

A NOVEL, RAPID ASSAY FOR DETECTION AND DIFFERENTIATION OF SEROTYPE-SPECIFIC ANTIBODIES TO VENEZUELAN EQUINE ENCEPHALITIS COMPLEX ALPHAVIRUSES

ERYU WANG,* SLOBODAN PAESSLER,* PATRICIA V. AGUILAR, DARCI R. SMITH, LARK L. COFFEY,
WENLI KANG, MARTIN PFEFFER, JAMES OLSON, PATRICK J. BLAIR, CAROLINA GUEVARA,
JOSE ESTRADA-FRANCO, AND SCOTT C. WEAVER

Center for Biodefense and Emerging Infectious Diseases, Department of Pathology, and Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas; Bundeswehr Institute of Microbiology, Munich, Germany; Naval Medical Research Center Detachment, Lima, Peru

Abstract. An epitope-blocking enzyme-linked immunosorbent assay was developed for the rapid differentiation of serologic responses to enzootic variety IE and ID versus epizootic variety IAB and IC strains of Venezuelan equine encephalitis (VEE) virus. Two monoclonal antibodies that differentially recognize epizootic versus enzootic VEE virus epitopes were used to measure the serotype-specific blocking abilities of antibodies in sera of naturally infected humans, equines, and bovines, as well as in experimentally infected equines. The assay is simple, species-independent, rapid, and sensitive, and will improve surveillance for VEE emergence. It could also be used to determine the epidemic potential of a VEE virus following an intentional introduction for bioterrorism.

INTRODUCTION

Venezuelan equine encephalitis viruses (VEEVs) include re-emerging epizootic and epidemic strains that belong to the genus *Alphavirus* in the family *Togaviridae*.¹ A group of closely related viruses including Everglades, Mucambo, Tonate, Cabassou, and Rio Negro, which were originally classified as subtypes in the VEE serocomplex,² are now considered distinct species.¹ Venezuelan equine encephalitis virus and related VEE complex viruses are naturally transmitted among vertebrate hosts by mosquitoes, but many are also highly infectious via the aerosol route and have caused many laboratory infections.³ Epizootic strains have caused numerous outbreaks of human and equine disease in the Americas since the 1920s, and recent epidemics indicate that VEEV continues to pose a serious threat to public health.⁴ Recently, attention and research has focused on the potential use of VEEV as a biologic weapon.⁵ Compounding the fears of health care providers, no effective treatment exists for infected equines or humans, although the attenuated vaccine virus strain TC-83, derived from an epizootic variety IAB strain, is used for equines in disease-endemic locations or during epizootics, and for at-risk humans.⁴

The VEE complex viruses have plus sense RNA genomes of approximately 11.4 kb in length. The 5' two-thirds of the genome encode four nonstructural proteins (nsP1–nsP4) that are involved in viral RNA replication.⁶ The three structural proteins (capsid and E2 and E1 envelope glycoproteins) are transcribed from a 26S subgenomic RNA that is identical to the 3' one-third of the genome. The major antigenic determinants that define VEEV subtypes and varieties are located on the E2 envelope glycoprotein, and include neutralization and several different, subtype- and variety-specific epitopes.⁷

The VEE complex alphaviruses are grouped into six antigenic subtypes based primarily on E2 protein epitopes. Subtypes II–VI and varieties ID–IF, many of which are now considered species distinct from VEEV, are enzootic viruses that are generally not associated with major epidemics or equine epizootics, but can cause fatal human disease.⁴ Subtype I,

varieties AB and C VEEV cause severe disease in both humans and equines, and are generally isolated only during epidemics and epizootics.⁸ Infection of humans usually produces a “flu-like” disease. The less common encephalitic form of human VEE is characterized by disorientation, ataxia, mental depression, and convulsions, and can be detected in up to 14% of infected individuals, especially children.⁹ Overall mortality rates during outbreaks rarely exceed 1%, but neurologic sequelae following human VEE are common.¹⁰ Long-term immunosuppression in patients who recover from VEEV infection has also been reported.¹¹

Diagnosis of VEE relies on virus isolation from acute phase serum or from spinal fluid of human or animal origin, or on detection of VEEV-specific IgM in the cerebrospinal fluid in cases of encephalitis.¹² Four-fold or greater increases in VEEV-specific antibodies can also be used to confirm an infection retrospectively. An IgM capture enzyme-linked immunosorbent assay (ELISA), as well as a monoclonal antibody (MAb)-based antigen-capture ELISA have been developed for detecting antibodies to alphavirus^{13–15} and are used for diagnosis of infections with encephalitic alphaviruses domestic to the United States such as EEEV and WEEV (<http://www.cdc.gov/ncidod/dvbid/arb/orbdet.htm>).

