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Identification of Anti-Premembrane Antibody as a Serocomplex-Specific Marker To Discriminate Zika, Dengue, and West Nile Virus Infections

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ABSTRACT Although transmission of Zika virus (ZIKV) in the Americas has greatly declined since late 2017, recent reports of reduced risks of symptomatic Zika by prior dengue virus (DENV) infection and increased risks of severe dengue disease by previous ZIKV or DENV infection underscore a critical need for serological tests that can discriminate past ZIKV, DENV, and/or other flavivirus infections and improve our understanding of the immune interactions between these viruses and vaccine strategy in endemic regions. As serological tests for ZIKV primarily focus on envelope (E) and nonstructural protein 1 (NS1), antibodies to other ZIKV proteins have not been explored. Here, we employed Western blot analysis using antigens of 6 flaviviruses from 3 serocomplexes to investigate antibody responses following reverse transcription-PCR (RT-PCR)-confirmed ZIKV infection. Panels of 20 primary ZIKV and 20 ZIKV with previous DENV infection recognized E proteins of all 6 flaviviruses and the NS1 protein of ZIKV with some cross-reactivity to DENV. While the primary ZIKV panel recognized only the premembrane (prM) protein of ZIKV, the ZIKV with previous DENV panel recognized both ZIKV and DENV prM proteins. Analysis of antibody responses following 42 DENV and 18 West Nile virus infections revealed similar patterns of recognition by anti-E and anti-NS1 antibodies, whereas both panels recognized the prM protein of the homologous serocomplex but not others. The specificity was further supported by analysis of sequential samples. Together, these findings suggest that anti-prM antibody is a flavivirus serocomplex-specific marker and can be used to delineate current and past flavivirus infections in endemic areas.

IMPORTANCE Despite a decline in Zika virus (ZIKV) transmission since late 2017, questions regarding its surveillance, potential reemergence, and interactions with other flaviviruses in regions where it is endemic remain unanswered. Recent studies have reported reduced risks of symptomatic Zika by prior dengue virus (DENV) infection and increased risks of severe dengue disease by previous ZIKV or DENV infection, highlighting a need for better serological tests to discriminate past ZIKV, DENV, and/or other flavivirus infections and improved understanding of the immune interactions and vaccine strategy for these viruses. As most serological tests for ZIKV focused on envelope and nonstructural

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protein 1, antibodies to other ZIKV proteins, including potentially specific antibodies, remain understudied. We employed Western blot analysis using antigens of 6 flaviviruses to study antibody responses following well-documented ZIKV, DENV, and West Nile virus infections and identified anti-premembrane antibody as a flavivirus serocomplex-specific marker to delineate current and past flavivirus infections in areas where flaviviruses are endemic.

KEYWORDS Zika virus, flavivirus, antibody, premembrane protein, serodiagnosis

Zika virus (ZIKV) belongs to the genus *Flavivirus* of the family *Flaviviridae*. After its discovery in 1947 and the first report of a human case in 1964, ZIKV received little attention until an outbreak on Yap Island in 2007 (1–4). This was followed by a large outbreak in French Polynesia from 2013 to 2014 and subsequent outbreaks in the Pacific Islands (5–7); the rapid spread of ZIKV in the Americas from 2015 to 2017 resulted in nearly 800,000 reported suspected or confirmed cases (8). While ZIKV infections are either asymptomatic or present as a self-limiting febrile illness (6, 7), the association of ZIKV infection with Guillain-Barré syndrome in adults and fetal microcephaly and other birth defects, known as congenital Zika syndrome (CZS), resulting from ZIKV infection during pregnancy, has raised global public health concerns and led to extensive research on ZIKV to better understand its epidemiology, transmission, and disease and promote the development of diagnostics, antivirals, and vaccines (9, 10).

In the genus *Flavivirus* of the family *Flaviviridae*, several mosquito-borne viruses belonging to different serocomplexes cause significant human diseases, including the four serotypes of dengue virus (DENV) in the DENV serocomplex, West Nile virus (WNV) and Japanese encephalitis virus (JEV) in the JEV serocomplex, yellow fever virus (YFV), and ZIKV (11). Antibodies that recognize a single member, members within the same serocomplex, or members of different serocomplexes are called type-specific, serocomplex-specific, or group-reactive antibodies, respectively. ZIKV contains a positive-sense, single-stranded RNA genome of about 10.7 kb in length, which encodes three structural proteins, the capsid (C), premembrane (prM), and envelope (E) at the 5′-end and seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 at the 3′-end (11). The E protein, a glycoprotein of approximately 55 kDa, is present on the surface of the virion and is the major target of neutralizing antibodies and vaccine development (11, 12). The ectodomain of E protein contains 3 domains, domain I, II, and III (DI, DII, and DIII); at the tip of DII is the fusion loop (FL), which together with the adjacent BC loop (BCL), contains several highly conserved residues (11). The prM protein, a glycoprotein of about 19 kDa present on immature virions, is cleaved to pr and M proteins by furin or furin-like protease during maturation in the *trans*-Golgi (11, 13, 14). While studies of DENV have reported that human anti-prM monoclonal antibodies (MAbs) recognize pr and cause ADE *in vitro* and *in vivo* (15–17), the role of anti-prM antibody in ZIKV pathogenesis remains unclear. The nonstructural protein 1 (NS1), a glycoprotein of approximately 40 kDa or more depending on its glycosylation status, is present intracellularly as a monomer, associated with the cell surface as a dimer and secreted outside of the cells as a hexamer (18). Studies of DENV and ZIKV have shown that NS1 protein is involved in viral RNA replication and immune evasion and can cause endothelial hyperpermeability and vascular leak through direct action on endothelial cells and triggering the release of vasoactive cytokines from immune cells (19–22).

Although the transmission of ZIKV in the Americas has greatly declined since late 2017, several questions remain unanswered, such as its surveillance, seroprevalence, potential reemergence, and effect on other flavivirus infections in regions where it is endemic (6–8). Previous studies have shown that DENV- or WNV-immune sera enhanced ZIKV replication *in vitro* and in mice (23–26); however, such enhancement was not consistently observed in nonhuman primates, highlighting the importance of human studies in the field (27–29). Two cohort studies reported that prior DENV infection was associated with reduced risk of symptomatic ZIKV infection (30, 31). A recent study in Nicaragua showed that one previous ZIKV infection or one prior DENV

followed by one ZIKV infection increased the risk of subsequent symptomatic DENV2 and severe disease, whereas a previous ZIKV with two or more DENV infections had protective effect (32). These observations not only demonstrate the complex interactions between DENV and ZIKV immunity in humans but also highlight a critical need for serological tests that can discriminate different DENV and ZIKV immune histories, including primary DENV (pDENV), secondary DENV (sDENV), primary ZIKV (pZIKV), and ZIKV infection with previous DENV (ZIKVwprDENV) infections, as well as other flavivirus infections, to better understand the epidemiology and pathogenesis of ZIKV and DENV in regions where these viruses are endemic.

Traditional E protein-based serological tests using either recombinant E protein, inactivated virions, or virus-like particles (VLPs) are complicated by cross-reactivity among various flaviviruses (33–35). Thus, further testing by the plaque reduction neutralization tests (PRNT) is recommended, though it is laborious and time-consuming (35). Several enzyme-linked immunosorbent assays (ELISAs), including a recently reported blockade of binding ELISA, and a microsphere immunoassay based on ZIKV NS1 protein have shown improved specificity (36–41). However, the durability of anti-NS1 antibodies in serum could be a challenge for seroprevalence studies. A study using recombinant DIII reported reduced cross-reactivity between ZIKV and DENV2, but cross-reactivity for sDENV infection during early convalescence remains (42). Other studies using FL/BCL-mutated recombinant E protein of DENV and ZIKV or FL-mutated VLPs showed improved specificity in IgM ELISA (43, 44) but not in IgG ELISA, which required preincubation with large amounts of heterologous recombinant E protein (44).

