CLINICAL AND VACCINE IMMUNOLOGY, Sept. 2011, p. 1486–1491 Vol. 18, No. 9
1556-6811/11/$12.00 doi:10.1128/CVI.05129-11

Use of Sindbis/Eastern Equine Encephalitis Chimeric Viruses in Plaque Reduction Neutralization Tests for Arboviral Disease Diagnostics▽

B. W. Johnson,[1]* O. Kosoy,[1] E. Wang,[2] M. Delorey,[1] B. Russell,[1] R. A. Bowen,[3] and S. C. Weaver[2]

Diagnostic and Reference Laboratory, Arboviral Diseases Branch, Division of Vector-Borne Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado[1]; Institute for Human Infections and Immunity, Center for Biodefense and Emerging Infectious Diseases, and Department of Pathology, University of Texas Medical Branch, Galveston, Texas[2]; and Department of Biomedical Sciences, Colorado State University, Fort Collins, Colorado[3]

Received 28 April 2011/Returned for modification 15 June 2011/Accepted 6 July 2011

Eastern equine encephalitis virus (EEEV) is a highly virulent, mosquito-borne alphavirus that causes severe and often fatal neurological disease in humans and horses in eastern North American, the Caribbean, and Mexico and throughout Central and South America. EEEV infection is diagnosed serologically by anti-EEEV-specific IgM detection, with confirmation by the plaque reduction neutralization test (PRNT), which is highly specific for alphaviruses. Live virus is used in the PRNT procedure, which currently requires biosafety level 3 containment facilities and select agent security in the case of EEEV. These requirements restrict the ability of public health laboratories to conduct PRNTs. Sindbis virus (SINV)/EEEV recombinant constructs have been engineered to express the immunogenic structural proteins from 2 wild-type EEEV strains in an attenuated form. These SINV/EEEVs, which are not classified as select agents, were evaluated as alternative diagnostic reagents in a PRNT using human, equine, and murine sera. The results indicate that the chimeric viruses exhibit specificity comparable to that of wild-type EEEV, with only a slight reduction in sensitivity. Considering their benefits in increased safety and reduced regulatory requirements, these chimeric viruses should be highly useful in diagnostic laboratories throughout the Americas.

Eastern equine encephalitis virus (EEEV) is a member of the family *Togaviridae*, genus *Alphavirus* (19). EEEV has been classified into one North American (NA) and three South American (SA) subtypes on the basis of antigenic and genetic analyses (5). Recently, Arrigo et al. (2) proposed that the South American variants be classified as a distinct species called *Madariaga virus* (MADV). In North America, EEEV is transmitted in an enzootic cycle between the ornithophilic mosquito vector *Culiseta melanura* and passerine birds (12, 18). However, humans and horses can become infected from the bite of an infected mosquito when they infringe on these enzootic foci or when bridge vectors transmit EEEV outside the enzootic swamp habitats. An average of 6 human cases of EEE is reported annually in the United States, primarily along the Atlantic and Gulf coasts (www.cdc.gov/easternequineencephalitis/Epi.html#map). Approximately 30 to 80% of apparent human EEE cases are fatal, and up to 30% of survivors have long-term neurological sequelae that can result in high costs for lifelong care (16). The burden of veterinary disease is much higher, with equids, swine, and domestic birds suffering fatal disease at higher rates (18).

Vaccines to prevent EEE are available for horses, but none has been licensed for human use, and there are no effective antiviral drug treatments. Personal protection from mosquito bites is the only effective prevention strategy during times of active transmission. Although large EEE outbreaks have been reported, especially during the mid-20th century, equine and human infections are generally sporadic. Laboratory-based surveillance is essential for detecting these cases and for implementing prevention and control strategies.

EEEV infection is diagnosed genetically by detection of viral RNA from acute-phase serum or cerebrospinal fluid or by virus isolation (10). Serological diagnosis relies on detection of anti-EEEV-specific IgM in the enzyme-linked immunosorbent assay (ELISA), with confirmation by the plaque reduction neutralization test (PRNT), the most specific serological test (4). In the PRNT procedure, infectious virus is mixed with serial dilutions of a serum sample, and if virus-specific neutralizing antibodies are present in the specimen, they bind to the virus. The mixture is then inoculated onto a monolayer of cells. Viruses bound to antibodies in these complexes are then prevented (neutralized) from infecting the cells.

