ELISA and IEM techniques for paired acute-phase and early-convalescent-phase samples, the NS1 BOB ELISA was significantly more sensitive and specific than the IEM ( $P = 0.0027$  and  $<0.0001$ , respectively).

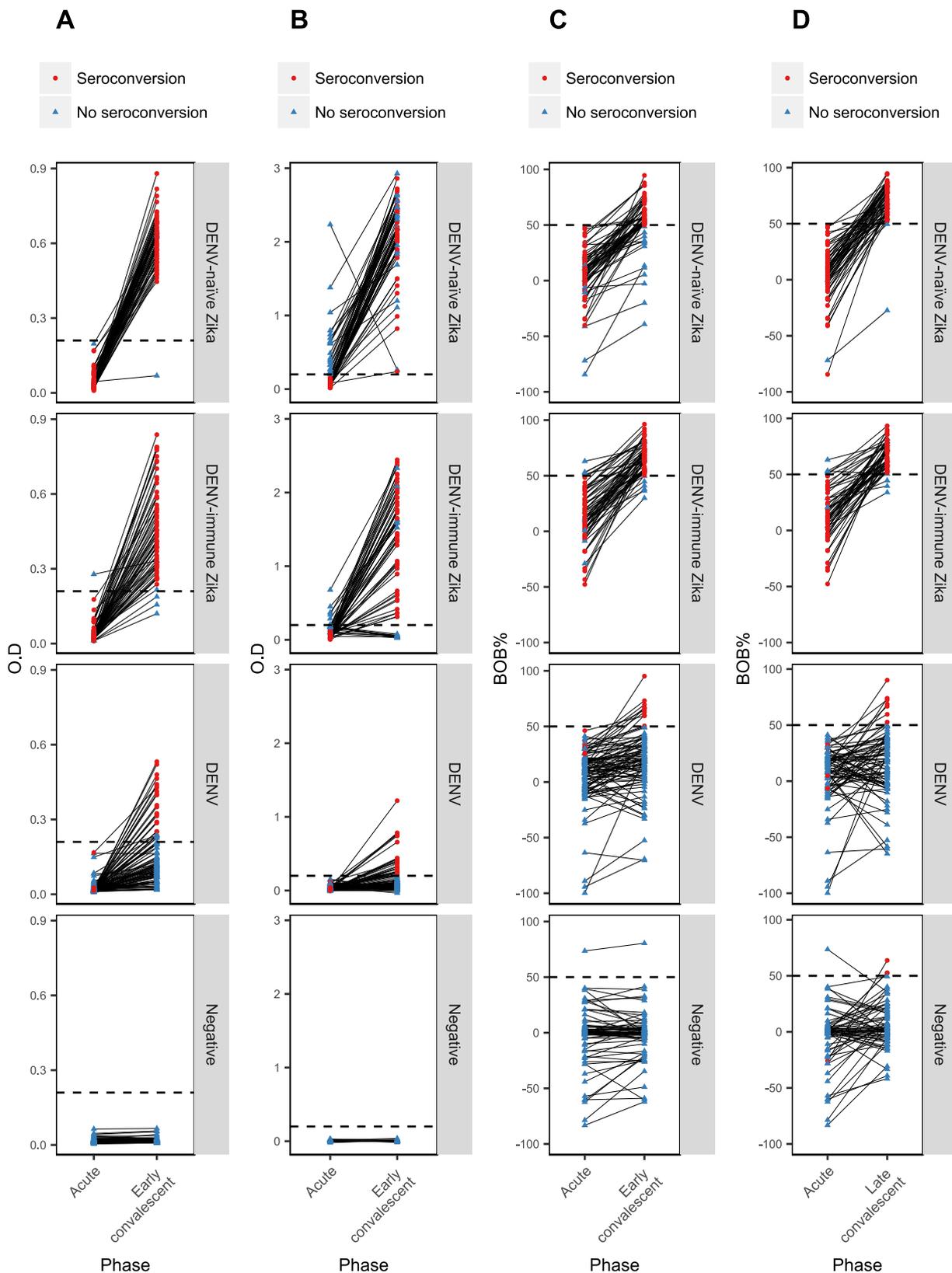
The two molecular methods performed similarly and demonstrated very good agreement ( $\kappa = 0.93$ ) for ZIKV detection in acute-phase samples. The overall sensitivity and specificity of each assay were 96.1% and 100%, respectively (Table 5). There were 10 samples with discordant rRT-PCR results, with 5 samples testing positive in only one of the two assays. All discordant samples had late threshold cycle ( $C_T$ ) values in the respective assays of  $>38$  for samples positive only in the ZCD assay and  $>35$  for samples positive only in the Triplex assay. However, all rRT-PCR-positive samples also tested positive by at least one serological method.