As most serological tests to ZIKV have focused on two viral antigens, E and NS1 proteins (35, 45), antibody responses to other ZIKV proteins, their relative abundance, and extent of cross-reactivity to different flaviviruses remain incompletely understood. Previously, we studied antibody responses following primary and secondary DENV2 infections by Western blot analysis using DENV1 to 4-infected cell lysates as antigens; we found anti-E antibody was the strongest, which cross-reacted to E proteins of all four DENV serotypes, followed by anti-prM and anti-NS1 antibodies (46). In this study, we used Western blot analysis, including antigens of 6 flaviviruses, to investigate antibody responses following ZIKV infection compared with ELISA. As a comparison, we also examined antibody responses following DENV and WNV infections. Our findings suggest that anti-prM antibody is a flavivirus serocomplex-specific marker and can be used to delineate current and past specific flavivirus infections in areas where these infections are endemic.

RESULTS

Antibody responses following ZIKV infection. The numbers, sampling times, and sources of serum or plasma samples from RT-PCR-confirmed ZIKV cases are summarized in Table 1. Samples collected <3 months or ≥ 3 months post-symptom onset (PSO) were designated as convalescent- or postconvalescent-phase samples, respectively. We first employed Western blot analysis using ZIKV, DENV1 to 4, and WNV-infected cell lysates as antigens, each of which contained individual viral proteins in equal molar ratio (except those structural proteins released with virions), to examine antibody responses 6 to 8 months PSO for 3 cases each from pZIKV and ZIKVwprDENV panels. The loading of similar amounts of antigens was confirmed by comparable intensity of E protein bands recognized by a mouse MAb FL0232, which recognized the E proteins of DENV1 to 4, ZIKV, and WNV similarly (Fig. 1C) (46, 47). As shown in Fig. 1A and B, anti-E antibodies cross-reactive to all 6 flaviviruses tested and anti-NS1 antibodies to ZIKV with some cross-reactivity to between one and four DENV serotypes were observed in both panels. In contrast, anti-prM antibodies were found to recognize ZIKV only in the pZIKV panel and both ZIKV and DENV in the ZIKVwprDENV panel as verified by long exposure (Fig. 1A; data not shown). A similar trend was observed in other samples (see Table S1 in the supplemental material); Table 2 summarizes different viral protein bands recognized by 18 pZIKV and 15 ZIKVwprDENV infection samples.

TABLE 1 Sampling time, numbers, and sources of different serum/plasma panels

Panel[a] (subgroup)	No. of subjects/ samples	Category (No. of samples)	Sampling time PSO[b] mean and (range)	Source(s) (no.) of samples, yr
Single or two time-point samples				
pZIKV	20/38[c]	Convalescent (20)	17 (14−24) days	Nicaragua, 2016
		Postconvalescent (18)	6.9 (6−8) mo	
ZIKVwprDENV	20/35[c]	Convalescent (20)	16 (14−19) days	Nicaragua, 2016
		Postconvalescent (15)	7.0 (6−8) mo	
pWNV	18/18	Convalescent	Not applicable[d]	U.S. ARC, 2006–2015
pDENV	21/21	Postconvalescent		
DENV1	15/15	Postconvalescent	5.3 (3−10) mo	Taiwan (2), 2006–2009; Hawaii (11), 2015; Nicaragua (2), 2006–2008
DENV2	3/3	Postconvalescent	24.7 (3−68) mo	Taiwan (1), 2006–2009; Nicaragua (2), 2006–2008
DENV3	3/3	Postconvalescent	8.3 (3−19) mo	Taiwan (1), 2006–2009; Nicaragua (2), 2006–2008
sDENV	21/21	Postconvalescent		
DENV2	15/15	Postconvalescent	7.3 (3−67) mo	Taiwan (11), 2006–2009; Nicaragua (4), 2006–2008
DENV1	4/4	Postconvalescent	3.6 (3−5.5) mo	Taiwan (2), 2006–2009; Hawaii (2), 2015
DENV3	2/2	Postconvalescent	18.0 (17−19) mo	Taiwan (2), 2006–2009
Flavivirus naive	10/10	Seroprevalence study	Not applicable	Taiwan (10), 2015–2016
Sequential samples				
pZIKV	5/14	Convalescent to postconvalescent	16.4 (0−43) days[e]	U.S. VRI, 2016
ZIKVwprDENV	5/15	Convalescent to postconvalescent	36.8 (0−97) days[e]	U.S. VRI, 2016
pWNV	6/17	Convalescent to postconvalescent	56.5 (5−182) days[e]	U.S. VRI, 2005–2006

[a]pDENV, primary DENV infection; sDENV, secondary DENV infection; pWNV, primary WNV infection; pZIKV, primary ZIKV infection; ZIKVwprDENV, ZIKV infection with previous DENV infection.
[b]PSO, post-symptom onset.
[c]Twenty subjects from each panel provided convalescent-phase samples and some provided postconvalescent-phase samples.
[d]Index samples tested positive for WNV transcription-mediated amplification, IgM and IgG from blood donors at the American Red Cross.
[e]Days after index day when ZIKV RT-PCR or WNV TMA was positive for blood donors at the Vitalant Research Institute.

Anti-prM antibodies can discriminate between three flavivirus infections. As a comparison, we further examined antibody responses during the postconvalescent-phase from 21 RT-PCR-confirmed pDENV and 21 RT-PCR-confirmed sDENV infections. Shown in Fig. 1D and E are the results of 3 samples from the pDENV panel (one from each serotype DENV1 to 3) and 3 from sDENV panel (one from each serotype DENV1 to 3). Anti-E antibodies cross-reactive to all 6 flaviviruses, anti-NS1 antibodies to one to four DENV serotypes with cross-reactivity to ZIKV (in 2 samples), and anti-prM antibodies to DENV1 to 4 without cross-reactivity to ZIKV or WNV were found in both panels (data not shown for long exposure). A similar pattern of viral protein recognition was observed in other pDENV and sDENV infection samples (Tables 1 and 2), except that the sDENV panel had a higher rate of cross-reactivity to ZIKV NS1 compared with that of the pDENV panel (66.7% versus 14.3%). We next examined antibody responses in the index donation of 18 blood donors found to be WNV transcription-mediated amplification (TMA) positive. As shown in Fig. 1F, anti-E antibodies cross-reactive to all 6 flaviviruses, anti-NS1 antibodies to WNV with cross-reactivity to one or two DENV serotypes, and anti-prM antibodies to WNV only without cross-reactivity to DENV or ZIKV were found in 3 samples (data not shown for long exposure). Notably, double bands of WNV prM protein were observed, presumably representing glycosylated and nonglycosylated forms, with the latter migrating faster than the former (11, 48). Compared with DENV, the more prominent double bands of WNV prM protein may suggest more nonglycosylated forms and thus incomplete use of the prM glycosylation site in WNV-infected cells. The viral protein bands recognized by 18 pWNV samples as well as 10 flavivirus-naive samples are summarized in Table 2.