Because live virus is required, confirmatory diagnostic testing by PRNT using wild-type (wt) EEEV and the closely related Venezuelan equine encephalitis virus (VEEV) poses challenges in public health laboratories. Wild-type EEEV and VEEV require biosafety level 3 (BSL-3) containment facilities and are classified as HHS and overlap (HHS and USDA) select agents, respectively (www.selectagents.gov/Select%20Agents%20and%20Toxins%20List.html). This prevents non-select-agent-registered diagnostic laboratories and those with only BSL-2 facilities from confirming EEEV and VEEV infection by PRNT. The attenuated VEEV strain TC-83 can be used to identify VEEV IAB, IC, and, to some extent, ID subtype

* Corresponding author. Mailing address: Diagnostic and Reference Laboratory, Centers for Disease Control and Prevention, 3150 Rampart Road, Fort Collins, CO 80521. Phone: (970) 266-3543. Fax: (970) 494-6631. E-mail: bfj9@cdc.gov.

▽ Published ahead of print on 13 July 2011.

infections, but it is not neutralized efficiently by antibodies elicited in infections by other VEEV subtypes. This has limited diagnostic testing for this group of medically important pathogens. Clearly, surrogate viruses with equivalent antigenic makeup that confer equivalent sensitivity and specificity to the wt viruses in these assays are needed. Pseudotypes have been described for this purpose (9) but are technically challenging to produce and thus unsuitable for many diagnostic labs, particularly in developing countries.

The Centers for Disease Control and Prevention, Division of Vector-Borne Diseases (CDC/DVBD), arbovirus diagnostic and reference laboratory has been validating assays and reagents that can enhance the arbovirus diagnostic testing capacity in public health laboratories with limited facilities. Previously, chimeric flaviviruses constructed from the attenuated yellow fever vaccine virus backbone, with the structural protein genes replaced with heterologous flaviviruses (ChimeriVax; Sanofi-Pasteur, Lyon, France [formerly Acambis Inc., Cambridge, MA]), were shown to be neutralized similarly to wt flaviviruses in diagnostic PRNTs (8, 15). These chimeric flaviviruses can be used under BSL-2 laboratory conditions without select agent registration, which is an advantage over the wt counterparts such as West Nile and St. Louis encephalitis viruses, which require BSL-3 facilities, and Japanese encephalitis (JE) virus, which additionally is under USDA select agent restrictions. Thus, the chimeric viruses have become important tools for laboratory diagnosis by PRNT.

Recombinant Sindbis virus (SINV)/VEEV and SINV/EEEV constructs have been engineered to express the immunogenic structural proteins from VEEV or EEEV in the relatively benign backbone of SINV (14, 17). These recombinant viruses are highly attenuated in mice and hamsters, are not regulated as select agents, and can be used under BSL-2 conditions according to current guidelines. Previously, SINV/VEEVs were shown to have comparable performance to wt VEEV strains in the PRNT (13). We report here on the evaluation of SINV/EEEV for use in diagnostic PRNT. We evaluated serostatus and measured neutralizing antibody titers using the SINV/EEEVs derived from wt North American and South American EEEV strains in the PRNT using sera from experimentally infected animals, humans who received an investigational new drug EEE vaccine administered by the U.S. Army Special Immunizations Program, and archived diagnostic specimens.

MATERIALS AND METHODS

Serum specimens. Sera were obtained from horses and mice experimentally infected with wt EEEV or SINV/EEEV at the University of Texas Medical Branch (UTMB) or Colorado State University (CSU). Human serum samples without personal identifiers from suspected EEEV or other arboviral infection cases and from vaccinees enrolled in the U.S. Army Special Immunizations Program who received an inactivated whole-virus preparation (3, 11) were obtained from the CDC/DVBD archived reference collection.