Identification of the subtype and variety of antibodies to VEEV in equines, humans, or rodent reservoir hosts can be critical for determining the potential of a naturally circulating or intentionally introduced strain to cause an epidemic via equine amplification. Although IgM assays readily differentiate infections with different alphaviruses, discrimination of subtype- or variety-specific antibodies to VEEV is difficult. The best methods currently available for discriminating enzootic- and epizootic-specific antibodies are plaque reduction neutralization tests (PRNTs). However, due to the similarity of the neutralization domains of closely related variety ID/IE and IAB/C VEEV strains, the degree of cross-neutralization is very high making the final diagnosis using the PRNT difficult. To overcome these problems, we developed an epitope-blocking-ELISA, similar to previously published assays for detection of antibodies to flaviviruses^{16–18} and alphavirus,¹⁹ which is able to distinguish between infections with enzootic, variety ID/E/F and epizootic, variety IAB/C VEEV strains.

* These authors contributed equally to this work.

Report Documentation Page				Form Approved OMB No. 0704-0188	
Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.					
1. REPORT DATE 2005		2. REPORT TYPE N/A		3. DATES COVERED -	
4. TITLE AND SUBTITLE A NOVEL, RAPID ASSAY FOR DETECTION AND DIFFERENTIATION OF SEROTYPE-SPECIFIC ANTIBODIES TO VENEZUELAN EQUINE ENCEPHALITIS COMPLEX ALPHAVIRUSES				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Center for Biodefense and Emerging Infectious Diseases, Department of Pathology, and Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Naval Medical Research Center Silver Spring, MD 20910				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release, distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT SAR	18. NUMBER OF PAGES 6	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			

MATERIALS AND METHODS

Viruses and cell cultures. Four representative VEEV strains were used: variety IAB vaccine virus strain TC-83,²⁰ enzootic variety ID strain 66637 from Venezuela,²¹ epizootic variety IC strain 243937 from Venezuela,²² and enzootic variety IE strain 68U201 from Guatemala.²³ All virus stocks were prepared in baby hamster kidney (BHK) cells or Vero cells obtained from the American Type Culture Collection (Manassas, VA) using Eagle's minimal essential medium supplemented with 5% fetal bovine serum.

Antigen preparation. Virus stocks purified from BHK cells using polyethylene glycol/NaCl precipitation and rate/zonal ultracentrifugation on sucrose density gradients.²⁴ They were stored at -80°C in EDTA-free, 50 mM bicarbonate buffer (pH 9.6) containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and used as antigens.

Monoclonal antibodies. The MAb 1A3A-5, which reacts only with epizootic variety IAB or IC VEEV, and MAb 1A1B-9, which reacts only with enzootic variety ID and IE in immunofluorescence assays, were described previously²⁵ (Table 1). All MAb stocks were diluted in water to a concentration of 1 mg/mL.

Monoclonal antibody-based epitope blocking ELISA. Optimal concentrations of viral antigens were estimated by titration with each MAb; 100 µL each of two-fold serial dilutions (beginning at 1:100) of virus in bicarbonate buffer were added to each well of a Nunc Immuno PolySorp 96-well plate (Nalge Nunc International, Rochester, NY) and incubated overnight at 4°C. The plate was then washed with buffer (phosphate-buffered saline with 0.1% Tween 20 [Sigma, St. Louis, MO]) and blocked with blocking buffer (phosphate-buffered saline, 0.1% Tween 20, 1% bovine serum albumin). Fifty microliters

of two-fold serial dilutions of each MAb (beginning at 1:100) were added and the plate was incubated for one hour at 37°C. After four additional washes, 50 µL of two-fold serial dilutions (beginning at 1:1,000) of horseradish peroxidase-conjugated rabbit anti-mouse IgG (Sigma) in blocking buffer was added to each well and incubated for one hour at 37°C, followed by four washes. Two hundred microliters of 3,3',5,5'-tetramethylbenzidine (Sigma) was added to each well and incubated for 30 minutes at 37°C. The reactions were stopped by adding 0.5 M H₂SO₄ and plates were read at 450 nm in an ELISA plate reader. The highest optical density (OD) values from each titration were determined as the optimal antigen and antibody dilutions.

