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MAJOR ARTICLE

Distinguishing Secondary Dengue Virus Infection From Zika Virus Infection With Previous Dengue by a Combination of 3 Simple Serological Tests

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Background. The explosive spread of Zika virus (ZIKV) and associated microcephaly present an urgent need for sensitive and specific serodiagnostic tests, particularly for pregnant women in dengue virus (DENV)–endemic regions. Recent reports of enhanced ZIKV replication by dengue-immune sera have raised concerns about the role of previous DENV infection on the risk and severity of microcephaly and other ZIKV complications.

Methods. Enzyme-linked immunosorbent assays (ELISAs) based on ZIKV and DENV nonstructural protein 1 (NS1) were established to test acute, convalescent phase, and post–convalescent phase serum/plasma samples from reverse-transcription polymerase chain reaction–confirmed cases including 20 primary ZIKV, 25 ZIKV with previous DENV, 58 secondary DENV, and 16 primary DENV1 infections.

Results. ZIKV-NS1 immunoglobulin M (IgM) and immunoglobulin G (IgG) ELISAs combined can detect ZIKV infection with a sensitivity of 95% and specificity of 66.7%. The ZIKV-NS1 IgG cross-reactivity by samples from secondary DENV infection cases ranged from 66.7% to 28.1% (within 1 month to 1–2 years post-illness, respectively). Addition of DENV1-NS1 IgG ELISA can distinguish primary ZIKV infection; the ratio of absorbance of ZIKV-NS1 to DENV1-NS1 IgG ELISA can distinguish ZIKV with previous DENV and secondary DENV infections with a sensitivity of 87.5% and specificity of 81.3%. These findings were supported by analysis of sequential samples.

Conclusions. An algorithm for ZIKV serodiagnosis based on 3 simple ELISAs is proposed to distinguish primary ZIKV, ZIKV with previous DENV, and secondary DENV infections; this could be applied to serodiagnosis for ZIKV, serosurveillance, and monitoring ZIKV infection during pregnancy to understand the epidemiology, pathogenesis, and complications of ZIKV in dengue-endemic regions.

Keywords. Zika virus; dengue virus; non-structural protein 1; serological test; cross-reactivity.

The explosive spread of Zika virus (ZIKV) and its association with microcephaly and other birth defects present an urgent need for diagnostic tests of high sensitivity and specificity, particularly for pregnant women [1–4]. ZIKV belongs to the family Flaviviridae, which includes several human pathogens such as the 4 serotypes of dengue virus (DENV1–4), West Nile virus (WNV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), and tick-borne encephalitis virus (TBEV) [5]. The current Centers for Disease Control and Prevention (CDC) guidelines for the laboratory diagnosis of ZIKV infection include a positive reverse-transcription polymerase chain reaction (RT-PCR) test within 2 weeks or as soon as possible after onset of symptoms to confirm ZIKV, and a negative immunoglobulin M (IgM) test to exclude ZIKV (Supplementary Figure 1) [6, 7]. Given that the majority (~80%) of ZIKV infections are asymptomatic, that many individuals seek ZIKV testing beyond the period with detectable RNA, and that ZIKV can be transmitted sexually including after asymptomatic infection, serological tests for ZIKV diagnosis is very important [1, 2, 8–11].

As the envelope (E) protein elicits the major antibody response after flaviviral infection, serological tests for flaviviruses have previously focused on the E protein, using either recombinant E protein, inactivated virions, or virus-like particles [5, 12–14]. Due to the antibody cross-reactivity of the E protein of ZIKV with other flaviviruses [14–16], positive or equivocal IgM tests based on E protein require confirmation by time-consuming

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plaque reduction neutralization tests (PRNTs) [6, 7], which can confirm infection in those acquiring ZIKV as their first flaviviral infection (primary ZIKV [pZIKV] infection) but not those who have experienced previous flaviviral infections. Several studies have shown that monoclonal antibodies (mAbs) against DENV E protein and dengue-immune sera can enhance ZIKV infection [17–20], known as antibody-dependent enhancement [21], raising the possibility that previous DENV infection may increase the risk and severity of congenital ZIKV infection and consequent fetal microcephaly. Given the spread of ZIKV and microcephaly in DENV-endemic regions, serological tests that can distinguish pZIKV infection from ZIKV infection with previous DENV infection (ZIKVwpDENV) are critically needed for diagnostics and to understand ZIKV pathogenesis and complications in pregnancy. A recent study using ZIKV nonstructural protein 1 (NS1) in IgM and immunoglobulin G (IgG) enzyme-linked immunosorbent assays (ELISAs) reported sensitivities of 58.8%, 88.2%, and 100% for IgM, IgG, and IgM/IgG combined, respectively, and a specificity of 99.8% based on 1015 healthy controls and 152 patients with other flaviviral infections including 93 travel-acquired DENV infection, mainly with primary DENV (pDENV) infection [22]. The current study investigated whether ZIKV-NS1 and DENV-NS1 ELISAs can distinguish pZIKV, ZIKVwpDENV, and secondary DENV (sDENV) infections.