Reference antibody controls. Virus-specific mouse hyperimmune ascitic fluid (MHIAF) produced in mice immunized with NA prototype EEEV strain NJ60, SA EEEV strain BeAn5122 (Brazil56), and SINVs were obtained from the CDC/DVBD arboviral reference collection. Antiserum from a horse experimentally infected with EEEV strain NJ60 at CSU was also included in the antibody-positive controls.

Viruses. EEEV strains FL93-939, isolated from a mosquito in Florida in 1993, and BeAr436087, a 1985 Brazilian mosquito isolate, are the parental strains of chimeric SINV/NA EEEV and SINV/SA EEEV, respectively (17). The parental SA and NA EEEV strains and the SINV/EEEVs were obtained from the WHO Collaborating World Reference Center for Emerging Viruses and Arboviruses at UTMB. NA EEEV strain NJ60, isolated from a *Culiseta melanura* mosquito in New Jersey in 1960, is the prototype NA EEEV strain at CDC/DVBD. NJ60 was therefore used as the reference challenge virus to evaluate the performance of the SINV/NA EEEV in the PRNT. NA prototype EEEV strain NJ60, SA EEEV strain BeAn5122 (Brazil56), and the SINVs (strains 140, Ar1055, 16260, EgAr339, Reed Warbler, and Michalovce; see Table 2) were obtained from the CDC/DVBD arboviral reference collection. Virus seeds were prepared in Vero cells in 25-cm^2 flasks at a multiplicity of infection (MOI) of 0.001 PFU/cell with Dulbecco's modified minimal essential medium (DMEM), 2% fetal bovine serum (FBS), gentamicin sulfate (50 mg/liter), and amphotericin B (1 mg/liter). The supernatants were harvested on the second day after infection; cellular debris was removed by centrifugation at 3,500 × *g* for 30 min. Bovine serum albumin was added to the clarified supernatant to a final concentration of 20%. Aliquots of the resultant bulk virus seeds were prepared and stored at −70°C until use. SINV/EEEVs were passaged once at the CDC/DVBD by the methods described above, with the exception that the virus was prepared in 150-cm^2 flasks and the supernatant was harvested on day 3.

PRNT. Neutralization assays were performed at UTMB or CDC/DVBD according to the respective laboratory standard protocols. The conditions and protocols of the tests conducted at CDC/DVBD simulated routine diagnostic testing conditions and protocols as much as possible (see Table 2). PRNT was completed by one Clinical Laboratory Improvement Amendments (CLIA)-certified microbiologist (O.K.), who routinely performs neutralization assays in the CDC/DVBD arboviral diseases diagnostic laboratory. Each sample was tested simultaneously with the prototype EEEVs and chimeric SINV/EEEVs. Samples were first heat inactivated at 56°C for 30 min to destroy the complement and to inactivate adventitious viruses. Serum samples initially diluted 1:5 were serially diluted 2-fold and then mixed with a constant concentration of virus. Normal control serum was added to the mixture at a 4% final concentration to provide a source of labile serum factor. The lower limit of quantification (LLOQ), therefore, was at a 1:10 dilution, except in testing with SINV/SA EEEV. Due to limited sample volumes, samples tested against SINV/SA EEEV had LLOQs ranging from 10 to 80. Following incubation at 37°C for 1 h, 100 μl of the mixture was inoculated onto a monolayer of Vero cells in 6-well plates using a 0.5% agarose double overlay, and on the next day the cells were visualized with neutral red staining of the second overlay (4). Each test run was validated with a standardized virus-specific MHIAF-positive control compared against the virus back-titration and negative (normal) control serum. Neutralizing antibody titers were calculated as the reciprocal of the highest serum dilution that reduced the challenge virus plaque count by 90%, on the basis of the back titration (6). Specimens were tested once; replicate testing was not done unless noted. The PRNT procedure used at UTMB to test the experimentally infected mouse and equine serum samples was similar to that at CDC, except the titer was calculated at 80% neutralization (Table 1).