Epitope-blocking ELISAs were performed as described previously¹⁸ with minor modifications. Briefly, purified virus was diluted in buffer (50 mM sodium carbonate, 50 mM sodium bicarbonate, pH 9.6). Each well of a 96-well plate surface was coated with 100 µL of diluted antigen. Coating buffers were used as controls for calculation of background OD. Coated plates were incubated overnight at 4°C and washed four times with 200 µL of wash buffer. Two hundred microliters of blocking buffer was then added to each well and incubated for one hour at 37°C to saturate non-specific binding sites. After four washes, 50 µL of diluted serum (1:4, 1:12, and 1:36) were added to each well and incubated for one hour at 37°C and then washed four times with wash buffer. The variety-specific murine MAbs were diluted in blocking buffer, added to the antigen, and incubated for one hour at 37°C in a final volume of 50 µL. Plates were washed again four times, then 50 µL of horseradish peroxidase-conjugated rabbit anti-mouse IgG (Sigma) at a 1:5,000 dilution in blocking buffer were added to each well and incubated for one hour at 37°C,

TABLE 1

Serologic reactions of human sera from Mexico and Peru using a blocking enzyme-linked immunosorbent assay and plaque reduction neutralization tests*

Serum number	Country, year, VEE variety	% Inhibition† of MAb 1A3A-5‡ binding (variety IAB/C-specific) at indicated serum dilution			% Inhibition† of MAb 1A1B-9§ binding (variety IE/ID-specific) at indicated serum dilution			80% Plaque reduction neutralization titer	
		1:4	1:12	1:36	1:4	1:12	1:36	Variety IE/ID¶	Variety IAB/C
503	Mexico, 2001	10.3	16.0	-6.6	94.3	81.7	68.6	320	40
525	Mexico, 2001	10.5	5.2	-8.5	82.0	64.6	47.0	320	160
528	Mexico, 2001	12.3	9.6	-10.7	62.1	28.5	18.1	320	160
545	Mexico, 2001	4.3	-1.1	-8.9	74.7	42.7	17.4	160	40
168	Mexico, 2001	8.7	4.5	-9.6	84.3	62.2	48.3	160	80
505	Mexico, 2001	-13.4	-12.7	-27.2	-6.1	-18.7	-11.8	< 20	< 20
470	Mexico, 2001	-9.1	-8.2	-12.2	0.8	8.9	22.0	< 20	< 20
486	Mexico, 2001	-6.2	-8.6	-10.7	18.7	9.8	14.9	< 20	< 20
504	Mexico, 2001	-9.3	14.4	-13.3	6.9	-16.2	-9.4	< 20	< 20
FSL0206	Peru, 2000, ID#	18.8	9.5	-8.0	65.3	51.0	29.0	320	80
IQT8231	Peru, 1998, ID#	7.8	4.1	-13.3	33.5	27.6	29.5	40	20
IQT7660	Peru, 1998, ID#	38.2	-4.3	-9.5	62.9	44.3	52.0	20	20
IQD4155	Peru, 1998, ID#	-1.1	4.5	-4.3	44.3	47.8	47.9	40	< 20
IQD2652	Peru, 2002, ID#	5.5	11.2	1.0	73.5	56.3	51.9	320	40
IQU1617	Peru, 1999, ID#	34.8	14.3	-3.9	85.2	77.2	51.5	40	20
IQT7172	Peru, 1998, ID#	18.8	14.1	4.2	11.1	69.0	55.9	80	20
FSL0241	Peru, 2000, ID#	16.4	1.4	1.7	50.1	16.7	18.9	320	20
UT445	Colombia, 2003, ID#	55.6	15.4	13.1	94.0	78.1	62.4	320	80
FSL0191	Peru, 2000, IID#	-5.8	-2.5	-24.6	18.4	14.0	13.3	< 20	< 20

* VEE = Venezuelan equine encephalitis.

† % inhibition values are means of duplicate or triplicate wells for each sample and positive results are indicated by **bold** numbers.

‡ Monoclonal antibody (MAb) 1A3A-5 binding to variety IAB/C strain TC-83 antigen.

§ MAb 1A1B-9 binding to variety IE strain 68U201 antigen for Mexican samples and to ID-66637 for Peruvian samples.

¶ Peruvian and Colombian samples were tested against the variety ID strain and Mexican samples were tested against the variety IE strain.

