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Immunoassay Targeting Nonstructural Protein 5 To Differentiate West Nile Virus Infection from Dengue and St. Louis Encephalitis Virus Infections and from Flavivirus Vaccination

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West Nile virus (WNV) is an emerging flavivirus that has caused frequent epidemics since 1996. Besides natural transmission by mosquitoes, WNV can also be transmitted through blood transfusion and organ transplantation, thus heightening the urgency of development of a specific and rapid serologic assay of WNV infection. The current immunoassays lack specificity because they are based on detection of antibodies against WNV structural proteins and immune responses to structural proteins among flaviviruses cross-react to each other. Here, we describe microsphere immunoassays that detect antibodies to nonstructural proteins 3 and 5 (NS3 and NS5). In contrast to immunoassays based on viral envelope and NS3 proteins, the NS5-based assay (i) reliably discriminates between WNV infections and dengue virus or St. Louis encephalitis virus infections, (ii) differentiates between flavivirus vaccination and natural WNV infection, and (iii) indicates recent infections. These unique features of the NS5-based immunoassay will be very useful for both clinical and veterinary diagnosis of WNV infection.

West Nile virus (WNV) is a member of the genus *Flavivirus*, which includes many significant human pathogens of global epidemiological importance, including four serotypes of dengue (DEN) virus, yellow fever (YF) virus, Japanese encephalitis (JE) virus, St. Louis encephalitis (SLE) virus, and tick-borne encephalitis (TBE) virus, as well as WNV (3). Among them, DEN virus, YF virus, TBE virus, JE virus, and WNV are listed by the National Institutes of Health as potential biodefense pathogens. Since its introduction into the United States in 1999, WNV has resulted in more than 4,156 known human cases, with 284 deaths (for updates, see http://www.cdc.gov/ncidod/dvbid/westnile/surv&controlCaseCount03.htm). Recent studies have shown that, besides natural transmission by mosquitoes, WNV can also be transmitted through blood transfusion, organ transplantation (9), breast feeding (8), intrauterine exposure (6), and laboratory-acquired infection (7). These findings have underlined the importance of developing an accurate serologic assay for diagnosis of WNV infection.

Flavivirus genomic RNA contains a single open reading frame encoding 10 viral proteins: three structural and seven nonstructural (NS) proteins (Fig. 1A). Viral envelope protein (E protein) (14), NS1 (18, 25, 26), and NS3 (24) are the most immunogenic proteins during flavivirus infection (15). The current serologic diagnosis of WNV infection is based on detection of antibodies against viral structural proteins, mainly the E protein (12, 21). Unfortunately, the high cross-reactivity of the E protein among flaviviruses limits the specificity of the assay. Positive sera or spinal fluids identified by the current assay must be verified by cross-species plaque reduction neutralization tests (PRNT) to exclude the possibility of infection with cross-reactive viruses such as SLE and DEN. These confirmatory tests have to be performed in level 3 biocontainment for many flaviviruses and substantially lengthen the overall time required for a definitive serologic test.

In this study, we used NS3 and NS5 as targets to develop a novel serologic assay for WNV diagnosis. NS3 and NS5 are key enzymes in flavivirus RNA replication (2). NS3 functions as a serine protease (in the presence of cofactor NS2b), 5′-RNA triphosphatase, nucleoside triphosphatase (NTPase), and helicase; NS5 functions as a methyltransferase and RNA-dependent RNA polymerase (RDRP) (19). Our results demonstrate that the NS5-based immunoassay reliably discriminates between WNV infections and DEN or SLE virus infections and that it differentiates between flavivirus vaccination and natural WNV infection.