Statistical analyses. Of primary importance was the serostatus of the sample under testing using the prototype and chimeric EEEVs. Because the serostatus outcome is binary, agreement between the results for the prototype wt and chimeric virus was assessed using the kappa statistic for intraclass correlation (7; see also, for example, p. 217 of reference 7). A secondary question was, were the titers of neutralizing antibody of a sample against either the prototype wt or chimeric EEEV challenge virus similar? For these comparisons, titers were modeled as a function of challenge virus strain and serum sample type (natural infection or vaccination) using a generalized linear mixed-effects model (glmm). In the EEEV PRNT comparison, the titers were modeled using a linear model. A linear model not only estimates the strength of the relationship between the titers of antibodies to the chimeric and wt viruses but also, unlike a simple correlation, can identify a scaling factor between the titers.

RESULTS

We assessed the ability of sera from experimentally and naturally infected horses, mice, and humans to neutralize SINV/NA EEEV compared to wt EEEV in the PRNT. Analyses of equine sera collected after experimental infection with NA EEEV strain FL93-939 revealed that all had neutralizing antibodies to both wt and chimeric challenge EEEVs 5 to 7 days after inoculation (Table 1). Endpoint titers were 4- to 16-fold higher using wt EEEV in 3 of the samples and equal in the 4th one. Murine sera collected ca. 4 to 5 weeks after

TABLE 1. Comparison of neutralizing antibody titers in sera from experimentally infected horses and mice using wt and chimeric challenge EEEVs in $PRNT_{80}$

Sample type (virus used in experimental infection)/sample no.	Neutralizing antibody titer[a] with the following challenge virus:			
	wt NA EEEV FL93-939	wt SA EEEV Br85	SINV/NA EEEV	SINV/SA EEEV
Horse serum (wt NA EEEV FL93-939)				
1 (D0)[b]	<20	NT[c]	<20	NT
1 (D5)	160	NT	40	NT
2 (D0)	<20	NT	<20	NT
2 (D5)	320	NT	20	NT
3 (D0)	<20	NT	<20	NT
3 (D7)	640	NT	80	NT
4 (D0)	<20	NT	<20	NT
4 (D7)	20	NT	20	NT
Mouse serum (SINV/NA EEEV)				
1	160	NT	160	NT
2	20	NT	20	NT
3	40	NT	80	NT
4	160	NT	160	NT
5	320	NT	320	NT
6	640	NT	80	NT
Mouse serum (SINV/SA EEEV)				
1	NT	320	NT	80
2	NT	320	NT	160
3	NT	40	NT	40
4	NT	320	NT	320
5	NT	160	NT	80
Normal mouse serum	<20	<20	<20	<20

[a] Neutralizing antibody titer is expressed as the reciprocal of the endpoint serum dilution that neutralized the challenge virus plaque count by 80%. The LLOQ was a neutralizing antibody titer of 20; <20, no detectable titer at the LLOQ.

[b] Values in parentheses are the number of days (D) postinoculation when serum was collected.

[c] NT, not tested.

infection with NA or SA SINV/EEEV had equivalent serostatus using either wt or chimeric EEEV strains in the PRNT and similar titers, with ≤8-fold differences being detected between endpoint titers (Table 1). Normal mouse serum showed no neutralization activity against either wt or chimeric viruses.

A sample set of 76 human serum samples and 12 reference antibodies was used in the side-by-side PRNT comparison of wt NA EEEV (NJ60) and NA and SA SINV/EEEV (Table 2). Thirty-two serum specimens (samples 1 to 32) had been submitted to the CDC/DVBD diagnostic laboratory and confirmed to be positive for EEEV infection, including 3 paired specimens (samples 27 to 32) (Table 2); 22 serum specimens had been submitted for monitoring of seroconversion in arbovirus vaccine recipients (samples 33 to 54) (Table 2); and 22 serum specimens were from suspected arbovirus infection cases which were submitted for testing but which did not have evidence of EEEV infection (samples 55 to 76) (Table 2). Neutralizing antibody titers had been determined previously using NA EEEV (NJ60) as the challenge virus (data not shown). Reference MHIAF samples, equine sera, and normal human control sera functioned as PRNT controls (samples 77 to 88) (Table 2). SINV reference MHIAF samples were included in the evaluation to determine if the SINV/EEEV was neutralized by SINV-specific antibodies.