## DISCUSSION

Since the introduction of ZIKV into the Americas in 2015, accurately differentiating ZIKV and DENV infections has been a challenge. Serological cross-reactivity among flaviviruses (8, 10, 11, 13) and the low ZIKV viral load and short period of detectable viremia (32, 33) have complicated serological diagnosis and molecular diagnosis, respectively. Hence, it is necessary to thoroughly evaluate multiple diagnostic assays. However, the evaluation of different assays has been hindered by limited access to adequate numbers of well-characterized samples. Over the past 14 years, the Nicaraguan PDCS, with an average size of  $\sim 3,600$  active participants each year, has accumulated a large collection of serum samples, including paired acute- and early-convalescent-phase samples from episodes of febrile illness together with annual healthy samples from subjects with well-documented histories of arbovirus infections (20). Hence, these samples are well-suited for diagnostic assay evaluation. In the

### FIG 1 Legend (Continued)

from the acute to convalescent phase in paired samples. Blue triangles represent results from convalescent-phase samples that did not undergo seroconversion. The dotted horizontal lines indicate the cutoff values for each method. For the CNDR MAC-ELISA, the mean cutoff value is depicted, while the other methods have a fixed cutoff value.



**FIG 2** Graphical representation of paired acute-phase and convalescent-phase samples for each serological method, stratified by sample set. (A) CNDR MAC-ELISA, acute-phase and early-convalescent-phase samples. (B) NIAID-BE1 MAC-ELISA, acute-phase and early-convalescent-phase samples. (C) Zika NS1 BOB ELISA, acute-phase and early-convalescent-phase samples. (D) Zika NS1 BOB ELISA, acute-phase and late-convalescent-phase samples. Red dots represent the acute/convalescent pairs that underwent seroconversion. Blue triangles represent the acute/convalescent pairs that did not undergo seroconversion. The dotted horizontal lines represent the cutoff values for each method. For the CNDR MAC-ELISA, the mean cutoff value is depicted, while the other methods have a fixed cutoff value.

**TABLE 3** Results of Zika NS1 BOB ELISA in early and late convalescent-phase samples for diagnosis and surveillance of ZIKV infections<sup>a</sup>

	NS1 BOB ELISA			
	Early convalescence		Late convalescence	
Patient population	Sens (95% CI)	Spec (95% CI)	Sens (95% CI)	Spec (95% CI)
Overall	85.0 (77.6–90.7)	91.4 (86.2–95.1)	96.5 (91.2–99.0)	92.6 (87.2–96.3)
ZIKV positive	85.0 (77.6–90.7)	NA	96.5 (91.2–99.0)	NA
DENV naïve	77.4 (65.0–87.1)	NA	98.3 (90.8–100.0)	NA
DENV immune	92.3 (83.0–97.5)	NA	94.5 (84.9–98.9)	NA
DENV positive	NA	88.7 (80.6–94.2)	NA	90.0 (81.2–95.6)
DENV1	NA	95.8 (78.9–99.9)	NA	85.0 (62.1–96.8)
DENV2	NA	85.3 (68.9–95.0)	NA	89.7 (72.2–97.8)
DENV3	NA	87.2 (72.6–95.7)	NA	93.5 (78.6–99.2)
Primary	NA	100.0 (92.6–100.0)	NA	89.5 (75.2–97.1)
Secondary	NA	77.6 (63.4–88.2)	NA	90.5 (77.4–97.3)
Negative	NA	94.8 (87.2–98.6)	NA	95.7 (87.8–98.1)