MATERIALS AND METHODS

Human sera. Six panels of human sera were used in this study. (i) WNV patient sera were from serum archives at the Wadsworth Center, New York State Department of Health. These sera had previously tested WNV positive by the immunoglobulin M (IgM) capture and indirect IgG enzyme-linked immunosorbent assay for antibodies reactive to noninfectious recombinant antigen (12, 17, 21). (ii) Paired acute- and convalescent-phase sera from DEN patients were provided by the National Microbiology Laboratory, Health Canada. The patients are Canadian residents who became infected with DEN during recent travels to

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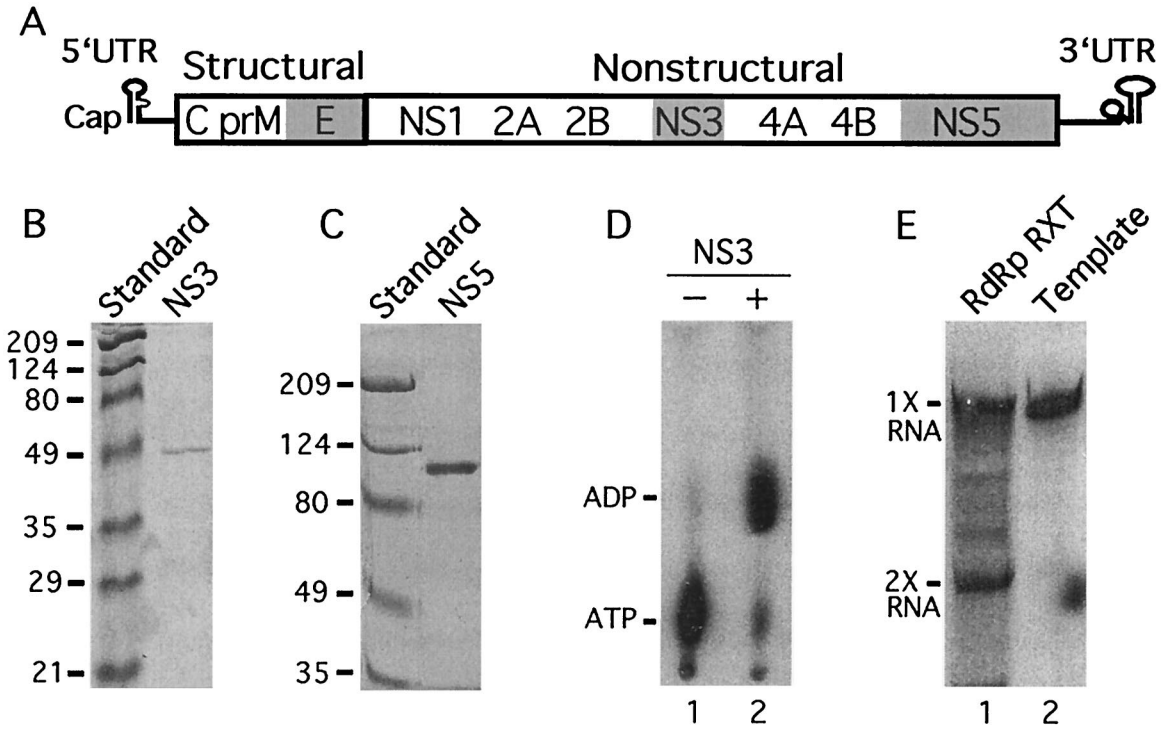

FIG. 1. (A) WNV genome structure. The recombinant proteins used in this study are shaded. (B and C) Purified NTPase/helicase domain of NS3 and full-length NS5 were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; gels were stained with Coomassie blue. (D) ATPase activity of the recombinant NTPase/helicase domain of WNV NS3. In the presence of recombinant NS3, [α-^{32}P]ATP was hydrolyzed to [α-^{32}P]ADP and phosphate (lane 2). No ATP is hydrolyzed in the absence of NS3 (lane 1). (E) RDRP activity of the recombinant NS5. The RDRP activity of NS5 was assayed with a WNV subgenomic RNA transcript (890 nt) containing a large deletion from nucleotide 269 to 10408. The reaction products (RXT) were labeled with [α-^{32}P]UTP, and the products of 1× and 2X forms of RNA were analyzed on a denaturing polyacrylamide gel followed by autoradiography (lane 1). A ^{32}P-labeled template RNA was loaded as a size control (lane 2).