There was 100% serostatus agreement between results generated with the wt NA EEEV (NJ60 strain) and SINV/NA EEEV in the human serum samples (samples 1 to 76) (Table 2). Using only the infection and vaccination data (samples 1 to 54) (Table 2), the kappa statistic for intraclass correlation was 1, with a 95% lower bound of 0.89. In sera from natural EEEV infections, there was no detectable neutralizing antibody to any of the wt or chimeric EEEVs in two samples (samples 31 and 32). In samples 55 to 76, in which there was no evidence of EEEV infection, there was no neutralizing antibody activity to any of the wt or chimeric EEEV strains.

The titers of neutralizing antibodies measured against wt NA EEEV were higher, on average, than those measured against SINV/NA EEEV. Results from the glmm indicate that the log titers of antibodies to wt EEEV were, on average, 1.3 times greater than the log titers of antibodies to the SINV/NA EEEV strain ($P < 0.01$). This result was confirmed when log titer SINV/NA EEEV data were regressed on log titer EEEV NJ60 data in a simple linear model. The slope for this model was 0.83 ($P < 0.01$), which implies that the log titers of antibodies to SINV/NA EEEV are, on average, 83% of the log titers of antibodies to EEEV NJ60 (the inverse of 0.83 is ≈1.2, which is close to the 1.3 obtained in the glmm). Additionally, the log titers among the infected sera were, on average, 3.2 times greater than the log titers among the vaccinated sera ($P < 0.01$).

There were three EEEV infection cases in which paired specimens had been collected (Table 2, samples 27 to 32). Samples 27 and 28 (pair A) were collected during the acute (6 days following onset of symptoms) and convalescent (13 days after onset) phases, respectively, and there was a corresponding >4-fold increase in titers of antibodies against both the wt and chimeric EEEV challenge viruses. There was a similar increase in titers between samples 29 and 30 (pair B), although there was only a 1-day interval between sample collections. Pair C sera (samples 31 and 32) were both obtained in the convalescent phase (9 and 21 days after onset of illness, respectively); the titers remained stable or dropped by 2-fold with challenge wt EEEV and SINV/NA EEEV, respectively.

Hyperimmune ascitic fluid from mice immunized with EEEV and SINV has been standardized for use as a positive control in the PRNT (Table 2). In the two NA EEEV (NJ60) MHIAF preparations (samples 77 and 78), the titers of neutralizing antibodies to SINV/SA EEEV ranged from 80 to 2,560, and those to wt NA EEEV and SINV/NA EEEV ranged from 10,240 to 20,480. MHIAF prepared with an SA EEEV (BeAn5122, Brazil56) inoculum (sample 79) had equivalent titers of 2,560 for antibodies to all the challenge viruses. MHIAF prepared with an SINV inoculum (samples 81 to 87) had titers of neutralizing antibody to challenge SINVs ranging from 160 to 40,960 but showed no neutralizing activity against either the wt NA EEEV or two SINV/EEEVs, confirming that neutralizing antibodies were elicited against the viral structural proteins only and not the SINV nonstructural proteins. There was no evidence of nonspecific activity to the challenge viruses in normal control human serum.

TABLE 2. Comparison of neutralizing antibody titers using wt and chimeric challenge NA and SA EEEVs in $PRNT_{90}$