Sera from patients from which virus was isolated and identified.

followed by four washes. Two hundred microliters of 3,3',5,5'-tetramethylbenzidine were then added to each well and incubated for 30 minutes at 37°C. Reactions were stopped by adding 100 µL of 0.5 M H₂SO₄ and plates were read at 450 nm in an ELISA plate reader. The percent inhibition of the colorimetric reaction caused by sample antibodies blocking binding of the MAb to the antigen was calculated for each serum at each dilution by using the formula % inhibition = 100 – [(TS – B)/CS – B] × 100, where TS = OD of the test serum, CS = OD of control serum, and B = background OD. The samples were analyzed in duplicate or triplicate using each antigen. Due to the small volumes available, some sera were only tested once with one or two antigens.

Serum samples. To determine the specificity of the epitope-blocking ELISA using variety-specific MAbs, we used well-characterized sera including samples from experimentally infected horses²⁶ and convalescent human serum samples from well characterized cases with viral etiologies determined by virus isolation and characterization. Additionally, human, bovine, and horse sera containing VEEV-specific neutralizing antibodies were obtained in regions of Mexico (Tabasco and Veracruz States) where only the IE variety circulates and where vaccination is prohibited. Informed consent was obtained from adult human participants and from the parents or legal guardians of minors. The University of Texas Medical Branch and the Naval Medical Research Center Detachment institutional review boards reviewed and approved the project.

Variety IAB/C- and IE-specific equine sera were also obtained from experimental horse infections previously performed.^{26,27} Sera were collected between 7 and 15 days postinfection and kept at -80°C until further processing. All sera were heated to 56°C for 30 minutes prior to serologic tests to inactivate complement.

Plaque reduction neutralization tests. The PRNT was performed to detect the ability of serum samples to neutralize VEEV strains as described previously.²⁸

RESULTS

Optimization of the blocking ELISA. The optimal concentrations of antigens and MAbs were determined based on maximizing colorimetric reactions and were as follows: IAB vaccine strain TC83 diluted 1:1,000 with IAB/C-specific MAb 1A3A-5 diluted 1:300; ID strain 66637 diluted 1:200 with MAb 1A1B-9 diluted 1:250; and IE virus strain 68U201 diluted 1:3,200 with MAb 1A1B-9 diluted 1:700. Based on previous ELISA blocking assays and negative control values of sera with known experimental infection histories, inhibition values ≥ 25% for the 1A1B-9 MAb and ≥ 30% for the 1A3A-5 MAb, at a 1:12 serum dilution, were chosen as cutoff values for the detection of variety-specific antibodies.

Serologic tests: human samples. Serum samples were obtained from convalescent Peruvian patients^{29,30} with a known history of VEEV infection (virus isolation) and from persons living in areas of Mexico with known variety IE VEEV activity.³¹

All human samples that tested positive in an 80% PRNT at a dilution ≥ 1:20 were also positive in the blocking ELISA at a dilution ≥ 1:12 (Table 1). Samples with PRNT titers below the limit of detection (< 1:20) did not have detectable blocking activity in the ELISA. One convalescent serum (FSL0191)

tested negative in our blocking assay; however, this sample was from a patient infected with a variety IIID strain in the VEE complex,²⁹ which is considered a different species of alphavirus (a variant of Mucambo virus).¹ This result indicates that our assay is specific for antibodies to VEE subtype I, and that the antigens we used in our assays (varieties IAB/C and ID) are probably not recognized by antibodies induced by variety IIID infection.

Some samples had cross-reactive inhibitory activity at a 1:4 dilution (IQT 7660, IQU 1617, UT445) when tested against the 1A3A-5 MAb (variety IAB/C-specific) in the blocking ELISA. However, at the next higher dilution (1:12), they were positive in the assay only against the 1A1B-9 (variety IE/D-specific) MAb, confirming the variety ID VEEV infection determined previously by virus isolation and genetic characterization.²⁹ All Mexican samples that tested positive for VEEV neutralizing antibodies were, in contrast to the PRNT, easily identified as variety IE/ID specific in the blocking ELISA. One sample (FSL0241) was positive at a 1:4 dilution, but negative at the 1:12 dilution in the variety ID-specific test. Because no cross-reactivity was found in the IAB/C-specific test and a variety ID virus was isolated from this patient, we considered it as weakly positive for variety ID. Overall, our serologic results correlated strongly with the clinical/microbiologic data obtained from patients and with the epidemiologic knowledge of VEEV circulation. The detection of enzootic VEEV-specific human antibodies using the blocking ELISA suggested a slightly lower sensitivity but higher specificity when compared with the PRNT.