various geographical regions. These sera had been tested by hemagglutination inhibition (HI) assays and PRNT for DEN, Powassan, or SLE virus. (iii) Forty SLE patient sera were generously provided by the Centers for Disease Control and Prevention (CDC). These samples had been previously confirmed by PRNT for SLE positive and WNV negative. (iv) JE-vaccinated human sera were from laboratory employees who had received three doses of formalin-inactivated JE vaccine. (v) Nineteen YF-vaccinated human sera were provided by the CDC. (vi) A panel of human sera from the Diagnostic Immunology Laboratory of the Wadsworth Center was used to examine the specificity of the WNV assays, including human specimens that were reactive in serologic assays for the agents of Lyme disease (*Borrelia burgdorferi* infection), ehrlichiosis (*Anaplasma phagocytophilum* infection), and syphilis (*Treponema pallidum* infection), human immunodeficiency virus (HIV), Epstein-Barr virus, cytomegalovirus, antinuclear antibodies, and rheumatoid factor. All samples were tested in a blinded fashion, with patient identifiers removed, according to guidelines of the National Institutes of Health and the Institutional Review Board of the New York State Department of Health.

Expression, purification, and enzyme assays of the NTPase/helicase domain of NS3 and full-length NS5. The NTPase/helicase domain of NS3 (amino acids 182 to 619) and full-length NS5 were cloned into the pET-21a and pET-28a vectors, respectively, and expressed in *Escherichia coli* BL21 cells upon induction with isopropyl-β-D-thiogalactopyranoside at 30°C for 3 to 4 h. The recombinant NS5 and NS3 NTPase/helicase domains contained a His_6 tag at the N and C termini, respectively, and were purified through a nickel column (Novagen, Madison, Wis.).

The NTPase assay was performed in a 10-μl reaction volume containing 20 mM Tris (pH 7.5), 2.5 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM cold ATP spiked with 1 μCi of corresponding [α-^{32}P]ATP (2,000 Ci/mmol) (Amersham, Piscataway, N.J.), and 0.8 μM recombinant NS3. The reaction mixture was incubated at 37°C for 30 min, and the reaction was terminated by addition of 1 μl of 0.5 M EDTA disodium salt. The reaction product (1 μl) was spotted onto a plastic-backed polyethyleneimine cellulose F sheet (J.T. Baker, Phillipsburg, N.J.) and analyzed by ascending thin-layer chromatography using 0.375 M potassium phosphate as a running buffer (pH 3.5). The thin-layer chromatogram was dried, visualized by autoradiography, and quantified with a phosphorimager analyzer. The RDRP assay was performed as previously described (1). The RDRP activity of NS5 was assayed by using a WNV subgenomic RNA transcript containing a large deletion from nucleotide 269 to 10408. The reaction products were labeled with [α-^{32}P]UTP and analyzed on a 4% denaturing polyacrylamide gel followed by autoradiography (1).

Cross-species PRNT and HI assays. Neutralizing antibodies were evaluated in PRNT with WN, SLE, or JE virus as previously described (20). Standard HI tests for DEN, Powassan, and SLE viruses and WNV were performed (4).

MIA. Approximately 50 μg of recombinant NS3, NS5, or E protein was covalently linked to the carboxylated surface of 6.25×10^6 microspheres through a two-step carbodiimide linkage protocol as described by the manufacturer (Luminex Corporation, Austin, Tex.). A two-step suspension microsphere immunoassay (MIA) was performed. A 96-well 1.2-μm filter plate (Millipore, Bedford, Mass.) was blocked for 2 min with 100 μl of PBN buffer (phosphate-buffered saline [pH 7.4] with 1% bovine serum albumin and 0.05% sodium azide), washed once with 150 μl of PBS-T buffer (phosphate-buffered saline [pH 7.4] with 0.05% Tween 20), and then wetted with 20 μl of PBN buffer. Serum samples (50 μl, diluted 1:100 in PBN buffer unless otherwise specified) and antigen-conjugated microspheres (2,500 in 50 μl of PBN buffer) were added to each well. The plate was incubated in the dark on a shaker at 37°C for 30 min and then washed three times with PBS-T using a vacuum manifold. Polyvalent goat anti-human immunoglobulins (IgG, IgA, and IgM; 50 μl of a 1:250 dilution in PBN buffer) conjugated with red-phycoerythrin (Bio-Source International, Camarillo, Calif.) were added. After incubation at 37°C for 30 min, the plate was washed twice with PBS-T. Microspheres were resuspended in 125 μl of PBN per well, and 75 μl of suspension was transferred to an opaque black enzyme immunoassay/radioimmunoassay 96-well plate (Costar, Corning, N.Y.). The microsphere fluorescence intensity (MFI) was quantified with a Luminex 100 flow analyzer (Luminex Corporation). The MFI of 100 microspheres was recorded for

each well. The mean for 20 normal sera plus 3 standard deviations (SD) was used as the cutoff value for each assay.