METHODS

Clinical Samples

The study of coded serum or plasma samples was approved by the institutional review board (IRB) of the University of Hawaii (protocol numbers 17568 and 23786). Forty convalescent-phase samples (14–24 days post-symptom onset [PSO]) from confirmed Zika cases that were DENV naive (n = 20) or previously DENV exposed (n = 20), designated as pZIKV and ZIKVwpDENV panels, respectively, were obtained from the Pediatric Dengue Cohort Study (PDCS) in Managua, Nicaragua, between July and September 2016. The PDCS is a community-based prospective study of children since 2004 [23]. At the Health Center Sócrates Flores Vivas, acute and convalescent blood samples are drawn for dengue, Zika, and chikungunya virus (CHIKV) testing from patients meeting the case definition for dengue or Zika or presenting with undifferentiated febrile illness. ZIKV infection was confirmed by positive RT-PCR in serum and/or urine using triplex assays [24], CDC Trioplex [25], or in some cases the CDC ZIKV monoplex assay [15], in parallel with a DENV-CHIKV multiplex assay [26]. In the PDCS, anti-DENV antibodies are measured annually using an inhibition ELISA [27, 28] and based on paired annual samples, infections are defined by seroconversion or a ≥4-fold rise in anti-DENV titers. DENV naive was defined for those who had neither detectable anti-DENV antibodies at entry nor during follow-up in the cohort; DENV immune for those who had either detectable anti-DENV antibodies at entry or during follow-up. The PDCS was approved by the IRBs of the University of California, Berkeley, and Nicaraguan Ministry of Health. Parents or legal guardians of all subjects provided written informed consent; subjects aged ≥6 years provided assent.

Nineteen convalescent-phase samples from patients who presented with symptoms compatible with Zika and had detectable anti-DENV IgG antibodies (Euroimmun) during the acute stage (3 ZIKV RT-PCR positive; Real Star, Altana Diagnostics), designated as probable ZIKVwpDENV panel, were from the Complexo Hospital at Federal University of Bahia, Brazil, between November 2015 and May 2016 (with approval by the Federal University of Bahia IRB). Convalescent-phase (within 35 days PSO) or post–convalescent phase (3 months to 2 years PSO) dengue samples were from RT-PCR–confirmed dengue cases including 40 from Kaohsiung, Taiwan, between 2001 and 2009 [29, 30], 18 from Nicaragua (3 cases with sequential samples) between 2006 and 2008 [31], and 12 from the Big Island, Hawaii, during the 2015 DENV1 outbreak. pDENV1 and sDENV infections were determined by IgM/IgG ratio or focus-reduction neutralization tests [29, 30]. Flavivirus-naive samples (n = 12) were from previous studies [29–31]. Fifteen sequential plasma samples (from the index day when ZIKV RT-PCR tested positive [32, 33] to 3 months post–index day [PID]) from 5 blood donors who had detectable anti-DENV IgG antibodies (InBios) on the index day between June and September 2016, and 9 sequential plasma samples (7 days to 6 months PID) from 4 blood donors who had sDENV infection between 2011 and 2013 based on PRNTs, were provided by Blood Systems Research Institute at San Francisco (with approval by the University of California, San Francisco IRB). Supplementary Table 1 summarizes the sampling time, serotype, and source of different panels.

Recombinant Nonstructural Protein 1

Codon-optimization NS1 gene (residues 1–352) of ZIKV (HPF2013 strain) with a His-tag at the C-terminus (Integrated DNA Technologies) was cloned into pMT-Bip vector. Comparing HPF2013 with ZIKV strains from Nicaragua and Brazil, there is only 1 amino acid difference (residue 100, Nicaragua strains). Drosophila S2 cells were cotransfected with ZIKV-NS1 construct plus pHygro and selected with hygromycin B to establish stable clones (Supplementary Figure 2). After induction with $CuSO_4$, ZIKV-NS1 protein in supernatants was verified by polyacrylamide gel electrophoresis and Western blot analysis, followed by purification with HisTrap column in a fast purification chromatography system (AKTA Pure, GE). Purified DENV1-NS1 protein was purchased from the Native Antigen (United Kingdom).