Sample type/sample no.	Virus strain inoculum	No. days from onset of illness to sample collection	Neutralizing antibody titer[a] with the following challenge virus:			
			wt NA EEEV (NJ60)	SINV/NA EEEV	SINV/SA EEEV	SINV (EgAr339)
Human serum, confirmed EEEV infection (n = 32)						
1			10,240	5,120	<80	
2			10,240	640	<80	
3[b]			5,120	2,560	<80	
4			5,120	1,280	<80	
5			2,560	1,280	80	
6			2,560	1,280	<80	
7			2,560	320	<80	
8			1,280	640	160	
9–10			1,280	640	<80	
11			1,280	320	<80	
12			1,280	160	<20	
13			640	320	20	
14–16			640	320	<20	
17			640	80	<20	
18			320	320	<10	
19			320	160	640	
20			320	160	40	
21			320	160	<20	
22–23			80	40	<10	
24			20	10	<10	
25–26[b]			<10	<10	<10	
Paired samples[c]						
27 (A, S1)		6	640	160	<20	
28 (A, S2)		13	5,120	2,560	320	
29 (B, S1)		7	160	40	<20	
30 (B, S2)		8	1,280	320	<80	
31 (C, S1)		9	2,560	1,280	<20	
32 (C, S2)		21	2,560	320	<80	
Human serum, EEEV vaccinated (n = 22)						
33			640	640	<10	
34–35			640	320	<10	
36–38			320	80	<10	
39–40			160	80	<10	
41–42			160	40	<10	
43–44			80	40	<10	
45			60	20	<10	
46			40	10	<10	
47–48			20	10	<10	
49–54			<10	<10	<10	
Human serum, no evidence of EEEV infection (n = 22), 55–76			<10	<10	<10	
Reference antibody controls (n = 12)						
77	MHIAF (EEEV NJ60)[d]		20,480	10,240	80	
78	MHIAF (EEEV NJ60)		20,480	20,480	2,560	
79	MHIAF (EEEV BeAn5122, Brazil56)		2,560	2,560	2,560	
80	Equine sera (EEEV NJ60)[d]		328,000	82,000	1,280	
81	MHIAF (SINV Ar1055)		<10	<10	<10	320
82	MHIAF (SINV Ar1055)		<10	<10	<10	640
83	MHIAF (SINV 16260)		<10	<10	<10	5,120
84	MHIAF (SINV EgAr339)		<10	<10	<10	160
85	(MHIAF/SINV EgAr339)[d]		<10	<10	<10	40,960
86	MHIAF (SINV Reed Warbler)		<10	<10	<10	2,560
87	MHIAF (SINV Michalovce)		<10	<10	<10	20,480
88	Normal human control serum		<10	<10	<10	<10

[a] Neutralizing antibody titer is expressed as the reciprocal of the endpoint serum or reference antibody dilution that neutralized the challenge virus plaque count by 90%. The LLOQ was a neutralizing antibody titer of 10, with the exception of the samples challenged with SINV/SA EEEV, in which the LLOQ ranged from 10 to 80, depending on the sample volume remaining. <10 to <80, no detectable titer at the LLOQ.

[b] CDC/DVBD EEEV IgM ELISA positive-control sera collected during acute phase of illness.

[c] S1 and S2, samples 1 and 2, respectively.

[d] PRNT positive control.

DISCUSSION

Awareness of the importance of laboratory-based surveillance for arboviruses in the United States has increased since the introduction of West Nile virus in 1999. In response, state public health laboratories have been called on to do more comprehensive testing for arboviruses, and as a result, the numbers of samples collected and tested and the number of different arboviruses routinely tested for have increased substantially. Subsequently, laboratory capacity has been enhanced through training and technical support, and many public health laboratories now have the capacity to do their own confirmatory testing by PRNT.

Because of the high rates of mortality associated with human NA EEEV infection and the sporadic nature of the outbreaks, laboratory-based surveillance is essential for detection of EEE cases, as well as vector control response. However, in the United States, use of all wt EEEV strains is restricted to select agent-registered laboratories with BSL-3 containment facilities, which has become a barrier to increasing diagnostic laboratory capacity, particularly in regard to the PRNT, which requires the use of live virus. Therefore, alternatives to using restricted viruses that require BSL-3 containment facilities have been developed for use in public health diagnostic laboratories, which often do not have these facilities. SINV/VEEV was previously shown to perform as well as wt VEEV for this purpose for diagnosis of suspected cases of VEEV complex alphavirus (13). We evaluated the performance of two SINV/EEEV strains in the diagnostic laboratory setting to determine if they could replace the wt EEEVs in the PRNT. Chimeric NA EEEVs were neutralized similarly to wt NA EEEVs in sera from experimentally infected horses and mice and from suspected human EEE cases, as well as from EEEV vaccinees. There was 100% serostatus agreement between the wt and chimeric EEEVs in all the types of samples, indicating that the anti-EEEV neutralizing antibodies in the test sera reacted effectively to both the wt and chimeric EEEV strains. Antibody recognition and neutralization are specific to the EEEV structural proteins of the chimeric viruses. Our results confirmed that sera contain no antibodies directed against alphavirus (in this case, SINV) nonstructural proteins, and there was no evidence that the SINV nonstructural protein genes present in the chimeras influenced the serological results.