RESULTS

Recombinant NS3 and NS5 of WNV retain NTPase and RDRP activities. The NTPase/helicase domain (amino acids 182 to 619) of NS3 (Fig. 1B) and full-length NS5 (Fig. 1C) of WNV were expressed and purified by using an *E. coli* expression system. The recombinant proteins were enzymatically active: NS3 exhibited an NTPase activity in hydrolyzing ATP to ADP and phosphate (Fig. 1D), and NS5 retained RDRP activity, using WNV RNA as a template to synthesize two types of RNA products: 1× form and 2× form (Fig. 1E). The activities of WNV NS3 and NS5 are comparable to those of DEN NS3 (11) and NS5 (1). The enzymatic activities indicate retention of native conformation by the recombinant NS3 and NS5.

Establishment of the NS3- and NS5-based MIAs. An MIA was selected to establish the NS3- and NS5-based serologic assays to detect antibodies induced by WNV infection. Recombinant NS3 or NS5 was covalently linked to microsphere beads and then reacted with patient serum followed by anti-human immunoglobulins with a fluorescent conjugate. The levels of reactive antibodies from the sera were quantified by a flow analyzer. Initially, 20 human sera from healthy individuals were used to establish cutoff levels for the assay. The MFI for NS3 was 909 (SD, 351), with an assay cutoff of 1,962; the mean MFI for NS5 was 1,810 (SD, 852), with an assay cutoff of 4,366. Analyses of five positive WNV sera, which had been previously confirmed by a subviral particle-based immunoassay (12) and PRNT, revealed that the NS5 MIA had an assay dynamic range of 32, from 100- to 3,200-fold serum dilutions. The NS3-based MIA did not exhibit consistent signals above the background level with these sera (see below).

NS5-based MIA reliably detects WNV infection and may indicate recent infections. A total of 61 sera from WNV patients with clinical symptoms and confirmation by PRNT were subjected to NS5- and NS3-based MIA, along with the recombinant-E-protein-based MIA for comparison (S. J. Wong et al., submitted for publication). In Fig. 2A to C, MFI values of individual serum samples collected from various WNV patients are plotted against days after symptom onset. The plot (Fig. 2A) shows that the NS5-reactive signals appeared on day 6, the MFIs for 35 of 38 (92%) sera collected from day 7 to 77 were positive, and the MFI dropped to a negative level for two sera collected on days 259 and 431. For the NS5-positive sera, the MFI ranged from 6,200 to 19,500 with a mean value of 14,400. The reactive pattern derived from the NS5-based assay correlated well with that from the E-protein-based assay, except that in the latter assay, reactive signals appeared around day 2 to day 6, and the MFI remained positive throughout the later time points, including day 259 and day 431 (compare Fig. 2A and C). Samples from days 29, 46, and 62 were negative in the NS5 MIA (Fig. 2A) but were positive in the E-protein MIA (Fig. 2C). The discrepancies between the NS5 and E-protein MIA results for these samples may be due to different immune responses to NS5 and E antigens in these individuals. On the other hand, the NS3 MIA did not exhibit consistent signals above the background level, with fewer than half of the sera showing positive MFIs (Fig. 2B); NS3 therefore was not further analyzed. These results demonstrate that the NS5-based MIA is a sensitive assay for detection of human WNV infection.

To examine the persistence of antibody against E protein and NS5 upon WNV infection, we examined a series of sera collected from a single patient at various times postinfection (Fig. 2D). Positive MFI signals were detected on day 17 after symptom onset in both E protein and NS5 MIAs. Signals from the E-protein-based MIA remained positive for sera collected on days 71, 259, and 431 after symptom onset (Fig. 2D). In contrast, signals from the NS5-based MIA were positive for sera collected on days 17 and 71 after symptom onset; however, the MFI declined to a negative range on day 259 and 431 after symptom onset (Fig. 2D). These results suggest that a positive NS5-based MIF indicates current or recent infection (see Discussion).