Table 3 summarizes the sensitivity and specificity of recognizing different NS1 and prM proteins to discriminate postconvalescent-phase ZIKV panel from DENV and WNV

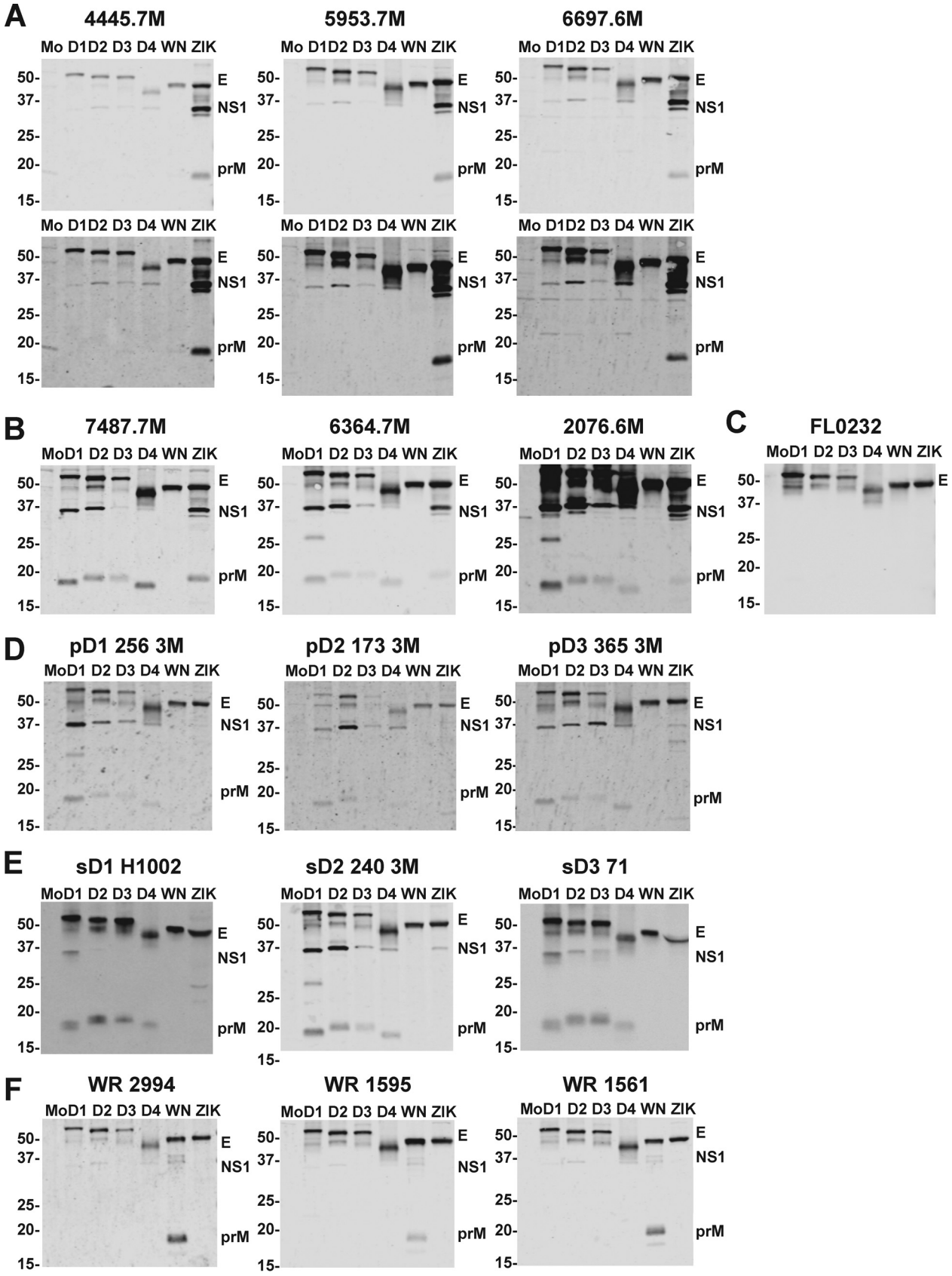

FIG 1 Antibody responses to 6 flavivirus antigens following ZIKV, DENV, and WNV infections. Lysates derived from mock-, DENV1 to 4-, WNV-, and ZIKV-infected Vero cells were subjected to SDS-12% polyacrylamide gel electrophoresis under nonreducing condition and Western blot

(Continued on next page)

TABLE 2 Summary of viral proteins recognized by different panels in Western blot analysis

	No. of positive/total samples (%) in different serum/plasma panels[a]							
		Postconvalescent phase					Convalescent phase	
Protein bands recognized[c,d]	Naive	pDENV	sDENV	pZIKV	ZIKVwprDENV	pWNV[b]	pZIKV	ZIKVwprDENV
D1, D2, D3, or D4 NS1	0/10 (0)	**20/21 (95.2)**	**21/21 (100)**	17/18 (94.4)	**15/15 (100)**	15/18 (83.3)	7/20 (35.0)	**20/20 (100)**
ZIKV NS1	0/10 (0)	3/21 (14.3)	14/21 (66.7)	**18/18 (100)**	**15/15 (100)**	5/18 (27.8)	**20/20 (100)**	**20/20 (100)**
WNV NS1	0/10 (0)	0/21 (0)	2/21 (9.5)	0/18 (0)	3/15 (20.0)	**9/18 (50.0)**	0/20 (0)	5/20 (25.0)
D1, D2, D3, or D4 prM	0/10 (0)	**20/21 (95.2)**	**21/21 (100)**	0/18 (100)	**10/15 (67.7)**	0/18 (0)	0/20 (0)	**16/20 (80.0)**
ZIKV prM	0/10 (0)	0/21 (0)	1/21 (4.8)	**18/18 (100)**	**15/15 (100)**	0/18 (0)	**15/20 (75.0)**	**11/20 (55.0)**
ZIKV C	0/10 (0)	0/21 (0)	0/21 (0)	0/18 (0)	0/15 (0)	0/18 (0)	**17/20 (85.0)**	**10/20 (55.0)**
WNV prM	0/10 (0)	0/21 (0)	0/21 (0)	0/18 (0)	3/15 (20.0)	**16/18 (88.9)**	0/20 (0)	4/20 (20.0)
ZIKV prM or C	0/10 (0)	0/21 (0)	1/21 (4.8)	18/18 (100)	15/15 (100)	0/18 (0)	**19/20 (95.0)**	**17/20 (85.0)**

[a]pDENV, primary DENV infection; sDENV, secondary DENV infection; pZIKV, primary ZIKV infection; ZIKVwprDENV, ZIKV infection with previous DENV infection; pWNV, primary WNV infection.
[b]Index samples tested positive for WNV transcription-mediated amplification, IgM and IgG from blood donors at the American Red Cross.
[c]NS1, nonstructural protein 1; prM, premembrane; C, capsid; D1, DENV1; D2, DENV2; D3, DENV3; D4, DENV4.
[d]No. (%) of homologous prM and NS1 proteins recognized by each panel are bold.

panels. The sensitivities/specificities of recognizing prM protein were 100/98.6%, 89.5/100%, and 88.9/96.5% for ZIKV, DENV, and WNV panels, respectively, suggesting that anti-prM antibodies could serve as a flavivirus serocomplex-specific marker. In contrast, the sensitivities/specificities of NS1 protein were 100/68.6%, 98.3/30.4%, and 50.0/94.1% for ZIKV, DENV, and WNV panels, respectively.