Equine samples. Serum samples were obtained from experimentally infected horses and from horses living in regions with known VEEV activity and vaccination policies and programs. All horses experimentally infected with variety IE VEEV had neutralizing antibodies exhibiting a high level of cross-reactivity, making it impossible to identify the variety of VEEV responsible for the infection. When the same samples were tested in the blocking ELISA, all but one (DP4) tested positive at the 1:12 dilution in the variety IE/ID assay while testing negative for variety IAB/C (Table 2).

Less uniform results were obtained with the samples from naturally exposed Mexican horses with no known history of vaccination. Three PRNT-positive horses (VER-15, VER-25, and VER-26) exhibited positive blocking activity in the IAB/C assay and all PRNT-positive horses also showed positive results in the IE/D assay. We could not rule out the possibility that some of the IAB/C-positive horses might have been moved from regions with strain TC-83 virus vaccination programs. All PRNT-negative samples were also negative in both blocking assays, indicating a concordance between the two assays (Table 3).

Serum samples from horses experimentally infected with variety IC VEEV, which had no detectable alphavirus-reactive antibodies prior to inoculation and which survived long enough to develop neutralizing antibodies, all tested positive in the IAB/C blocking assay. The same samples did not show blocking activity in the IE/D assay, indicating a high specificity of the assay and its ability to detect epizootic-specific seroconversion early after infection.

Bovine samples. Unlike equines, cattle are not vaccinated against VEE because they do not develop overt disease de-

TABLE 2

Blocking enzyme-linked immunosorbent assay and plaque reduction neutralization test results from horses infected experimentally with Venezuelan equine encephalitis virus (VEEV)

Serum number	Days after VEEV infection (strain variety)	% Inhibition† of Mab 1A3A-5‡ binding (variety IAB/C-specific) at indicated serum dilution			% Inhibition† of Mab 1A1B-9§ binding (variety IE/ID-specific) at indicated serum dilution			80% Plaque reduction neutralization titer*		
		1:4	1:12	1:36	1:4	1:12	1:36	Variety IC	Variety ID	Variety IE
DP1	14 days (IE)	23.7	7.6	13.3	40.4	27.6	10.0	320	640	640
DP2	14 days (IE)	38.5	17.2	12.2	47.0	37.6	24.9	640	640	640
DP3	14 days (IE)	14.0	7.5	3.1	57.2	51.1	41.7	320	640	640
DP4	14 days (IE)	18.3	13.2	11.3	46.2	23.8	19.0	160	320	640
DP29	14 days (IE)	17.5	16.6	4.0	42.3	32.0	19.5	80	160	640
DP30	14 days (IE)	34.5	13.3	11.2	48.9	36.1	27.9	160	320	640
DP31	14 days (IE)	19.7	5.4	4.4	40.6	34.5	15.6	80	320	640
836	10 days (IC)	81.0	74.1	75.6	14.1	-3.4	-11.0	640	< 20	NT
744	8 days (IC)	86.3	82.3	79.4	-12.5	21.3	3.5	640	< 20	NT
968	15 days (IC)	81.0	78.7	75.9	40.3	24.9	-8.3	80	640	NT
876	15 days (IC)	80.1	73.1	65.8	-7.1	-5.1	-5.1	80	80	NT

* NT = not tested.

† % inhibition values are means of duplicate or triplicate wells for each sample and positive results are indicated by **bold** numbers.

‡ Monoclonal antibody (Mab) 1A3A-5 binding to variety IAB/C strain TC-83 antigen.

§ Mab 1A1B-9 binding to variety IE strain 68U201 antigen.

spite being naturally infected.^{32,33} They therefore represent excellent sentinels for VEEV surveillance. Bovine sera were collected from Veracruz State, Mexico, where variety IE VEEV-positive equine samples were previously obtained. Based on the results with equine samples (three variety IAB/C-positive horses), we wanted to investigate possible VEEV transmission to other animal species living close to horses. Five of 10 bovine sera had detectable VEEV neutralizing antibodies (Table 4). All but one (VB-14) of these five PRNT-positive samples tested positive in the variety IE/D/F blocking assay, while all 10 samples were negative in the IAB/C assay. These results indicated previous enzootic VEEV infections, probably caused by variety IE, and a slightly lower sensitivity of the blocking ELISA compared with the PRNT.