NS5-based MIA differentiates WNV infection from nonflavivirus infections or diseases and from flavivirus vaccination. The specificity of the NS5-based MIA was demonstrated by challenging 120 sera from patients with various infections, autoimmune conditions, JE vaccination, YF vaccination, or good health (Table 1). Only one patient with HIV infection showed an MFI (7,517) above the cutoff level of the NS5 MIA (4,366). It should be noted in particular that none of the sera from the JE vaccine recipients reacted with the WNV NS5 antigen; only 1 of 19 (5%) YF vaccine recipients exhibited a positive MFI signal. By contrast, all 10 (100%) JE-vaccinated sera and 10 of the 19 (53%) YF-vaccinated sera showed positive MFIs in the E-protein-based MIA (data not shown). These results demonstrated that the NS5-based assay can be used to differentiate between WNV infection and vaccinations with either an inactivated (JE virus) or a live attenuated (YF virus) flavivirus.

NS5-based MIA differentiates WNV infection from DEN or SLE virus infections. The cross-reactivity of WNV NS5 and E protein with DEN infection was tested with 17 pairs of acute- and convalescent-phase sera from DEN-infected individuals (Table 2). The DEN patient sera reacted with WNV E protein. The MFI signal and the titer of the E-protein MIA correlated well with the HI titer of the sera. Twenty-four of the 34 (71%) DEN sera tested positive in the E-protein-based MIA; 8 samples with negative E-protein MIA results either were HI negative or showed low HI titers. For the NS5-based MIA, only 3 of the 34 (9%) DEN sera were marginally positive (samples 3A, 4B, and 11A), with MFI values very close to the cutoff value. Next, we examined the potential cross-reactivity of WNV NS5 and E protein with SLE patient sera. Among the 20 pairs of SLE sera that had been previously confirmed by PRNT, only 2 (5%) sera were MFI positive (samples 3A and 3B) in the WNV NS5-based assay, while 11 of the 40 (27.5%) SLE sera were positive in the E-protein-based assay (Table 3). These results suggest that, compared with the E-protein-based MIA, the NS5-based MIA exhibits substantially improved discrimination between DEN/SLE virus and WNV infections.

DISCUSSION

WNV structural proteins have been used to serologically detect a broad range of flavivirus infections (12). Various approaches have been taken to improve the specificity of the diagnosis of flavivirus infection (13, 16, 25). In this study, we