<sup>a</sup>Sens, sensitivity. Sensitivity values were calculated for each assay based on ZIKV-positive samples, stratified by those who had been previously infected with DENV (DENV immune) and those who were DENV naïve. Spec, specificity. Specificity values were calculated for each assay based on two groups of ZIKV-negative samples: (i) ZIKV-negative DENV-positive samples, stratified by DENV subtype (DENV1 to DENV3) and primary and secondary infection, and (ii) ZIKV-negative DENV-negative controls. 95% CI, 95% confidence interval. NA, not applicable: data from ZIKV-positive cases served only for determination of sensitivity and were not used for the specificity calculation. Similarly, data from ZIKV-negative cases served only for determination of specificity and were not used for the sensitivity calculation.

present study, we assessed four serological and two molecular methods for the diagnosis and surveillance of ZIKV infection using sample sets from a total of 301 patients, including flavivirus-immune and flavivirus-naïve individuals with Zika, dengue, or other febrile illnesses.

Among the serological methods evaluated, two detect anti-ZIKV IgM antibodies; one detects total antibodies against the ZIKV NS1 protein, and the other measures total antibodies against ZIKV. The CNDR MAC-ELISA was significantly more sensitive and

**TABLE 4** Results of IEM for diagnosis of Zika cases and determination of ZIKV infection incidence<sup>a</sup>

	Inhibition ELISA			
	Diagnosis		Infection incidence	
Patient population	Sens (95% CI)	Spec (95% CI)	Sens (95% CI)	Spec (95% CI)
Overall	68.3 (59.4–76.3)	58.8 (51.0–66.3)	94.0 (88.0–97.5)	98.6 (94.9–98.8)
Zika virus positive	68.3 (59.4–76.3)	NA	94.0 (88.0–97.5)	NA
DENV naïve	55.7 (42.4–68.5)	NA	96.6 (88.1–99.6)	NA
DENV immune	80.0 (68.2–88.9)	NA	90.9 (80.0–97.0)	NA
Dengue virus positive	NA	40.6 (30.7–51.1)	NA	97.2 (90.3–99.7)
DENV1	NA	41.7 (22.1–63.4)	NA	100.0 (82.4–100.0)
DENV2	NA	38.2 (22.2–56.4)	NA	100.0 (86.3–100.0)
DENV3	NA	42.1 (26.3–59.2)	NA	92.3 (76.5–99.1)
Primary	NA	60.4 (45.3–74.2)	NA	100.0 (89.4–100.0)
Secondary	NA	20.8 (10.5–35.0)	NA	94.9 (82.7–99.4)
Negative	NA	82.4 (71.8–90.3)	NA	100.0 (94.7–100.0)

<sup>a</sup>Sens, sensitivity. Sensitivity values were calculated for each assay based on ZIKV-positive samples, stratified by those who had been previously infected with DENV (DENV immune) and those who were DENV naïve. Spec, specificity. Specificity values were calculated for each assay based on two groups of ZIKV-negative samples: (i) ZIKV-negative DENV-positive samples, stratified by DENV subtype (DENV1 to DENV3) and primary and secondary infection, and (ii) ZIKV-negative DENV-negative controls. 95% CI, 95% confidence interval. NA, not applicable: data from ZIKV-positive cases served only for determination of sensitivity and were not used for the specificity calculation. Similarly, data from ZIKV-negative cases served only for determination of specificity and were not used for the sensitivity calculation.

**TABLE 5** Evaluation of real-time RT-PCR methods<sup>a</sup>

Patient population	RT-PCR			
	ZCD		Trioplex	
	Sens (95% CI)	Spec (95% CI)	Sens (95% CI)	Spec (95% CI)
Overall	96.1 (91.1–98.7)	100 (97.9–100)	96.1 (91.1–98.7)	100 (97.9–100)
Zika virus positive	96.1 (91.1–98.7)	NA	96.1 (91.1–98.7)	NA
DENV naïve	93.5 (84.3–98.2)	NA	95.2 (86.5–99)	NA
DENV immune	98.5 (91.7–100)	NA	96.9 (89.3–99.6)	NA
Dengue virus positive	NA	100 (96.3–100)	NA	100 (96.3–100)
DENV1	NA	100 (85.8–100)	NA	100 (85.8–100)
DENV2	NA	100 (89.7–100)	NA	100 (89.7–100)
DENV3	NA	100 (91–100)	NA	100 (91–100)
Negative	NA	100 (95.3–100)	NA	100 (95.3–100)