DISCUSSION

We developed an epitope-blocking ELISA that will be useful for distinguishing the humoral immune responses to en-

zootic versus epizootic VEEV infections in a variety of animals and in humans. In all cases in which we had prior information about the source of VEEV infection, we were able to make a specific serologic diagnosis. Some of the field serum samples obtained from horses, which are vaccinated with the IAB-derived TC-83 strain in some regions of Mexico, reacted positively in the epizootic variety IAB/C blocking test. However, the majority of the samples were positive only in the enzootic IE/D-specific test, indicating natural exposure. All seropositive bovines from the same region, which are never vaccinated, showed a positive reaction only in the enzootic IE/D assay. These discordant results suggest that 1) equines in some non-endemic regions of Mexico where vaccination is not permitted are vaccinated illegally with strain TC-83; 2) some of the IAB/C-positive horses were moved from the Pacific coast where vaccination is encouraged, or 3) equines develop higher levels of cross-reactive antibodies after field (possibly multiple) exposure to variety IE strains. Horses experimentally infected with variety IE VEEV from Mexico

TABLE 3

Serologic reactions of horse sera collected in 2000 from Veracruz State, Mexico using a blocking enzyme-linked immunosorbent assay and plaque reduction neutralization tests

Serum number	% Inhibition† of Mab 1A3A-5‡ binding (variety IAB/C-specific) at indicated serum dilution			% Inhibition† of Mab 1A1B-9§ binding (variety IE/ID-specific) at indicated serum dilution			80% Plaque reduction neutralization titer	
	1:4	1:12	1:36	1:4	1:12	1:36	Variety IE/ID	Variety IAB/C
VER-6	43.1	15.3	11.1	86.2	71.8	63.7	640	40
VER-12	46.0	28.4	12.1	92.5	74.9	63.2	320	< 20
VER-20	NT	NT	NT	83.6	87.5	63.2	320	40
VER-05	24.4	26.5	7.7	51.9	35.2	22.2	320	< 20
VER-15	50.0	34.9	10.0	63.6	34.8	36.3	160	40
VER-16	37.2	13.9	-3.7	79.2	59.4	21.8	160	160
VER-25	45.8	31.8	8.9	77.4	45.0	30.9	320	80
VER-26	58.3	37.2	12.9	71.9	65.0	37.6	320	20
VER-21	-4.0	3.9	-13.4	-2.3	-12.4	-11.5	< 20	< 20
VER-22	-7.0	5.7	-0.6	3.8	-18.9	-16.1	< 20	< 20
VER-24	6.2	-8.3	-7.1	19.8	8.7	1.8	< 20	< 20
VER-34	0.2	-3.0	-11.4	-6.1	-3.9	12.6	< 20	< 20
VER-41	-25.1	-25.1	-47.0	-3.4	-5.5	13.1	< 20	< 20
VER-36	13.2	-6.9	0.0	0.0	1.3	0.0	< 20	< 20

† % inhibition values are means of duplicate or triplicate wells for each sample and positive results are indicated by **bold** numbers.

‡ Monoclonal antibody (Mab) 1A3A-5 binding to variety IAB/C strain TC-83 antigen.

§ Mab 1A1B-9 binding to variety IE strain 68U201 antigen.

TABLE 4

Blocking enzyme-linked immunosorbent assay and plaque reduction neutralization test results from Mexican bovine sera collected in 2003

Serum number	State	% Inhibition* of MAb 1A3A-5† binding (variety IAB/C-specific) at indicated serum dilution			% Inhibition* of MAb 1A1B-9‡ binding (variety IE/ID-specific) at indicated serum dilution			80% Plaque reduction neutralization titer	
		1:4	1:12	1:36	1:4	1:12	1:36	Variety IE	Variety IAB/C
VB-1	Veracruz	-7.4	-16.7	-13.3	24.3	26.6	5.2	160	20
VB-3	Veracruz	6.8	-22.1	-27.8	60.6	50.2	14.6	320	160
VB-7	Veracruz	2.5	-26.3	-34.4	51.9	33.2	6.7	160	40
VB-13	Veracruz	36.1	13.8	9.8	59.2	51.1	4.6	320	320
VB-14	Veracruz	-3.4	-1.8	-1.7	19.1	14.5	-10.6	160	40
TB-1	Tabasco	9.4	14.1	12.0	8.0	14.5	15.6	< 20	< 20
TB-2	Tabasco	5.8	11.4	11.3	17.5	23.2	11.0	< 20	< 20
TB-3	Tabasco	-1.9	0.0	0.3	10.0	20.5	6.9	< 20	< 20
TB-5	Tabasco	4.1	6.6	5.4	15.2	23.1	-5.7	< 20	< 20
TB-6	Tabasco	-12.4	-11.1	-13.6	-4.3	3.9	-6.3	< 20	< 20

* % inhibition values are means of duplicate or triplicate wells for each sample and positive results are indicated by **bold** numbers.