Anti-C antibody to ZIKV during convalescent-phase of ZIKV infection. We further examined the antibody responses during the convalescent phase, 14 to 24 days PSO, for 3 cases each from pZIKV and ZIKVwprDENV panels in comparison with their postconvalescent-phase samples. As shown in Fig. 2A, anti-E antibodies cross-reactive to 6 flaviviruses, anti-NS1 antibodies to ZIKV with faint cross-reactivity to DENV2 in 2 samples, and anti-prM antibodies to ZIKV only were seen in the pZIKV panel (data not shown for long exposure). Interestingly, antibodies recognizing an ~15-kD protein band corresponding to ZIKV C protein were found in 2 samples; this was supported by the DENV1 to 3 C protein bands at a corresponding position recognized by a mouse MAb to DENV C protein and a rabbit serum against DENV2 and DENV4 C proteins (Fig. 2C and D). However, the anti-C antibodies disappeared 6 to 8 months later (Fig. 2A). For ZIKVwprDENV panel, anti-E antibodies cross-reactive to 6 flaviviruses, anti-NS1 antibodies to ZIKV and DENV1 to 4, and anti-prM antibodies to DENV1 to 4 and ZIKV (in one sample) were observed (Fig. 2B, data not shown for long exposure). Anti-C antibody was seen in one sample and disappeared 6 to 8 months later. Table 2 summarizes the viral protein bands recognized by 20 pZIKV and 20 ZIKVwprDENV infection samples and reveals the detection of anti-C antibodies and lower rate of anti-prM antibodies during the convalescent-phase of ZIKV infection.

Table 4 summarizes the sensitivity and specificity of different ZIKV proteins to distinguish the convalescent-phase ZIKV panels from DENV and WNV panels. While the sensitivities/specificities of prM and C protein alone for ZIKV panels were 65.0/98.6% and 67.5/100%, respectively, the sensitivity/specificity of prM and/or C protein for ZIKV panels was 90.0/98.6%, suggesting anti-ZIKV prM and/or C antibodies could be a ZIKV-specific marker for convalescent-phase ZIKV samples.

Sequential samples. To better understand the development of antibodies to different viral proteins following ZIKV infection, we further examined sequential samples from 10 blood donors, including 5 each with pZIKV and ZIKVwprDENV infections from the index day, when ZIKV RT-PCR was positive, to 43 to 97 days later (Table 1). As shown in Fig. 3A, weak anti-E antibodies to all 6 flaviviruses plus anti-NS1 and anti-prM

FIG 1 Legend (Continued)
analysis probed with different serum or plasma samples or anti-E MAb FL0232 (C). Postconvalescent-phase samples from 3 cases each with primary ZIKV (A), ZIKV infection with previous DENV (B), primary DENV (D), secondary DENV (E), and primary WNV (F) infections. Both short (upper) and long (lower) exposures of each gel in panel A are shown. The positions of E, NS1, and prM protein bands are indicated. The size of molecular weight markers is shown in kDa. Mo, mock; D1, DENV1; D2, DENV2; D3, DENV3; D4, DENV4; WN, WNV; ZIK, ZIKV.

TABLE 3 Sensitivity and specificity of different viral proteins recognized by postconvalescent-phase samples in Western blot analysis

Viral proteins recognized[d]	Group	% Sensitivity (95% CI)[a,b]	% Specificity (95% CI)[a,b]
D1, D2, D3, or D4 NS1	Overall	98.3 (94.8–100)	30.4 (17.1–37.2)
	Subgroup	pDENV, 95.2; sDENV, 100; ZIKVwprDENV, 100	Naive, 100; pZIKV, 5.6; pWNV, 16.7
ZIKV NS1	Overall	100 (100–100)	68.6 (57.7–74.1)
	Subgroup	pZIKV, 100; ZIKVwprDENV, 100	Naive, 100; pDENV, 85.7; sDENV, 33.3; pWNV, 72.2
WNV NS1	Overall	50.0 (26.9–61.8)	94.1 (89.1–96.7)
	Subgroup	pWNV, 50.0	Naive, 100; pDENV, 100; sDENV, 90.5; pZIKV, 100; ZIKVwprDENV, 80.0
D1, D2, D3 or D4 prM	Overall	**89.5 (81.5–93.5)**[c]	**100 (100–100)**[c]
	Subgroup	pDENV, 95.2; sDENV, 100; ZIKVwprDENV, 66.7	Naive, 100; pZIKV, 100; pWNV, 100
ZIKV prM	Overall	**100 (100–100)**[c]	**98.6 (95.8–100)**[c]
	Subgroup	pZIKV, 100; ZIKVwprDENV, 100	Naive, 100; pDENV, 100; sDENV, 95.2; pWNV, 100
WNV prM	Overall	**88.9 (74.4–96.3)**[c]	**96.5 (92.6–98.5)**[c]
	Subgroup	pWNV, 88.9	Naive, 100; pDENV, 100; sDENV, 100; pZIKV, 100; ZIKVwprDENV, 80.0

[a]CI, confidence interval; pDENV, primary DENV infection; sDENV, secondary DENV infection; pZIKV, primary ZIKV infection; ZIKVwprDENV, ZIKV infection with previous DENV infection; pWNV, primary WNV infection. Comparison with postconvalescent-phase samples (6 to 8 months post-symptom onset) for pZIKV and ZIKVwprDENV panels.
[b]For simplicity, the 95% CIs in the subgroup are not shown.
[c]The sensitivity and specificity of recognizing prM are bold.
[d]NS1, nonstructural protein 1; prM, premembrane; D1, DENV1; D2, DENV2; D3, DENV3; D4, DENV4.

antibodies to ZIKV appeared on day 29 and became stronger on day 42 in a blood donor with pZIKV infection (data not shown for long exposure). A similar trend was observed in the other 4 donors with pZIKV infection (see Table S2 in the supplemental material). In contrast, strong anti-E antibodies to 6 flaviviruses and anti-prM antibodies to DENV1 to 4 were found on day 1 in two donors with ZIKVwprDENV infection (Fig. 3B); strong anti-NS1 antibodies to DENV1 to 4/ZIKV plus anti-prM antibodies to ZIKV were seen on day 1 in one donor and on days 45 to 93 in another donor. A similar pattern of strong anti-E and anti-NS1 antibodies plus anti-prM antibodies to ZIKV during the postconvalescent phase was found in the other 3 donors with ZIKVwprDENV infection (Table S2). These observations suggest that strong anti-E antibodies cross-reactive to flaviviruses together with anti-NS1 and anti-prM antibodies to ZIKV developed quicker during ZIKVwprDENV infection than with pZIKV infection. We also examined sequential samples from 6 blood donors with pWNV infection 6 to 182 days after the index day (Table 1). As shown in Fig. 3C, anti-E antibodies to 6 flaviviruses plus anti-prM antibody to WNV were found since day 21 or 28, whereas faint anti-NS1 antibodies were found since day 28 or 49. A similar pattern was observed in another 4 donors with pWNV infection. The observation that anti-NS1 antibodies to WNV developed slowly and were weaker than with anti-prM antibodies was in agreement with the lower detection rate of anti-NS1 antibodies (50.0%) than that of anti-prM antibodies (88.9%) in single time point samples (Table 2).

Comparison of detection of anti-NS1 antibodies by two assays. Previous studies using recombinant NS1 protein in ELISA have reported good sensitivity and specificity of anti-NS1 antibodies to distinguish ZIKV and DENV infections (36–40, 49). Our observations that anti-NS1 antibodies in Western blot analysis cross-reacted to DENV in pZIKV and pWNV panels (35 to 94.4% and 83.3%, respectively) and to ZIKV in pDENV and pWNV panels (14.3 and 27.8%, respectively) were unexpected (Table 2). To further clarify this, we tested available samples from three primary infection panels (pZIKV, pDENV, and pWNV) by using three NS1-based ELISAs (coated with mixed DENV1 to 4, ZIKV, or WNV NS1 protein) as described previously (40). As shown in Fig. 4A and B, postconvalescent-phase samples from pDENV and pWNV panels recognized their homologous NS1 proteins with limited cross-reactivity (1 out of 14 and 18, respectively) in ELISAs, whereas both panels showed cross-reactivity to heterologous NS1 proteins in Western blot analysis (3/21 in pDENV panel; 15/18 and 5/18 in pWNV panel).