<sup>a</sup>Sens, sensitivity. Sensitivity values were calculated for each assay based on ZIKV-positive samples, stratified by those who had been previously infected with DENV (DENV immune) and those who were DENV naïve. Spec, specificity. Specificity values were calculated for each assay based on two groups of ZIKV-negative samples: (i) ZIKV-negative DENV-positive samples, stratified by DENV subtype (DENV1 to DENV3) and primary and secondary infection, and (ii) ZIKV-negative DENV-negative controls. 95% CI, 95% confidence interval. NA, not applicable: data from ZIKV-positive cases served only for determination of sensitivity and were not used for the specificity calculation. Similarly, data from ZIKV-negative cases served only for determination of specificity and were not used for the sensitivity calculation.

more specific than the NIAID-BEI MAC-ELISA. The use of a conjugated MAb, ZKA64, which is highly neutralizing and is specific for domain III of the ZIKV E protein (14), in the CNDR MAC-ELISA, compared to the NIAID-BEI MAC-ELISA, which uses a less-specific anti-flavivirus MAb, could explain the differences between the assays. Importantly, there are substantial differences in processing time between the two IgM ELISAs. The CNDR MAC-ELISA takes 3 to 4 h, whereas the NIAID-BEI MAC-ELISA BEI takes 2 to 3 days. The shorter processing time is very important for epidemiological surveillance during epidemics, as evidenced in Nicaragua during the 2016 Zika virus disease epidemic.

Overall, the CNDR and NIAID-BEI MAC-ELISAs performed well. In another report, Safronetz et al. (34) evaluated 5 commercial Zika immunoassays and concluded that most of them had adequate (higher than 90%) specificity in returned Canadian travelers but low sensitivity (37% to 65%), except for the InBios ELISA, which had 100% sensitivity but low specificity in samples from dengue cases. Another study reported sensitivity for Zika IgM detection by the Euroimmun assay of 39.5%, which improved to 83% when the assay was combined with IgG detection (19). The specificity was 92%; however, only samples from flavivirus-naïve or likely primary DENV infections were evaluated, whereas our study measured specificity by using samples from both primary and secondary DENV infections as well as from flavivirus-naïve individuals.

In contrast to the MAC-ELISA, the Zika NS1 BOB ELISA can be used for both diagnosis and seroprevalence studies as it likely primarily measures levels of IgG antibodies. However, as it shows higher sensitivity in later convalescence, the optimal use is for surveillance and seroprevalence studies. The Zika NS1 BOB method was initially evaluated in a multicenter study involving laboratories from 5 countries, yielding sensitivity and specificity values of 92% and 96%, respectively (22). In the present evaluation, similar results were obtained, with higher sensitivity in the late-convalescent-phase samples (mean of 223 days postinfection) than in the early-convalescent phase samples (mean of 16.7 days). This is consistent with the previous results, where the sensitivity of the NS1 BOB ELISA was increased at >20 days post-onset of symptoms (22). We also noted that the sensitivity in early convalescence was greater in DENV-immune patients than in DENV-naïve Zika patients, suggesting more rapid kinetics of the antibody response in secondary flavivirus infections, as expected. Thus, unlike DENV or CHIKV infections, where antibody titers rise sufficiently by days 14 to 17 postinfection, it appears that anti-NS1 antibodies rise more slowly in ZIKV infections, with even slower kinetics in DENV-naïve Zika patients. One possible explanation is the lower

viremia of ZIKV than of DENV and CHIKV, which could result in lower antibody titers and/or slower antibody kinetics.

Regarding specificity, Balmaseda et al. (22) reported a specificity of 89% for the NS1 BOB ELISA in evaluation of dengue cases, coinciding with the current study, which yielded 88.7% specificity for dengue cases. The reported specificity increased to 96% when samples from patients infected with other flaviviruses or other viruses and healthy donors were included (22); in the present study, the specificity increased to 91.4 to 92.6% when samples from flavivirus-negative patients were included. In general, these results indicate that the NS1 BOB ELISA can be useful for seroprevalence studies of anti-ZIKV antibodies at the population level, differentiating individuals infected by ZIKV from those infected by other flaviviruses.