† Monoclonal antibody (MAb) 1A3A-5 binding to variety IAB/C strain TC-83 antigen.

‡ MAb 1A1B-9 binding to variety IE strain 68U201 antigen.

tested positive only in the IE/D assay, indicating that primary infection produces more enzootic IE-specific antibodies. Additionally, horses experimentally infected with variety IC VEEV developed antibodies detected only in the IAB/C assay, exhibiting no cross-reactivity in the enzootic IE/D assay.

In addition to being considerably faster than the PRNT, a major advantage of the blocking ELISA is the species-independent nature of the test. Unlike some other ELISA formats, antibodies from any species can be tested using the same reagents. Because VEEV infects a wide variety of animals during outbreaks, this advantage can be exploited to use a number of different animals as sentinels. Bovines offer several advantages as described above, but other domestic animals such as dogs and pigs also become infected and seroconvert,³⁴ and could be used as surrogates for human exposure.

In general, our blocking ELISA exhibited slightly lower sensitivity than the PRNT. This lower sensitivity may reflect that the blocking ELISA relies on only one epitope for antibody binding, and/or that some individuals do not produce antibodies against that specific epitope. When maximum sensitivity is needed, sera should be prescreened using the PRNT prior to testing with the blocking ELISA.

Both the blocking ELISA and PRNT are highly specific for a given alphavirus or species. However, the blocking ELISA is superior in distinguishing infections with different VEEV subtypes and varieties that are known to have dramatically different abilities to amplify and spread via equine viremia and mosquito transmission. Our data indicated that the MAbs described by Roehrig and Bolin,²⁵ which distinguish epizootic from enzootic VEEV varieties, are useful in serologic assays in detecting variety-specific seroconversion in humans and animals. Establishing these assays using recombinant Sindbis/VEE viruses³⁵ and VEEV pseudotypes,³⁶ which are non-infectious or highly attenuated in animal models, and not regulated as select agents, would lower the risk of laboratory infections associated with VEEV use and simplify diagnostic procedures. These assays are under development in our laboratory.

Received October 28, 2004. Accepted for publication December 14, 2004.

Acknowledgments: We thank Richard Bowen (Colorado State Uni-

versity, Fort Collins, CO) for the equine serum samples, and John Roehrig and Ann Powers (Centers for Disease Control and Prevention, Fort Collins, CO) for providing the monoclonal antibodies.

Financial support: Slobodan Paessler was supported by a National Institutes of Health K08 award AI059491. Patricia V. Aguilar and Lark L. Coffey were supported by the James W. McLaughlin Fellowship Fund. Darci R. Smith was supported by the TO1/CCT622892 Fellowship Training Grant in Vector-Borne Infectious Diseases from the Centers for Disease Control and Prevention. Martin Pfeffer was supported by Fraunhofer Gesellschaft grant 0499-V-4302. This work was supported by a grant from the National Institute of Allergy and Infectious Diseases (to Scott C. Weaver) through the Western Regional Center of Excellence for Biodefense and Emerging Infectious Diseases Research, NIH grant number U54 AI057156, and by grants AI39800 and AI48807 and contract N01-AI25489 from the National Institutes of Health.

Authors' addresses: Eryu Wang, Slobodan Paessler, Darci R. Smith, Lark L. Coffey, Wenli Kang, Jose Estrada-Franco, and Scott C. Weaver, Department of Pathology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-0609, Telephone: 409-747-0758, Fax: 409-747-2415, E-mail: sweaver@utmb.edu. Patricia V. Aguilar, Department of Microbiology and Immunology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-1019. Martin Pfeffer, Bundeswehr Institute of Microbiology, Neuherbergstrasse 11, D-80937 Munich, Germany. James Olson, Patrick Blair, and Carolina Guevara, U.S. Naval Medical Research Center Detachment, Unit 3800, American Embassy, Lima, Peru.

Reprint requests: Scott C. Weaver, Department of Pathology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-0609.