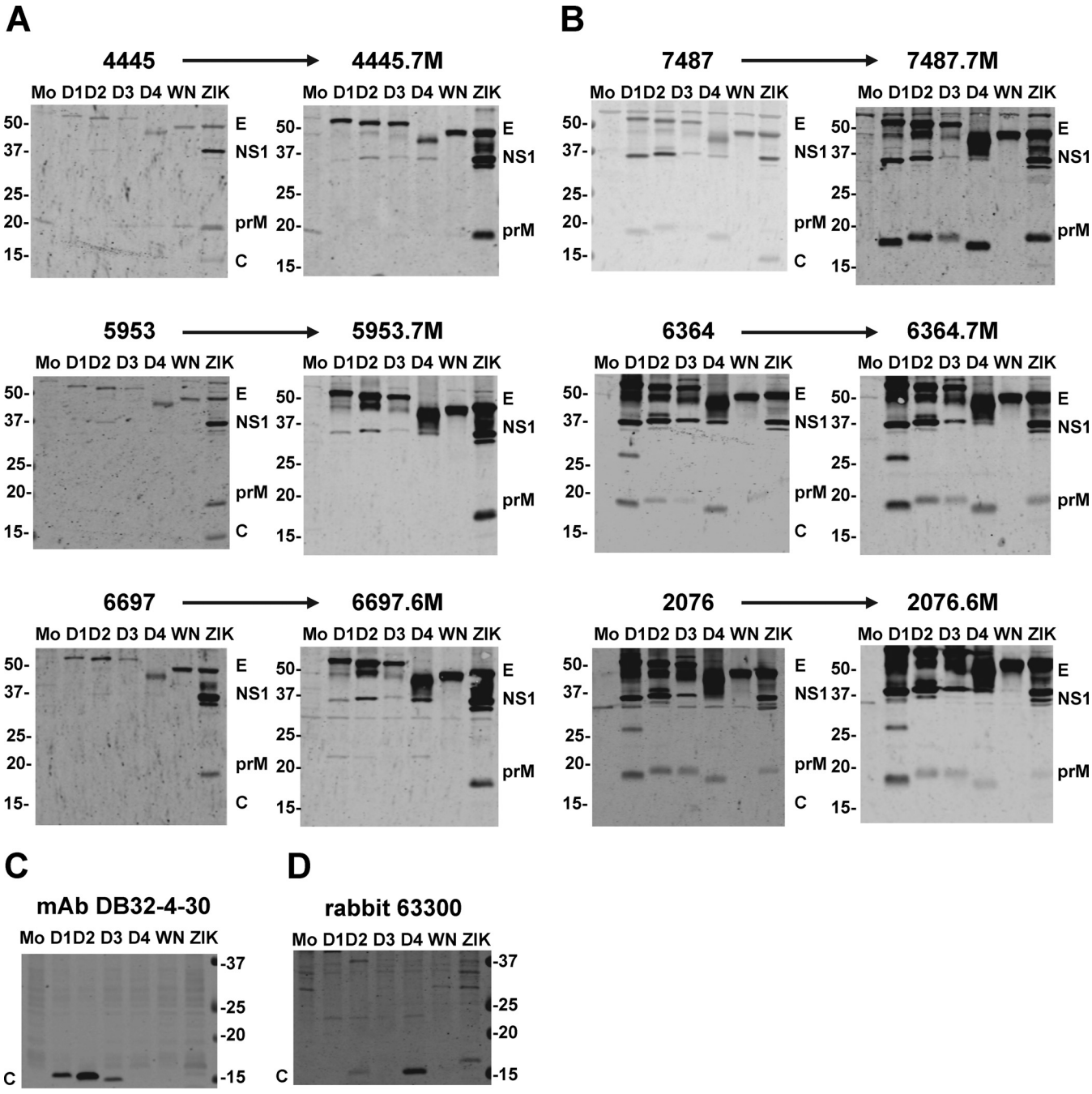

FIG 2 Antibody responses during convalescent-phase following ZIKV infection. Lysates derived from mock-, DENV1 to 4-, WNV-, and ZIKV-infected Vero cells were subjected to SDS-12% polyacrylamide gel electrophoresis under nonreducing condition and Western blot analysis probed with convalescent- and postconvalescent-phase samples from 3 cases each with primary ZIKV (A) and ZIKV infection with previous DENV (B) infections and with a mouse MAb DB-32-4-30 recognizing DENV1 to 3 C proteins (C) and a rabbit serum against DENV2 and DENV4 C proteins (D). The positions of E, NS1, prM, and C protein bands are indicated. The size of molecular weight markers is shown in kDa. Mo, mock; D1, DENV1; D2, DENV2; D3, DENV3; D4, DENV4; WN, WNV; ZIK, ZIKV.

Similarly, postconvalescent-phase samples from pZIKV panel recognized ZIKV NS1 protein but not DENV or WNV NS1 protein in ELISAs, whereas they cross-reacted to DENV NS1 protein (17/18) in Western blot analysis (Fig. 4C). Consistent with previous reports of slow development of anti-NS1 antibody in ELISA among patients with pZIKV infection (38, 39), the detection rate of anti-NS1 antibody to ZIKV increased from 5% (1/20) in convalescent-phase samples to 100% (18/18) in postconvalescent-phase samples based on ELISA. Interestingly, anti-NS1 antibody to ZIKV can be detected in all

TABLE 4 Sensitivity and specificity of different ZIKV proteins recognized by convalescent-phase ZIKV samples in Western blot analysis

Viral proteins recognized[d]	Group	% Sensitivity (95% CI)[a,b]	% Specificity (95% CI)[a,b]
ZIKV NS1	Overall	100 (100–100)	68.6 (57.7–74.1)
	Subgroup	pZIKV, 100; ZIKVwprDENV, 100	Naive, 100; pDENV, 85.7; sDENV, 33.3; pWNV, 72.2
ZIKV prM	Overall	65.0 (50.2–72.5)	98.6 (95.8–100)
	Subgroup	pZIKV, 75.0; ZIKVwprDENV, 55.0	Naive, 100; pDENV, 100; sDENV, 95.2; pWNV, 100
ZIKV C	Overall	67.5 (53.0–74.9)	100 (100–100)
	Subgroup	pZIKV, 85.0; ZIKVwprDENV, 50.0	Naive, 100; pDENV, 100; sDENV, 100; pWNV, 100
ZIKV prM or C	Overall	**90.0 (80.7–94.7)**[c]	**98.6 (95.8–100)**[c]
	Subgroup	pZIKV, 95.0; ZIKVwprDENV, 85.0	Naive, 100; pDENV, 100; sDENV, 95.2; pWNV, 100

[a]CI, confidence interval; pDENV, primary DENV infection; sDENV, secondary DENV infection; pZIKV, primary ZIKV infection; ZIKVwprDENV, ZIKV infection with previous DENV infection; pWNV, primary WNV infection. Comparison with convalescent-phase samples (14 to 24 days post-symptom onset) for pZIKV and ZIKVwprDENV panels.
[b]For simplicity, the 95% CIs in the subgroup are not shown.
[c]The sensitivity and specificity of recognizing ZIKV prM or C are bolded.
[d]NS1, nonstructural protein 1; prM, premembrane; C, capsid; D1, DENV1; D2, DENV2; D3, DENV3; D4, DENV4.

convalescent-phase samples (20/20) by Western blot analysis, though with 35% (7/20) cross-reactivity to DENV NS1 (Fig. 4C). As a comparison, ZIKVwprDENV panel recognized both ZIKV and DENV NS1 proteins by ELISA and Western blot analysis with cross-reactivity to heterologous WNV NS1 protein ranging from 25 to 70% (5/20 to 14/20) to 20 to 40% (3/15 to 6/15) in convalescent-phase and postconvalescent-phase, respectively (Fig. 4D). Taken together, these findings suggest different sensitivity and specificity of detecting anti-NS antibodies by ELISA and Western blot analysis, in which the latter is more sensitive but less specific than the former.