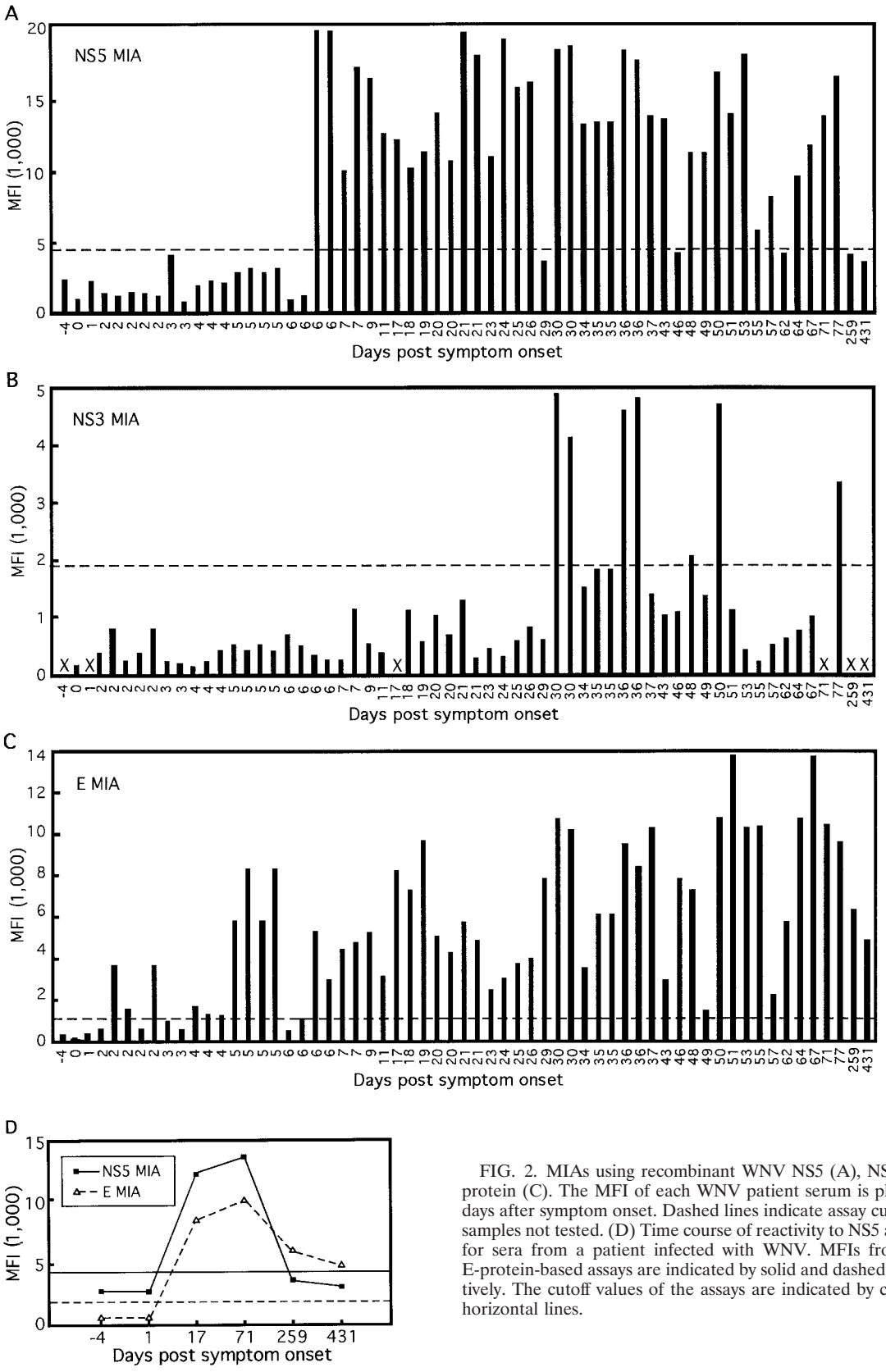

FIG. 2. MIAs using recombinant WNV NS5 (A), NS3 (B), and E protein (C). The MFI of each WNV patient serum is plotted against days after symptom onset. Dashed lines indicate assay cutoff levels. X, samples not tested. (D) Time course of reactivity to NS5 and E protein for sera from a patient infected with WNV. MFIs from NS5- and E-protein-based assays are indicated by solid and dashed lines, respectively. The cutoff values of the assays are indicated by corresponding horizontal lines.

TABLE 1. Specificity of the NS5-based MIA tested against various human sera

Specimen type	No. of sera	Mean MFI (range)	SD	No. positive[a]
Syphilis (*T. pallidum* positive)	10	1,862 (7–3,375)	1,241	0
B. burgdorferi infection	10	2,312 (1,567–2,768)	563	0
HIV infection	10	2,009 (299–7,517)	2,127	1
A. phagocytophilum infection	10	2,030 (1,046–3,427)	825	0
Antinuclear antibody positive	10	1,680 (477–3,723)	1,680	0
Rheumatoid factor positive	6	730 (85–1,377)	730	0
Herpes simplex virus positive	5	1,902 (1,031–2,797)	1,902	0
Cytomegalovirus infection	5	1,613 (7–3,480)	1,492	0
Epstein-Barr virus infection	5	2,002 (1,111–2,727)	631	0
JE virus vaccine recipients	10	1,633 (638–3,316)	984	0
YF virus vaccine recipients	19	2,563 (966–5,056)	1,179	1
Normal	20	1,811 (970–3,878)	853	0
Total	120			2

[a] The cutoff for positivity for NS5 is 4,366.

aimed to exploit the unique features of viral NS proteins, which are mainly involved in viral RNA replication, for serologic diagnosis of WNV infection. Enzymatically active NTPase/helicase domain of NS3 and full-length NS5 were expressed and purified (Fig. 1). To our surprise, NS5, but not the NS3 NTPase/helicase domain, reacted consistently with WNV patient sera (Fig. 2A and B). Compared with the recombinant-E-protein-based assays, the immunoassay targeting NS5 has three major diagnostic advantages.