Enzyme-Linked Immunosorbent Assays

For NS1-IgG ELISA, purified NS1 proteins (16 ng per well) were coated on 96-well plates overnight, followed by blocking and incubation with primary (serum or plasma at 1:400 dilution)

and secondary (anti-human IgG conjugated with horseradish peroxidase, Jackson ImmunoResearch) antibodies [30, 31]. The optical density at 450 nm (OD_{450}) was read with a reference wavelength of 630 nm. Each ELISA plate utilized the inner 60 wells and included 2 positives (OD >1; 2 confirmed Zika and 2 confirmed dengue samples for ZIKV- and DENV-NS1 ELISAs, respectively), 8 negatives (4 flavivirus-naive sera and 4 flavivirus-naive plasma), and samples (all in duplicates). The cutoff was defined by the mean OD value of negatives plus 12 standard deviations, which gave a confidence level of 99.9% from 4 negative controls [34, 35]. The OD values were divided by the mean OD value of 1 positive control (OD close to 1) in the same plate to calculate the relative OD (rOD) values for comparison between plates. NS1-IgM ELISA was performed similarly, except each sample was incubated with Gullsorb reagent (Meridian Bioscience), an IgG absorbent, for 10 minutes before adding to wells [36]. Each ELISA (containing samples in duplicates) was run twice. Two-tailed Mann-Whitney test was used for comparisons between 2 groups. Subsets of samples (Table 1) were tested by E protein–based IgM ELISAs including ZIKV- and DENV-detect IgM capture (MAC) ELISAs (InBios) to identify those qualified for analysis in the CDC testing algorithm.

RESULTS

Zika Virus-Nonstructural Protein 1 Enzyme-Linked Immunosorbent Assays

We first used ZIKV-NS1 IgM ELISA to test convalescent-phase serum or plasma samples from RT-PCR–confirmed cases with pZIKV, ZIKVwpDENV, pDENV, and sDENV infections. As shown in Table 1, 90% of pZIKV, 55% of ZIKVwpDENV, 0% of pDENV, and 4% of sDENV in the panels were positive, suggesting that ZIKV-NS1 IgM ELISA can distinguish ZIKV from DENV infection with a sensitivity of 72.5% and specificity of 97.4% (Table 2). When testing with ZIKV-NS1 IgG ELISA, 5% of pZIKV, 95% of ZIKVwpDENV, 0% of pDENV, and 66.7% of sDENV panels were positive, demonstrating that nearly 67% of cases with sDENV infection contain IgG cross-reactive to ZIKV-NS1. Probably due to the relatively early sampling time of these convalescent-phase samples, only 1 sample in the pZIKV panel was positive for ZIKV-NS1 IgG ELISA. Combining IgM and IgG ELISAs together, the sensitivity for ZIKV infection was 95%, but the specificity was only 66.7% (Table 2). The 2 ZIKV-NS1 ELISAs combined cannot distinguish between pZIKV and ZIKVwpDENV infections.

Addition of Dengue Virus Nonstructural Protein 1 Immunoglobulin G Enzyme-Linked Immunosorbent Assay

For those samples with either ZIKV-NS1 IgM or IgG positivity, we employed a DENV1-NS1 IgG ELISA to distinguish pZIKV, ZIKVwpDENV, and sDENV infections. As all the cases with pDENV infection in this study were DENV1, DENV1-NS1 ELISA was chosen to increase the sensitivity of detection. This is not a concern for samples from those with sDENV infection, which commonly cross-reacted to DENV NS1 proteins of 3–4 serotypes based on our previous study of anti-NS1 antibodies in 50 cases with sDENV infection [14]. As shown in Table 1, none (0%) of the pZIKV panel cross-reacted to DENV1-NS1, whereas 85% of ZIKVwpDENV and 95.8% of sDENV panel reacted to DENV1-NS1, suggesting that negative results in the DENV1-NS1 IgG ELISA can be used to distinguish pZIKV from ZIKVwpDENV and sDENV infections.

The high positive rates to DENV1-NS1 in sDENV and ZIKVwpDENV panels were not surprising, considering both groups have been exposed to DENV. As all samples from cases with sDENV infection were collected before 2013 from regions without ZIKV activity, the high rate (66.7%) of cross-reactivity to ZIKV-NS1 was unexpected. Despite no difference in the rOD values of DENV1-NS1 IgG ELISA between the 2 groups (Figure 1A), the rOD values of ZIKV-NS1 IgG ELISA in the sDENV panel were significantly lower than those in the ZIKVwpDENV panel ($P < .0001$, 2-tailed Mann-Whitney test) (Figure 1B). For those positive in both ZIKV-NS1 and DENV1-NS1 IgG ELISAs, the rOD ratio of ZIKV-NS1 to DENV1-NS1 was significantly lower in the sDENV panel compared with the ZIKVwpDENV panel ($P < .0001$, 2-tailed Mann-Whitney test) (Figure 1C). Interestingly, using a cutoff of the

Table 1. Results of Enzyme-Linked Immunosorbent Assays in Different Serum/Plasma Panels