The small differences in PRNT endpoint titers that we detected between wt and chimeric EEEV strains were unexpected because the structural protein compositions of these viruses should be identical. Although this difference could occasionally result in a low-titer seropositive sample testing negative with the chimeric SINV/EEEV, we believe that this occurrence will be rare, because in natural EEEV infections, human and equine titers are usually well above the cutoff of 20 (Tables 1 and 2). One of the criteria for laboratory confirmation of recent arbovirus infection is a 4-fold or greater rise in virus-specific antibody titer in serum collected during the acute and convalescent phases of illness (4), and the paired sera included in the study had the typical rise in titers of antibodies against both the wt and chimeric challenge EEEVs. Although a very small paired sample set was tested, SINV/EEEV would likely be neutralized similarly with other appropriately timed specimens.

The difference in titer could be more important for testing seroconversion in vaccinees, where titers generally remain low because equine and human EEE vaccines are inactivated. However, equine titers are not generally tested in response to vaccination, and the U.S. Army Special Immunizations Program, which administers the human vaccine being tested under an investigational new drug application, is equipped to handle wt EEEV.

The reasons for the slightly reduced seroreactions to SINV/EEEV compared to wt EEEV are unknown. It is possible that protein-RNA interactions in some way determine the structure of alphaviruses and that the small differences in antibody neutralization that we detected are due to slight conformational changes in the chimeric viruses caused by unnatural interactions between the SINV portion of the chimeric RNA genome and the EEEV proteins. Structural studies of the chimeras and wt EEEV strains using cryo-electron microscopy are needed to test this hypothesis. However, despite slightly higher endpoint neutralization titers of antibodies against the wt NA EEEV compared to the SINV/NA EEEV, for diagnostic purposes, the titers of antibodies against both viruses were sufficiently high. It is possible that in certain cases, when serum samples are collected at very early or late time points after infection, neutralizing antibody titers might fall below the LLOQ of the PRNT by using the chimeric EEEVs, resulting in false negatives. However, in our opinion, this small chance is outweighed by the enhanced safety and reduced regulatory requirements for use of the chimeras.

Among EEEV subtypes and lineages, the North American subtype (subtype I) is primarily associated with human illness. This EEEV lineage occurs in eastern North America, the Caribbean, and Mexico. The SA EEEVs, which include lineages II to IV, are restricted to South and Central America (2, 5). Although the SA EEEVs are not often associated with human illness (1), they do cause equine disease, occasionally involving large numbers of horses. There are considerable antigenic and sequence differences between the SA and NA EEEV lineages, and as shown in Table 2, there is little cross-reactivity of neutralizing antibodies to the challenge SA and NA EEEVs. Therefore, to increase sensitivity where SA and NA EEEVs may cocirculate, such as in southern Mexico, it is essential to test sera by PRNT using both SINV/NA EEEV and SINV/SA EEEV.

In summary, the chimeric SINV/EEEV strains appear to produce qualitatively identical results in the critical PRNT required to diagnose EEEV infections in humans and domestic animals. The use of these attenuated, chimeric viruses will facilitate diagnostic testing of EEE in public health laboratories that do not have the high-level biosafety facilities and select agent certification required for working with wt EEEV. Their use will therefore enhance arboviral disease surveillance as well as improve biodefense preparedness.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Institute of Allergy and Infectious Diseases (NIAID) through the Western Regional Center of Excellence for Biodefense and Emerging Infectious Disease Research, National Institutes of Health (NIH) grant U54 AIO57156.