First, unlike the E-protein-based MIA, the NS5 MIA reliably discriminates between WNV infection and DEN (Table 2) or SLE infections (Table 3): only 3 of the 34 DEN sera and 2 of the 40 SLE sera showed weak NS5 MFI signals. On the other hand, WNV E protein cross-reacts with both DEN (26 of 34) and SLE (11 of 40) patient sera. These results are in agreement with a previous report that NS antigens were virus-type specific, whereas structural antigens were cross-reactive among flaviviruses (23). The low reactivity of WNV NS5 with anti-DEN virus and anti-SLE virus sera could be due to virus-type specificity of the NS5 antigen among flaviviruses. Although the amino acid sequence homology of NS5 between WNV and DEN virus (75%) or SLE virus (82%) is higher than that of E protein between WNV and DEN virus (62%) or SLE virus (78%), epitopes (either structure or sequence) presented by E protein could be more conserved than those in the NS5, resulting in cross-reactivity in the E-protein-based assay. Alternatively, the specificity of the WNV NS5-based assay may be due to a failure of an anti-NS5 response during DEN and SLE infections. This is unlikely, because partially purified NS proteins of DEN virus, SLE virus, and WNV were demonstrated to be reactive only with homologous sera, not with heterologous sera, indicating the production of antibodies against the NS proteins during infections (23). The anti-NS5 response has been demonstrated in sera from DEN-infected patients by Western blotting (10). Nevertheless, the specificity of the NS5-based assay may eliminate the need for PRNT, and therefore the requirement of level 3 biocontainment, to discriminate among infecting flaviviruses. Quick and accurate differentiation between WNV and DEN/SLE infections will be important in diagnosing specimens where WNV cocirculates with DEN and/or SLE viruses.

Second, the NS5 MIA differentiates between flavivirus vaccination and natural WNV infection. None of the JE-vaccinated sera reacted with the WNV NS5 (Table 1). This feature was expected, because only replicative viruses produce NS proteins, while inactivated JE vaccines could not replicate and produce NS proteins. No or very little NS protein may exist in the inactivated JE virus vaccines, since the vaccines are prepared through an extensive purification procedure (5). Moreover, only 1 of 19 YF vaccine recipients exhibited a positive MFI signal. Since the YF vaccine is a live attenuated virus, this result reiterates the specificity of the NS5-based assay. Distinguishing between vaccination and natural viral infection is important for WNV diagnosis in geographic regions where JE and/or YF vaccinations are performed and in vaccinated military personnel or travelers. For the same reason, the NS5 MIA may be useful for testing whether horses previously vaccinated with inactivated WNV (12, 22) have encountered a new round of WNV infection.

Lastly, the NS5 MIA could potentially be used to indicate the timing of WNV infection. Time course analysis of WNV

TABLE 2. Cross-reactivity of WNV NS5 and E protein with DEN patient sera

Sample[a]	MFI		Titer	
	NS5[b]	E protein[c]	E protein MIA[d]	HI
1A	1,225	280	<100	10
1B	1,368	2,016	200	160
2A	2,325	1,440	100	20
2B	2,614	2,951	400	80
3A	5,677	6,587	25,600	10,240
3B	2,472	4,894	3,200	320
4A	1,348	180	<100	–
4B	5,750	1,554	200	640
5A	674	235	<100	–
5B	715	1,497	200	40
6A	810	113	<100	–
6B	953	1,081	100	160
7A	2,432	289	<100	–
7B	4,935	2,860	100	80
8A	720	875	<100	20
8B	829	558	<100	80
9A	864	3,459	400	160
9B	1,864	4,826	1600	160
10A	1,832	1,366	100	20
10B	1,755	6,686	6,400	10,240
11A	4,658	7,474	51,200	10,240
11B	1,723	5,013	6,400	1,280
12A	841	5,344	3200	640
12B	794	6,105	12,800	2,560
13A	3,833	825	100	80
13B	2,761	1,549	800	80
14A	678	5,578	6400	2,560
14B	757	4,720	1600	80
15A	1,548	4,807	1600	160
15B	1,587	8,626	51,200	10,240
16A	945	6,159	3,200	640
16B	1,128	6,417	6,400	80
17A	1,427	225	<100	–
17B	1,554	3,108	800	800