	Serum/Plasma Panel				
ELISA[a]	Flavivirus Naive (n = 12)	pDENV1[b] (n = 16)	pZIKV[c] (n = 20)	sDENV[d] (n = 24)	ZIKVwpDENV[c] (n = 20)
ZIKV-NS1 IgM^+	0/8 (0%)	0/9 (0%)	18/20 (90%)	1/24 (4%)	11/20 (55%)
ZIKV-NS1 IgG^+	0/12 (0%)	0/16 (0%)	1/20 (5%)	16/24 (66.7%)	19/20 (95%)
ZIKV-NS1 IgM^+ or IgG^+	0/8 (0%)	0/16 (0%)	18/20 (90%)	16/24 (66.7%)	20/20 (100%)
DENV1-NS1 IgG^+	0/12 (0%)	13/16 (81.3%)	0/20 (0%)	23/24 (95.8%)	17/20 (85%)

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; IgM, immunoglobulin M; NS1, nonstructural protein 1; pDENV1, primary DENV1 infection; pZIKV, primary ZIKV infection; sDENV: secondary DENV infection; ZIKVwpDENV, ZIKV infection with previous DENV infection.

[a]Results were based on data from 2 experiments (each in duplicates) as described in Methods.

[b]For the pDENV1 panel, 12 were from Hawaii and 4 from Taiwan (Supplementary Table 1). Only those collected within 4 months post-symptom onset were tested for IgM.

[c]The pZIKV and ZIKVwpDENV panels were from Nicaragua as described in the Methods.

[d]The sDENV panel was from Taiwan (Supplementary Table 1).

Table 2. Sensitivity and Specificity of Enzyme-Linked Immunosorbent Assays

ELISA	Comparisons[a]	Sensitivity[b]	Specificity[b]
ZIKV-NS1 IgM$^+$	ZIKV vs DENV	72.5%	97.4%
ZIKV-NS1 IgG$^+$	ZIKV vs DENV	50%	66.7%
ZIKV-NS1 IgM$^+$ or IgG$^+$	ZIKV vs DENV	95%	66.7%

Abbreviations: DENV, dengue virus; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; IgM, immunoglobulin M; NS1, nonstructural protein 1; ZIKV, Zika virus.

[a]ZIKV includes primary ZIKV and ZIKV with previous DENV infections. DENV includes primary DENV1 and secondary DENV infections.

[b]Data were based on Table 1.

rOD ratio at 0.24, we could distinguish ZIKVwpDENV from sDENV infection with a sensitivity of 87.5% and specificity of 81.3% (Figure 1C).

We further tested another 19 samples from cases of probable ZIKVwpDENV infection from Brazil. Consistent with the observations in the ZIKVwpDENV panel from Nicaragua, the rOD values of the ZIKV-NS1 IgG ELISA and the rOD ratio of ZIKV-NS1 to DENV1-NS1 were significantly lower in the sDENV panel compared with the probable ZIKVwpDENV panel from Brazil (P = .03 and P < .001, respectively, 2-tailed Mann-Whitney test) (Figure 1B). Using a rOD ratio cutoff at 0.24 to compare sDENV panel with ZIKVwpDENV and probable ZIKVwpDENV panels together, we could distinguish them with a sensitivity of 81.3% and specificity of 81.3%.

Cross-reactivity of Secondary Dengue Virus Infection Panel to Zika Virus-Nonstructural Protein 1 Over Time

It is worth noting that the sampling time for the sDENV panel was compatible with that for the pZIKV and ZIKVwpDENV panels (Supplementary Table 1). To investigate whether the IgG cross-reactivity to ZIKV-NS1 by sDENV panel is limited to convalescent phase, we examined 38 post–convalescent phase samples (3 months to 2 years PSO) from cases with sDENV infection. The rOD values of DENV1-NS1 and ZIKV-NS1 IgG ELISAs in these later time-point samples were not significantly different from those in the convalescent-phase samples (P = .87 and P = .23, respectively, 2-tailed Mann-Whitney test) (Figure 1A vs 1D, 1B vs 1E). Notably, the positivity rates of ZIKV-NS1 IgG ELISA in the sDENV panel decreased from 83.3% (3–6 months PSO) to 27.8% and 28.6% (1 year and 1.5–2 years PSO, respectively) (Figure 1F).

We further examined sequential samples. For 3 Nicaraguan cases and 4 blood donors with sDENV infection, DENV1-NS1 IgG ELISA was positive at all time points, and ZIKV-NS1 IgG changed from positive to negative in the 3 cases (12–18 months PSO, Figure 2A) and 1 blood donor (2–6 months PID, Figure 2B). For the 5 blood donors with ZIKVwpDENV infection, DENV1-NS1 IgG ELISA was positive starting from the index day, which is consistent with their previous DENV infection (Figure 2C). The ZIKV-NS1 IgG ELISA showed 2 patterns. Three seroconverted at 1.5–3 months PID; 2 had ZIKV-NS1 IgG starting from the index day, which was also positive for Zika MAC-ELISA (data not shown), suggesting later timepoint of infection. Despite the gradual decline in rOD values over time, the rOD ratios of ZIKV-NS1 to DENV1-NS1 calculated for samples positive for both were >0.24 in all 15 samples from those with ZIKVwpDENV infection and <0.24 in 10 of 12 samples from those with sDENV infection (data not shown). Taken together, the results of sequential samples were generally in agreement with those of cross-sectional samples.