Two applications of the Zika IEM were evaluated, based on our experience with the dengue IEM (35–37). To diagnose febrile episodes, a  $\geq 4$ -fold increase in titer or seroconversion between the acute-phase samples and early-convalescent-phase samples was used; however, the Zika IEM performed poorly in this setting. Again, this was potentially due to low ZIKV viremia resulting in delayed and lower antibody responses. The other scenario is to detect incidence of ZIKV infections by comparing titers in paired annual samples bounding the intervening year. Here, the IEM performed better, with a sensitivity value of 94% and specificity of 98.6%. The IEM has been used successfully for 14 years for anti-DENV antibody detection in the PDCS and other Nicaraguan studies (18, 30, 35–37). A similar method was adapted for chikungunya diagnosis and surveillance, with sensitivity and specificity greater than 95% (26).

For Zika diagnosis, using paired acute-phase and early-convalescent-phase samples, the IgM-based CNDR MAC-ELISA technique is recommended, since it is based on a domain III ZIKV E monoclonal antibody and yielded 94.5% sensitivity and 85.6% specificity, performing better than the NIAID-BEI MAC ELISA, the NS1 BOB ELISA, and the IEM. For paired acute-phase and late-convalescent-phase samples, as well as seroprevalence studies, the NS1 BOB ELISA is recommended, as it was highly sensitive (96.5%) and specific (92.6%) and has a shorter processing time than the IEM. If paired annual samples are available (as in a cohort study), the IEM technique is recommended for determination of infection incidence.

Regarding molecular methods, two multiplex rRT-PCR assays were evaluated, the ZCD method targeting the ZIKV NS4B gene and the Triplex assay directed to the E gene of the ZIKV genome. Both rRT-PCR assays demonstrated high sensitivity and specificity for ZIKV (96.1% and 100%, respectively), which is consistent with previous results for the ZCD assay evaluated against a monoplex rRT-PCR (16). Thus, the multiplex design of the two assays evaluated here does not appear to affect the sensitivity of ZIKV detection. As there is no gold standard for Zika diagnosis, the current study utilized a composite reference standard that required at least one positive molecular result and one positive serological result for a case to be considered positive. This, coupled with the use of samples from confirmed dengue cases and from cases of nondengue, non-Zika febrile illnesses, provides strong evidence that these rRT-PCR assays provide sensitive and specific ZIKV detection in the acute setting.

Overall, the strengths of this study were the sample size and the use of well-characterized cases. Importantly, equal numbers of DENV-immune and DENV-naïve Zika cases were evaluated, as well as equal numbers of primary and secondary infections with DENV1, DENV2, and DENV3. Data corresponding to prior DENV exposure were not determined by preinfection antibody titers, which can potentially be misleading, but rather by inclusion of children who had entered the cohort study in a DENV-naïve state and who had had no DENV infections (DENV-naïve children) or had experienced a documented DENV infection or had entered the PDCS with serological evidence of prior DENV infection (DENV-immune children). Of note, all the evaluations were performed in Nicaragua. One limitation was that there is very little circulation of DENV4 in Nicaragua and that clinical illness due to DENV4 is very rare; therefore, this analysis focused on DENV serotypes 1, 2, and 3.

In conclusion, four serological methods were evaluated, confirming their usefulness

for the diagnosis of ZIKV infections (e.g., both IgM MAC-ELISAs) and/or for surveillance and seroprevalence studies (e.g., NS1 BOB ELISA, Zika IEM), including two in-house serological assays and the NS1 BOB ELISA, which provide low-cost alternatives to commercial kits. Overall, together with the two molecular assays that performed well, we have established and evaluated both molecular and serological methods that allow discrimination of current and past ZIKV infection in regions where infections by other flaviviruses, such as DENV, are also endemic.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JCM.01785-17>.

**SUPPLEMENTAL FILE 1**, PDF file, 0.1 MB.

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We report no conflicts of interest.

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