DISCUSSION

In this study, we employed viral antigens of 6 viruses from 3 different flavivirus serogroups in Western blot analysis to examine antibody responses following 3 different flavivirus infections and identified anti-prM antibody as a flavivirus serocomplex-specific marker with sensitivities/specificities of 100/98.6%, 89.5/100%, and 88.9/96.5% for ZIKV, DENV, and WNV panels, respectively. These observations have important applications for serodiagnosis and serosurveillance of ZIKV, DENV, and WNV infections in regions where multiple flaviviruses cocirculate.

In agreement with the flavivirus cross-reactivity reported previously (33–35, 46), anti-E antibodies cross-reactive to all 6 flaviviruses were found following different ZIKV, DENV, and WNV infections. Compared with those following pZIKV infection during the convalescent phase, anti-E antibodies were stronger and developed quicker after ZIKVwprDENV infection (Fig. 1). Among the postconvalescent-phase samples, anti-prM antibodies to ZIKV could be detected in all (33/33) of the pZIKV and ZIKVwprDENV panels, resulting in a sensitivity/specificity of 100/98.6% (Table 2). Comparing pZIKV and ZIKVwprDENV panels, anti-prM antibodies to any DENV serotype were detected in 10/15 of the ZIKVwprDENV panel but in 0/18 of the pZIKV panel ($P < 0.0001$, Fisher exact test) (Table 2), suggesting that anti-DENV prM antibodies can distinguish these two panels. For the convalescent-phase samples, anti-prM antibodies to ZIKV were detected in 26/40 ZIKV samples (pZIKV and ZIKVwprDENV panels together) with a sensitivity/specificity of 65.0/98.6%. Similarly, anti-C antibodies to ZIKV were detected in 27/40 ZIKV samples with a sensitivity/specificity of 67.5/100%; a combination of anti-prM and anti-C antibodies resulted in a sensitivity/specificity of 90.0/98.6% (Table 4). Comparing pZIKV and ZIKVwprDENV panels revealed that anti-DENV prM antibodies can be detected in 16/20 of the ZIKVwprDENV panel but not in the pZIKV panel (0/20, $P < 0.0001$, Fisher exact test) (Table 2).

We found that compared with ELISA, Western blot analysis is more sensitive but less specific in detecting anti-NS1 antibodies (Fig. 4). It is worth noting that recombinant NS1 protein is present as a hexamer in solution, whereas NS1 protein in Western blot analysis is a monomer under detergent treatment. It is possible that these cross-reactive anti-NS1

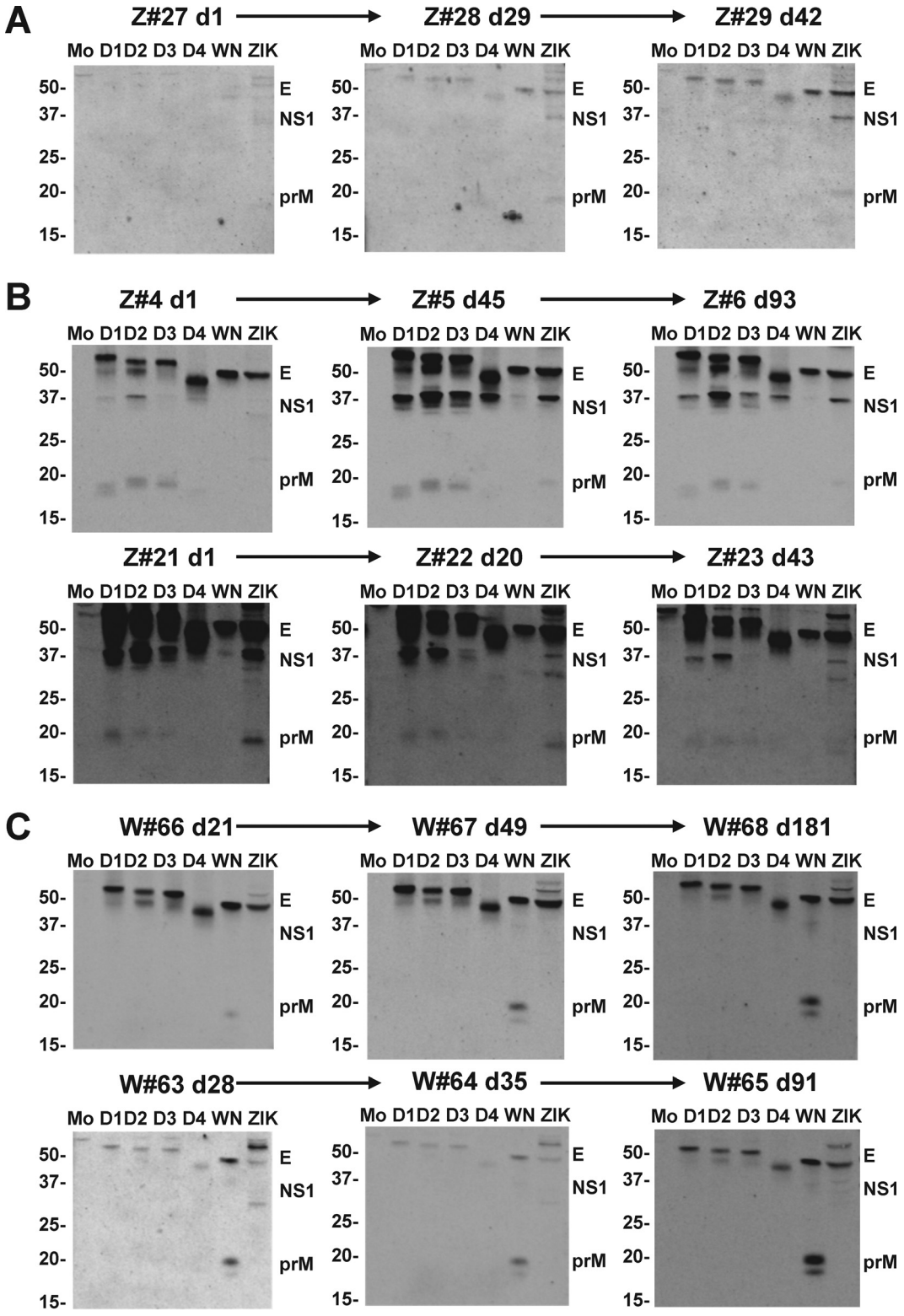

FIG 3 Antibody responses of sequential samples following ZIKV and WNV infections. Lysates derived from mock-, DENV1 to 4-, WNV-, and ZIKV-infected Vero cells were subjected to SDS-12% polyacrylamide gel electrophoresis under nonreducing condition and Western blot analysis probed with sequential samples of blood donors from the index day when ZIKV RT-PCR or WNV TMA was positive (day 1) to day 181. One donor with primary ZIKV (A), two donors with ZIKV infection with previous DENV (B), and two donors with primary WNV (C) infections. The positions of E, NS1, and prM protein bands are indicated. The size of molecular weight markers is shown in kDa. Mo, mock; D1, DENV1; D2, DENV2; D3, DENV3; D4, DENV4; WN, WNV; ZIK, ZIKV.