Proposed Algorithm to Distinguish Zika Virus and Dengue Virus Infections

Based on the above observations, we propose an algorithm using 3 ELISAs to distinguish pZIKV, ZIKVwpDENV, and sDENV infections (Figure 3). Based on the result of InBios ZIKV- and DENV-detect MAC-ELISAs, both E protein–based IgM ELISAs, for all samples in Table 1, only those tested positive or equivocal by either test are included in the algorithm.

DISCUSSION

In this study, we report high rates of IgG cross-reactivity to ZIKV-NS1 protein by samples from participants with sDENV infection, which ranged from 83.3% (3–6 months) to 66.7% (<1 month) and 28.1% (1–2 years PSO), suggesting that after IgM antibody wanes, serodiagnosis or serosurveillance for ZIKV by NS1 IgG assay needs to rule out sDENV infection. Moreover, combination of 3 simple ELISAs could distinguish pZIKV, ZIKVwpDENV, and sDENV infections; differentiation between ZIKV and DENV is urgently needed for monitoring pregnant women in regions where ZIKV and DENV co-circulate.

Partly due to the presence of absolutely conserved fusion loop residues in the E protein and its immunodominance reported in human sera following DENV infection, traditional E protein–based serological tests to detect a specific flaviviral infection has been hampered by extensive cross-reactivity among diverse flaviviruses [14–16], especially in regions where 2 or more flaviviurses co-circulate. Thus, under the CDC guidelines, positive or equivocal IgM tests based on E protein require PRNTs [6, 7], which can confirm pZIKV infection but not those who experienced previous flaviviral infections, including sDENV and ZIKVwpDENV infections. Instead of performing PRNTs by reference laboratories, our algorithm proposes performing 3 ELISAs for those with positive or equivocal IgM tests (Figure 3) to distinguish pZIKV, ZIKVwpDENV, and sDENV infections with high sensitivity and improved specificity compared with 2 ZIKV-NS1 ELISAs combined. The estimated time for PRNTs is 5–6 days, whereas that for ELISAs within 7 hours. To our knowledge, this is the first report suggesting that combination of 3 serological tests, in the absence of neutralization tests, could delineate past and present flaviviral infections.

A recent study revealed that most anti-NS1 mAbs derived from patients with pZIKV infection were specific to ZIKV