[a] Seventeen pairs of acute-phase (A) and convalescent-phase (B) sera from DEN-infected individuals were tested.
[b] The cutoff for positivity for NS5 is 4,366. There were 3 positive samples out of 34 (8.8%).
[c] The cutoff for positivity for E protein is 1,084 (Wong et al., submitted). There were 24 positive samples out of 34 (71%).
[d] E protein MIA titers represent the maximal dilutions of patient sera that were reactive in the E-protein-based MIA above the MFI cutoff of 1,084.

TABLE 3. Cross-reactivity of WNV NS5 and E protein with SLE patient sera

Sample[a]	MFI		PRNT titer	
	NS5[b]	E protein[c]	SLE virus	WNV
1A	550	953	640	40
1B	892	1,347	1,280	40
2A	1,081	437	320	<10
2B	606	272	320	<10
3A	7,314	492	320	20
3B	5,894	982	640	40
4A	1,157	522	640	10
4B	2,315	828	1,280	40
5A	643	1,582	640	<10
5B	576	1,185	1,280	<10
6A	924	329	10	<10
6B	2,093	1,020	1,280	10
7A	858	456	20	<10
7B	738	214	320	10
8A	215	59	40	<10
8B	324	323	640	20
9A	834	378	80	<10
9B	631	550	160	10
10A	751	196	10	<10
10B	1,272	284	40	<10
11A	778	688	160	10
11B	691	715	320	20
12A	733	864	640	40
12B	1,148	1,388	640	20
13A	734	966	320	<10
13B	1,731	1,645	320	<10
14A	931	409	160	10
14B	802	415	160	10
15A	1,241	522	40	<10
15B	586	678	320	10
16A	980	3,057	5,120	640
16B	1,420	2,740	2,560	640
17A	1,328	1,490	5,120	1,280
17B	1,912	2,845	1,280	2,560
18A	175	1,679	40	<10
18B	188	1,476	80	<10
19A	398	489	40	<10
19B	628	687	160	<10
20A	1,281	591	640	10
20B	2,296	637	1,280	<10

[a] Twenty pairs of acute-phase (A) and convalescent-phase (B) sera from SLE-infected individuals were tested.
[b] The cutoff for positivity for NS5 is 4,366. There were 2 positive samples out of 40 (5%).
[c] The cutoff for positivity for E protein is 1,084 (Wong et al., submitted). There were 11 positive samples out of 40 (28%).

patient sera showed that, after serum conversion at approximately day 6 after symptom onset, the anti-E-protein antibody signal remained highly positive up to 431 days after symptom onset (Fig. 2C and D), while antibodies against NS5 diminished to a negative level between 71 and 259 days after symptom onset (Fig. 2A and D). More clinical samples from late time points postinfection are required to confirm this conclusion. Since NS5 is present only during viral replication and associates with the replication complex located at the cytoplasmic side of the endoplasmic reticulum, NS5 may be more accessible to protein degradation, resulting in a shorter half-life in cells than the membrane-spanning E protein. It is also possible that antibodies generated in response to NS5 are of shorter duration than the anti-E-protein antibodies.

Overall, the unique features of the NS5-based immunoassay will be very useful for both clinical and veterinary diagnosis of WNV infection. The MIA assay format used in this study is highly sensitive (flow-cytometry based), has a rapid turnaround time (3 to 4 h for testing 96 specimens), and is cost effective (approximately 50 tests per microgram of recombinant protein). More importantly, the MIA format allows the performance of multiplex assays to detect antibodies against E protein and NS5 in a single tube. E protein and NS5 can be covalently linked to microsphere beads containing different fluorochromes. During the assay readout, the first laser excites the intrinsic fluorochrome in the antigen-bearing microspheres, allowing identification of each bead in the assay mixture. The second laser excites the fluorochrome tag of the reporter molecule, measuring the level of antibodies that bind to the specific antigen. The multiplex assay should allow simultaneous primary and confirmatory diagnosis of WNV infections.

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