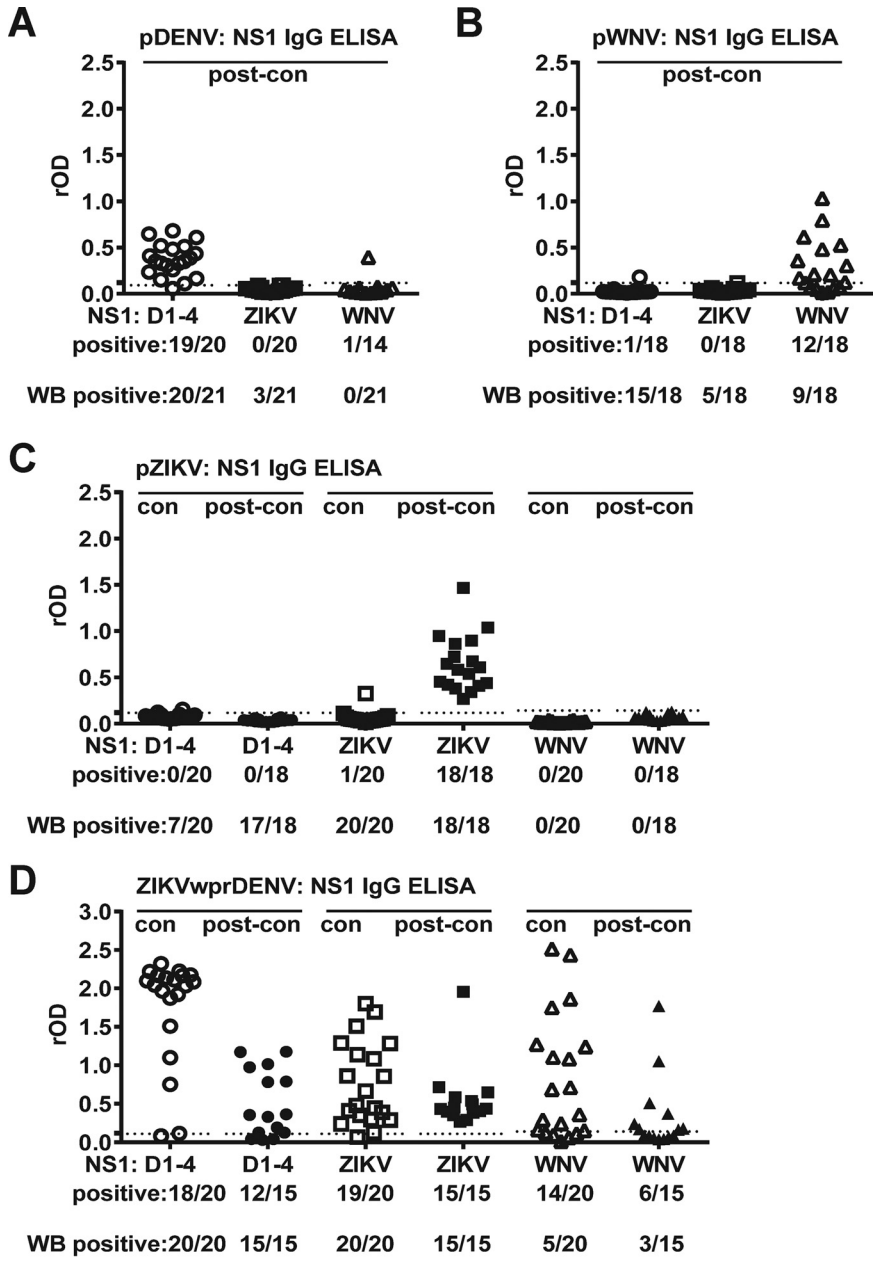

FIG 4 Detection of anti-NS1 antibodies in primary DENV, ZIKV, and WNV panels by ELISA compared with Western blot analysis. DENV1 to 4, ZIKV, and WNV-NS1 IgG ELISAs were tested for pDENV (A), pWNV (B), pZIKV (C), and ZIKVwprDENV (D) panels. Dotted lines indicate cutoff rOD values. The positive rates of ELISA were compared with those of Western blot analysis in Table 1. Data are means of two experiments (each in duplicate). D1 to 4, DENV1 to 4; con, convalescent; post-con, postconvalescent.

antibodies can only recognize linear epitopes in detergent-treated NS1 monomers but not recombinant NS1 hexamers in ELISA, thus resulting in reduced cross-reactivity in ELISA. Although anti-NS1 antibodies in Western blot analysis could not distinguish ZIKV and DENV infections, in particular sDENV and ZIKVwprDENV panels, anti-ZIKV prM and/or C antibodies could distinguish these two panels with a sensitivities/specificities of 100/95.2% and 85.0/95.2% for the postconvalescent-phase and convalescent-phase samples, respectively (Table 2).

Within the DENV serocomplex, we found anti-NS1 antibodies, though cross-reactive to all four DENV serotypes, recognized the homologous serotype more strongly than

the heterologous serotypes (Fig. 1D); this is consistent with our previous reports of higher detection rate of anti-DENV NS1 antibodies to homologous serotype than heterologous serotypes for the pDENV panel in IgG ELISA and in Western blot analysis (40, 46). Due to the small sample size in our pDENV panel and variability of anti-NS1 cross-reactivity, it is difficult to use anti-NS1 antibodies in Western blot analysis to discriminate infecting DENV serotypes. Comparing pDENV and sDENV infection panels, a higher rate of cross-reactive anti-ZIKV NS1 antibodies was found in the sDENV panel than in the pDENV panel (14/21 versus 3/21; $P = 0.001$, Fisher exact test) (Table 2).

PRNT is generally regarded as a gold standard for serological tests for ZIKV and other flaviviruses (35). PRNT can confirm ZIKV infection for those who acquire ZIKV as the first flavivirus infection, known as pZIKV infection; however, for those who have experienced other flavivirus infections before, PRNT results can only be interpreted as unspecified flavivirus infection, greatly restricting the application of PRNT for ZIKV surveillance in endemic regions. With the high specificity of anti-prM antibodies to different serocomplexes in Western blot analysis, our assay can discriminate different ZIKV (pZIKV and ZIKVwprDENV), DENV, and WNV infections. A recent study reported different cross-neutralization patterns between ZIKV and DENV infections (50); however, the sensitivity and specificity of neutralizing antibody titers to discriminate DENV and ZIKV infections, in particular sDENV and ZIKVwprDENV panels, remains to be defined. Neutralization tests are time-consuming and can be performed only in reference laboratories (35). Compared with PRNT, our Western blot analyses require less time (18 h for 6 viral antigens versus 5 to 6 days for PRNT for each virus) and less sample volume (5 μl versus 128 μl for PRNT for 6 antigens or viruses). Moreover, Western blot analysis using precoated membranes/strips is simple, inexpensive, and readily applicable to regional laboratories in developing countries, as exemplified by the first-generation HIV immunoassays (51). Due to the IgG-based detection in Western blot analysis, our assay focused on IgG rather than IgM antibodies and was not intended for serodiagnosis during the acute phase of infection. It has been reported that neutralizing antibodies following primary flavivirus infections become more type specific over a period of 3 months (50, 52). Analysis of sequential antibodies following primary ZIKV or WNV infection did not reveal increased type specificity during a period of 3 months (Fig. 3A and C; see also Table S2 in the supplemental material), suggesting different characteristics between neutralizing antibody and binding antibody detected by Western blot analysis.