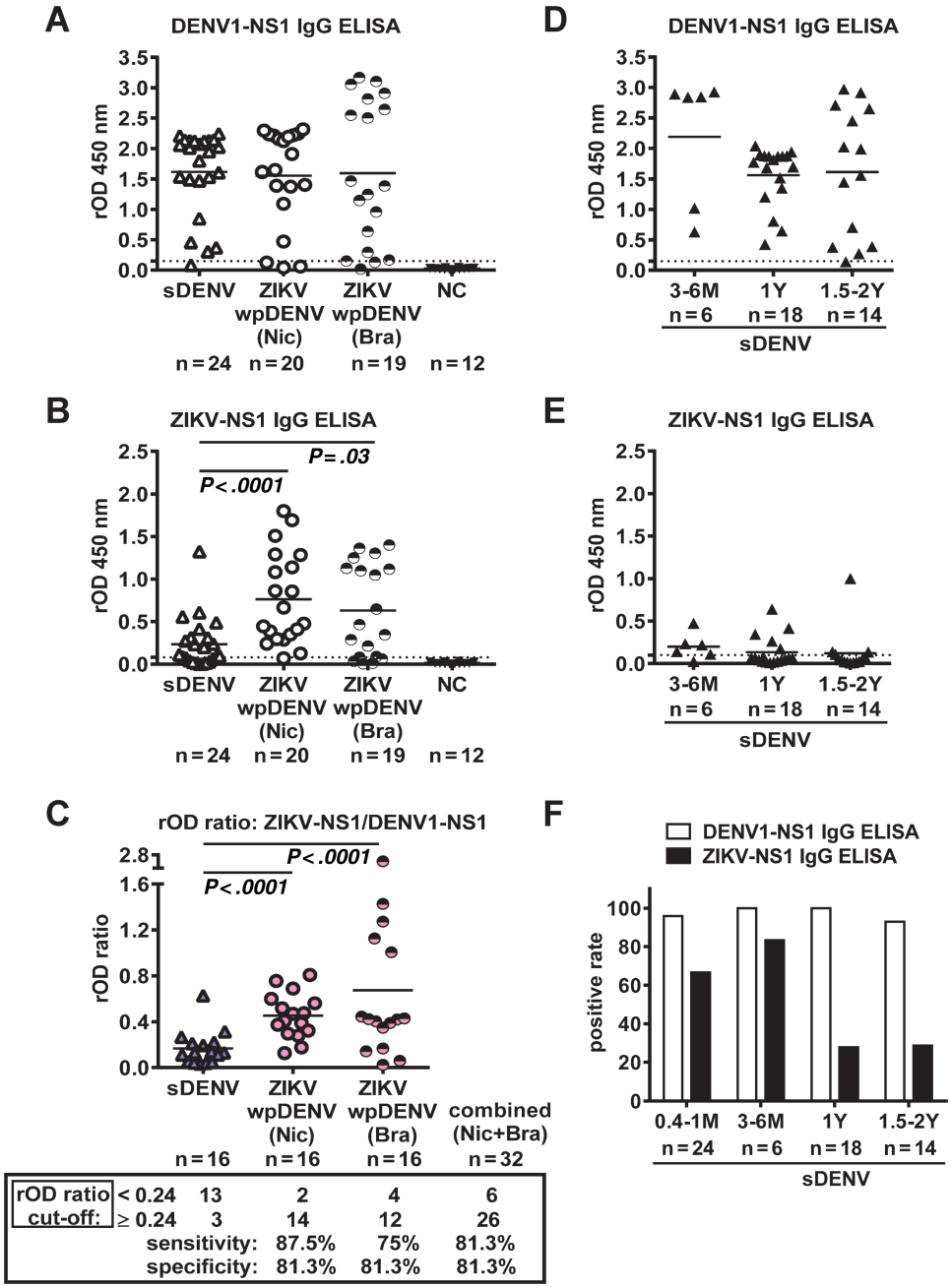

Figure 1. Results of nonstructural protein 1 (NS1) immunoglobulin G (IgG) enzyme-linked immunosorbent assays (ELISAs) for secondary dengue virus (sDENV) and Zika virus (ZIKV) with previous (wp) DENV infection panels. DENV1-NS1 (*A*) and ZIKV-NS1 (*B*) IgG ELISAs in convalescent-phase samples from sDENV, ZIKVwpDENV (Nicaragua [Nic]), probable ZIKVwpDENV (Brazil [Bra]) and negative control (NC) panels. *C*, Relative optical density (rOD) ratio of ZIKV-NS1 to DENV1-NS1. The sensitivity and specificity are shown based on a cutoff rOD ratio at 0.24. DENV1-NS1 (*D*) and ZIKV-NS1 (*E*) IgG ELISAs in post–convalescent phase samples (3 months to 2 years post–symptom onset) from sDENV panels. *F*, Positive rates of DENV1- and ZIKV-NS1 IgG ELISAs in sDENV panels over time. Dotted lines indicate cutoff rOD values for ELISAs. Data are mean of 2 experiments (each in duplicate). Two-tailed Mann-Whitney test was used to compare the 2 groups.

and >50% of those from patients of ZIKVwpDENV infection reacted to DENV [20]. Our findings on polyclonal sera are generally in agreement with these observations. Compared with the sensitivity (100%) and specificity (99.8%) of the Euroimmun kits (combined ZIKV-NS1 IgM and IgG ELISAs) [22], the sensitivity and specificity of our combined ZIKV-NS1 IgM and IgG ELISAs are 95% and 66.7%, respectively. The high specificity of the Euroimmun kits is likely due to the inclusion of controls with large number of flavivirus-naive individuals plus travel-acquired pDENV infection. The low specificity of our combined 2 ELISAs is due to the inclusion of many cases of sDENV infection as controls (Table 2). The Euroimmun kits cannot distinguish pZIKV, ZIKVwpDENV, and sDENV infections, whereas our combined ELISAs can.