REFERENCES

1. **Aguilar, P. V., et al.** 2007. Endemic eastern equine encephalitis in the Amazon region of Peru. Am. J. Trop. Med. Hyg. **76:**293–298.
2. **Arrigo, N. C., A. P. Adams, and S. C. Weaver.** 2010. Evolutionary patterns of eastern equine encephalitis virus in North versus South America suggest ecological differences and taxonomic revision. J. Virol. **84:**1014–1025.
3. **Bartelloni, P. J., R. W. McKinney, T. P. Duffy, and F. E. Cole, Jr.** 1970. An inactivated eastern equine encephalomyelitis vaccine propagated in chick-embryo cell culture. II. Clinical and serologic responses in man. Am. J. Trop. Med. Hyg. **19:**123–126.
4. **Beaty, B. J., C. H. Calisher, and R. E. Shope.** 1995. Arboviruses, p. 189–212. *In* E. T. Lennete and D. A. Lennete (ed.), Diagnostic procedures for viral, rickettsial and chlamydial infections, 7th ed. American Public Health Association, Washington, DC.
5. **Brault, A. C., et al.** 1999. Genetic and antigenic diversity among eastern equine encephalitis viruses from North, Central, and South America. Am. J. Trop. Med. Hyg. **61:**579–586.
6. **Calisher, C. H., et al.** 1989. Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. J. Gen. Virol. **70:**37–43.
7. **Fleiss, J. L.** 1981. Statistical methods for rates and proportions, 2nd ed. John Wiley & Sons, New York, NY.
8. **Johnson, B. W., et al.** 2009. Evaluation of chimeric Japanese encephalitis and dengue viruses for use in diagnostic plaque reduction neutralization tests. Clin. Vaccine Immunol. **16:**1052–1059.
9. **Kolokoltsov, A. A., E. Wang, T. M. Colpitts, S. C. Weaver, and R. A. Davey.** 2006. Pseudotyped viruses permit rapid detection of neutralizing antibodies in human and equine serum against Venezuelan equine encephalitis virus. Am. J. Trop. Med. Hyg. **75:**702–709.
10. **Lambert, A. J., D. A. Martin, and R. S. Lanciotti.** 2003. Detection of North American eastern and western equine encephalitis viruses by nucleic acid amplification assays. J. Clin. Microbiol. **41:**379–385.
11. **Maire, L. F., III, R. W. McKinney, and F. E. Cole, Jr.** 1970. An inactivated eastern equine encephalomyelitis vaccine propagated in chick-embryo cell culture. I. Production and testing. Am. J. Trop. Med. Hyg. **19:**119–122.
12. **Morris, C. D.** 1988. Eastern equine encephalomyelitis, p. 1–36. *In* T. P. Monath (ed.), The arboviruses: epidemiology and ecology, vol. III. CRC Press, Boca Raton, FL.
13. **Ni, H., et al.** 2007. Recombinant alphaviruses are safe and useful serological diagnostic tools. Am. J. Trop. Med. Hyg. **76:**774–781.
14. **Paessler, S., et al.** 2003. Recombinant Sindbis/Venezuelan equine encephalitis virus is highly attenuated and immunogenic. J. Virol. **77:**9278–9286.
15. **Pugachev, K. V., et al.** 2004. High fidelity of yellow fever virus RNA polymerase. J. Virol. **78:**1032–1038.
16. **Villari, P., A. Spielman, N. Komar, M. McDowell, and R. J. Timperi.** 1995. The economic burden imposed by a residual case of eastern encephalitis. Am. J. Trop. Med. Hyg. **52:**8–13.
17. **Wang, E., et al.** 2007. Chimeric Sindbis/eastern equine encephalitis vaccine candidates are highly attenuated and immunogenic in mice. Vaccine **25:**7573–7581.
18. **Weaver, S. C.** 2001. Eastern equine encephalitis, p. 151–159. *In* M. W. Service (ed.), The encyclopedia of arthropod-transmitted infections. CAB International, Wallingford, United Kingdom.
19. **Weaver, S. C., et al.** 2005. *Togaviridae*, p. 999–1008. *In* C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Ball (ed.), Virus taxonomy. VIIIth report of the ICTV. Elsevier/Academic Press, London, United Kingdom.