Our assay can be applied to identify the immune background of potential participants in a vaccine trial or a seroepidemiological study and to confirm the presence of three flaviviruses in endemic regions. Given the complex interactions between DENV and ZIKV immunity, previous investigations of the effects of DENV immunity on ZIKV outcomes and ZIKV immunity on DENV outcomes have relied exclusively on cohort studies (30–32). Our assay could be applied in retrospective studies of pregnant women with CZS or normal babies to understand the effects of different ZIKV, DENV, and/or WNV immune status on pregnancy outcomes. These together would enhance our understanding of the epidemiology, pathogenesis, and complications of ZIKV in endemic regions (6, 7, 9, 10).

This study had several limitations. First, the sample size in each panel of RT-PCR-confirmed flavivirus infection is small; future studies involving larger sample size are needed to verify these observations. Second, convalescent-phase samples were available from pZIKV, ZIKVwprDENV, and pWNV panels but not from pDENV and sDENV panels. Future studies involving convalescent-phase samples from pDENV and sDENV panels are needed to confirm these findings. Third, as most human anti-prM MAbs to DENV recognize pr protein, the possibility of using recombinant pr protein as antigen in serological tests to distinguish different flaviviruses remains to be explored. It is worth noting that flavivirus prM protein has been shown to serve as a chaperone for proper folding of E protein and does not express well in the absence of E protein (53, 54). Fourth, compared with previous studies of antibody responses to DENV using Western blot analysis, which

included antigens from one to two DENV serotypes (55–57), our study including 6 flavivirus antigens provides new information about the extent of cross-reactivity. Serological tests involving more flavivirus antigens to distinguish ZIKV from other medically important flaviviruses, such as JEV, YFV, and tick-borne encephalitis virus (TBEV) are warranted (45, 58). Furthermore, it is important to compare Western blot analysis and neutralizing antibodies for their specificity and sensitivity to distinguish convalescent antibodies from primary flavivirus infections using a blinded test format. In addition, given the introduction of several flaviviral vaccines and vaccine trials in endemic regions, serological tests that can distinguish ZIKV infection from immunization with flavivirus vaccines including DENV, JEV, YFV, and TBEV vaccines remain to be explored (45, 58).

MATERIALS AND METHODS

Human samples. This study was approved by the Institutional Review Boards (IRB) of the University of Hawaii (CHS number 17568, CHS number 23786). Table 1 summarizes the numbers, sampling times, and sources of different panels of serum or plasma samples. Samples from RT-PCR-confirmed Zika cases including DENV-naive ($n = 20$) or previously DENV-exposed ($n = 20$), designated as pZIKV and ZIKVwpDENV panels, respectively, were collected between July and March 2017 from the Pediatric Dengue Cohort Study and the Pediatric Dengue Hospital-based Study in Managua, Nicaragua (59, 60). These studies have been approved by the IRBs of the University of California, Berkeley, and the Nicaraguan Ministry of Health. Eighteen plasma samples from blood donors who tested positive for WNV TMA, IgM, and IgG antibodies between 2006 and 2015, designated as primary WNV (pWNV) infection, were provided by the American Red Cross at Gaithersburg, Maryland (61). Postconvalescent-phase samples from RT-PCR-confirmed cases with pDENV infection (including primary DENV1, DENV2, or DENV3 infection) or sDENV infection (including secondary DENV1, DENV2, or DENV3 infection) were from Taiwan, Hawaii, and Nicaragua prior to the 2015 to 2016 Zika outbreak; 10 flavivirus-naive samples from a seroprevalence study were included as controls (47, 60–62). pDENV or sDENV infection was determined by IgM/IgG ratio or focus-reduction neutralization tests as described previously (47, 62–64). Sequential plasma samples from 10 blood donors including five with pZIKV and five with ZIKVwprDENV infections between June and September 2016, and sequential plasma samples from six blood donors with pWNV infection between 2005 and 2006 (Table 1) were provided by the Vitalant Research Institute at San Francisco (with approval by IRB of the University of California, San Francisco) (39).

Western blot analysis. Vero cells infected with mock, DENV1 (Hawaii strain), DENV2 (NGC strain), DENV3 (CH53489 strain), DENV4 (H241 strain), ZIKV (PRVABC59 strain), or WNV (NY99 strain) were lysed with NP-40 lysis buffer (1% NP-40, 50 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, and 1 mM Na_3VO_4) when cytopathic effects were observed in 50% of cells. The cell lysates were subjected to SDS-12% polyacrylamide gel electrophoresis under nonreducing conditions (2% SDS, 0.5 M Tris, pH 6.8, 20% glycerol, 0.001% bromophenol blue, final) (46, 47), followed by transfer to nitrocellulose membrane (Hybond-C Extra; GE Healthcare), hybridization with human serum/plasma samples (1:200 dilution), mouse MAb, or rabbit serum against C protein and secondary antibody (IRDye 800CW-conjugated goat anti-human IgG at 1:10,000). The signal was detected by Li-Cor Odyssey classic (Li-Cor Biosciences) and analyzed by Image Studio software with both short and long exposures (65). Each gel was read independently by 3 researchers with the results summarized in Tables S1 and S2 in the supplemental material.

Recombinant NS1 proteins. The NS1 gene (residues 1 to 352) of ZIKV (HPF2013 strain) with a His-tag at the C terminus was codon optimized (Integrated DNA Technologies, Skokie, IL), cloned into pMT-Bip vector, stably expressed in *Drosophila* S2 cells, and purified by a fast-purification chromatography system (AKTA Pure; GE Health Care Bio-Science, Pittsburg, PA) (39). Purified DENV1 to DENV4 and WNV NS1 proteins were purchased from the Native Antigen (Oxford, UK).

ELISAs. Briefly, purified recombinant NS1 proteins (16 ng for ZIKV or WNV NS1 protein per well and 8/4/8/4 ng for mixed DENV1/2/3/4 NS proteins per well) were coated onto 96-well plates at 4°C overnight, followed by blocking (StartingBlock blocking buffer; Thermo Scientific, Waltham, MA) at room temperature for 1 h, incubation with primary antibody (serum or plasma at 1:400 dilution) at 37°C for 2 h, wash with washing buffer (0.5% Tween 20 in 1× PBS) 4 times, incubation with secondary antibody (anti-human IgG conjugated with horseradish peroxidase [HRP] at 1:10,000 dilution; Jackson Immune Research Laboratory, West Grove, PA) at 37°C for 1 h, and washed with washing buffer 6 times (39, 40). After incubation with tetramethylbenzidine substrate (Thermo Scientific, Waltham, MA) at room temperature for 15 min and stop solution, the optical density (OD) at 450 nm was read with a reference wavelength of 630 nm. Each ELISA plate contained two positive controls (OD higher than 1; two confirmed-ZIKV, DENV, or WNV samples), four negative controls (flavivirus-naive serum or plasma), and test samples (all in duplicate). For comparison between plates, the relative OD (rOD) values were calculated by the OD values divided by the mean OD value of one positive control (OD close to 1) in the same plate. The cutoff rOD was defined by the mean rOD value of negatives plus 12 standard deviations, which gave a confidence level of 99.9% from 4 negatives (66). Each ELISA was performed twice (each in duplicate).

Statistical analysis. The sensitivity, specificity, and 95% confidence interval (CI) was calculated by Excel. The two-tailed Fisher's exact test was employed to compare detection rates (categorical variable) between two groups (GraphPad Prism 6).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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