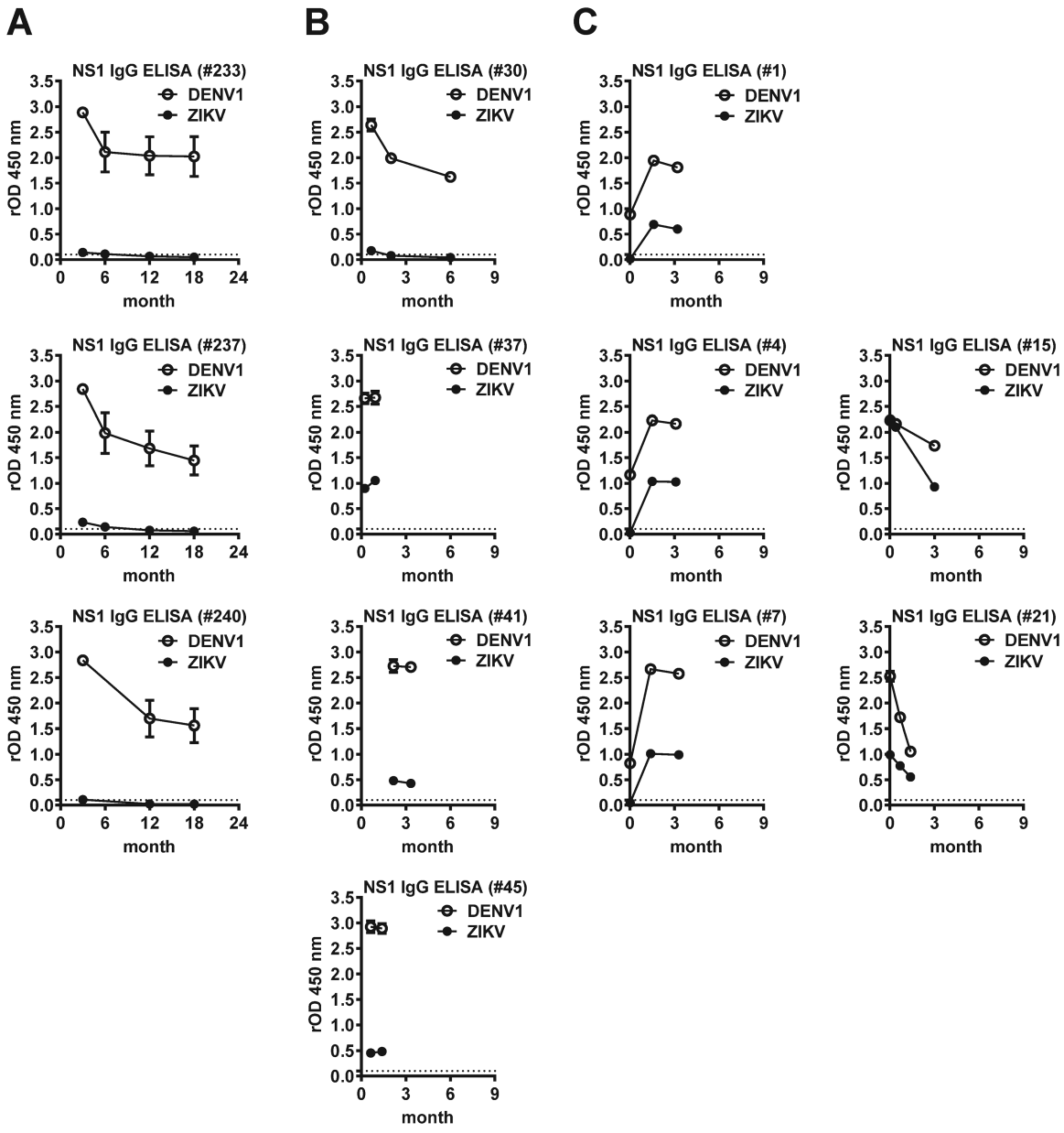

Figure 2. Results of nonstructural protein 1 (NS1) immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISAs) in sequential samples from individuals with secondary dengue virus (sDENV) and Zika virus (ZIKV) infection with previous (wp) DENV infections. *A*, Three cases with sDENV infection, 3–18 months post–symptom onset. *B*, Four blood donors with sDENV infection, 7 days to 6 months post–index day (PID). *C*, Five blood donors with ZIKVwpDENV infection, from index day to 3 months PID. Three cases (1, 4, and 7) seroconverted to ZIKV-NS1; 2 cases (15 and 21) had ZIKV-NS1 IgG starting from the index day. Dotted lines indicate cutoff relative optical density (rOD) values for ELISAs. Data are mean of 2 experiments (each in duplicates).

The proposed algorithm of ZIKV serodiagnosis using 3 ELISAs could be applied in clinical laboratories as potential routine serological tests for ZIKV infection. This is relevant for pregnant women in dengue-endemic regions and for people in dengue-nonendemic regions who have previous dengue or frequently travel to dengue-endemic regions. The algorithm could also be an important research tool for serosurveillance and Zika pregnancy studies to better understand the epidemiology of ZIKV as well as risk and spectrum of ZIKV complications in pregnancy. Increasing evidence reveals both symptomatic and asymptomatic ZIKV infections in pregnancy are associated with fetal microcephaly [37]. The protean manifestations of congenital Zika syndrome include not only structural anomalies but also functional disabilities, which may affect both microcephalic and normacephalic babies during growth and development [38, 39]. Our IgG-based NS1 ELISAs to distinguish pZIKV, ZIKVwpDENV, and sDENV infections could be useful in retrospective studies to investigate the relationship of pZIKV infection alone or ZIKVwpDENV infection to the full-spectrum of congenital Zika syndrome. Notably, these

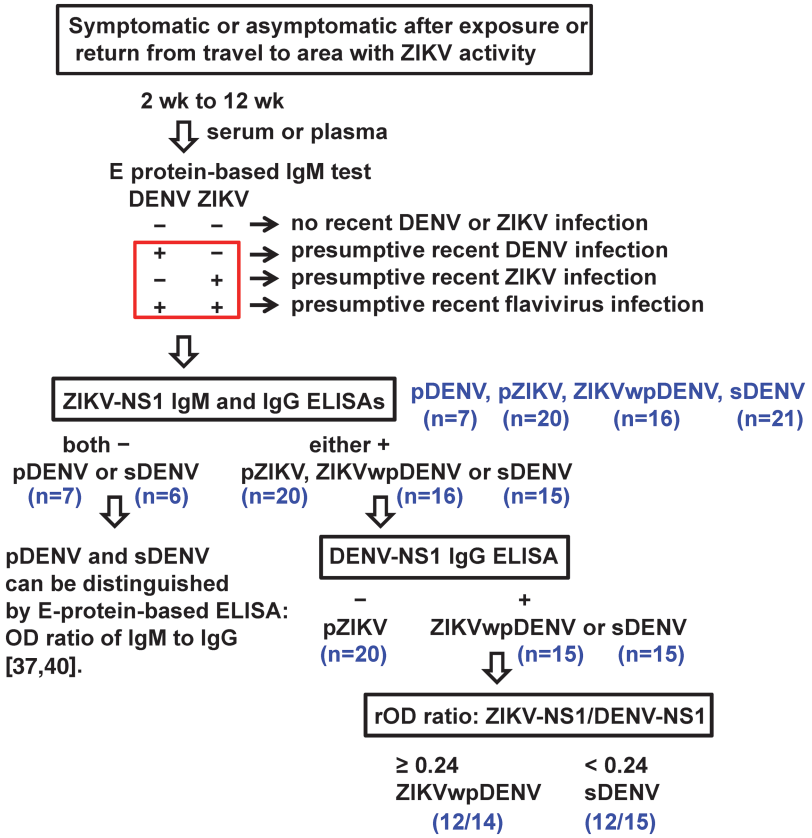

Figure 3. Proposed algorithm of using 3 serological tests (without neutralization tests) to distinguish different Zika virus (ZIKV) and dengue virus (DENV) infections in dengue- and Zika-endemic regions in the framework of Centers for Disease Control and Prevention guidelines for laboratory diagnosis of ZIKV infection [6, 7]. Only samples tested positive or equivocal by ZIKV or DENV E protein–based immunoglobulin M (IgM) enzyme-linked immunosorbent assays (ELISAs) are included in the algorithm. The total numbers from each panel and the numbers of positive or negative based on the 3 Nonstructural Protein 1 (NS1) ELISAs are shown in parentheses. Abbreviations: IgG, immunoglobulin G; OD, optical density; pDENV, primary dengue virus infection; pZIKV, primary Zika virus infection; rOD, relative OD; sDENV, secondary dengue virus infection; ZIKVwpDENV, Zika virus infection with previous dengue virus infection.

ELISAs are convenient, cost-effective, and readily applicable to field sites in developing countries. They can also be developed into various high-throughput formats or rapid tests for different clinical or field studies.

There are several limitations to this study. First, the sample size is small; future studies involving larger sample size are needed to further validate these observations. Second, only limited numbers of sequential samples were tested. Future studies involving more sequential samples following well-documented infections (such as pZIKV, ZIKVwpDENV, and sDENV) are needed to better understand the performance of these assays. Third, the specificity of 81.3% to distinguish ZIKVwpDENV and sDENV infections remains to be improved by inclusion of other newly identified parameters or biomarkers. Fourth, based on our previous report of the cross-reactivity of anti-DENV NS1 antibodies within the DENV serocomplex [14], only DENV1-NS1 IgG ELISA was chosen in this study to test if combination with ZIKV-NS1 IgM and IgG ELISAs can distinguish pZIKV, ZIKVwpDENV, and sDENV. Future studies involving NS1 proteins of DENV2–4 will help to determine if NS1 protein of other serotype or a mixture of NS1 proteins of DENV1–4 performs better. Additionally, the cutoff value (0.24) for the rOD ratio was based on the sDENV panel in Figure 1C (all DENV2 cases); the cutoff value for other serotype remains to be determined. Fifth, developing serodiagnostic assays to distinguish ZIKV from other medically important flaviviruses such as JEV, WNV, YFV, and TBEV in future studies is relevant, in light of the global spread of ZIKV to regions where these flaviviruses are prevalent. Furthermore, serodiagnostic assays to distinguish ZIKV infection from immunizations by different formulations of various flaviviral vaccines including DENV, JEV, YFV, and TBEV vaccines remain to be explored.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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