REFERENCES

- Weaver SC, Dalgarno L, Frey TK, Huang HV, Kinney RM, Rice CM, Roehrig JT, Shope RE, Strauss EG, 2000. Family Togaviridae. van Regenmortel MHV, Fauquet CM, Bishop DHL, Carstens EB, Estes MK, Lemon SM, Maniloff J, Mayo MA, McGeogh DJ, Pringle CR, Wickner RB, eds. *Virus Taxonomy Classification and Nomenclature of Viruses. Seventh Report of the International Committee on Taxonomy of Viruses*. San Diego, CA: Academic Press, 879-889.
- Calisher CH, Karabatsos N, 1988. Arbovirus serogroups: definition and geographic distribution. Monath TP, ed. *The Arboviruses: Epidemiology and Ecology*. Volume I. Boca Raton, FL: CRC Press, 19-57.
- U.S. Department of Health and Human Services, 1999. *Biosafety in Microbiological and Biomedical Laboratories*. Fourth edition. Washington, DC: U.S. Government Printing Office.
- Weaver SC, Ferro C, Barrera R, Boshell J, Navarro JC, 2004.

- Venezuelan equine encephalitis. *Annu Rev Entomol* 49: 141–174.
5. Hawley RJ, Eitzen EM Jr, 2001. Biological weapons—a primer for microbiologists. *Annu Rev Microbiol* 55: 235–253.
 6. Strauss JH, Strauss EG, 1994. The alphaviruses: gene expression, replication, and evolution. *Microbiol Rev* 58: 491–562.
 7. Roehrig JT, 1993. Immunogens of encephalitis viruses. *Vet Microbiol* 37: 273–284.
 8. Rivas F, Diaz LA, Cardenas VM, Daza E, Bruzon L, Alcala A, De la Hoz O, Caceres FM, Aristizabal G, Martinez JW, Revelo D, De la Hoz F, Boshell J, Camacho T, Calderon L, Olano VA, Villarreal LI, Roselli D, Alvarez G, Ludwig G, Tsai T, 1997. Epidemic Venezuelan equine encephalitis in La Guajira, Colombia, 1995. *J Infect Dis* 175: 828–832.
 9. Johnson KM, Martin DH, 1974. Venezuelan equine encephalitis. *Adv Vet Sci Comp Med* 18: 79–116.
 10. Leon CA, 1975. Sequelae of Venezuelan equine encephalitis in humans: a four year follow-up. *Int J Epidemiol* 4: 131–140.
 11. Tsai TF, Weaver SC, Monath TP, 2002. Alphaviruses. Richman DD, Whitley RJ, Hayden FG, eds. *Clinical Virology*. Washington, DC: American Society for Microbiology Press, 1177–1210.
 12. Deresiewicz RL, Thaler SJ, Hsu L, Zamani AA, 1997. Clinical and neuroradiographic manifestations of eastern equine encephalitis. *N Engl J Med* 336: 1867–1874.
 13. Calisher CH, el-Kafrawi AO, Al-Deen Mahmud MI, Travassos da Rosa AP, Bartz CR, Brummer-Korvenkontio M, Haksosudo S, Suharyono W, 1986. Complex-specific immunoglobulin M antibody patterns in humans infected with alphaviruses. *J Clin Microbiol* 23: 155–159.
 14. Sahu SP, Alstad AD, Pedersen DD, Pearson JE, 1994. Diagnosis of eastern equine encephalomyelitis virus infection in horses by immunoglobulin M and G capture enzyme-linked immunosorbent assay. *J Vet Diagn Invest* 6: 34–38.
 15. Martin DA, Muth DA, Brown T, Johnson AJ, Karabatsos N, Roehrig JT, 2000. Standardization of immunoglobulin M capture enzyme-linked immunosorbent assays for routine diagnosis of arboviral infections. *J Clin Microbiol* 38: 1823–1826.
 16. Blitvich BJ, Bowen RA, Marlenee NL, Hall RA, Bunning ML, Beaty BJ, 2003. Epitope-blocking enzyme-linked immunosorbent assays for detection of West Nile virus antibodies in domestic mammals. *J Clin Microbiol* 41: 2676–2679.
 17. Blitvich BJ, Marlenee NL, Hall RA, Calisher CH, Bowen RA, Roehrig JT, Komar N, Langevin SA, Beaty BJ, 2003. Epitope-blocking enzyme-linked immunosorbent assays for the detection of serum antibodies to West Nile virus in multiple avian species. *J Clin Microbiol* 41: 1041–1047.
 18. Hall RA, Broom AK, Hartnett AC, Howard MJ, Mackenzie JS, 1995. Immunodominant epitopes on the NS1 protein of MVE and KUN viruses serve as targets for a blocking ELISA to detect virus-specific antibodies in sentinel animal serum. *J Virol Methods* 51: 201–210.
 19. Passler S, Pfeffer M, 2003. Detection of antibodies to alphaviruses and discrimination between antibodies to eastern and western equine encephalitis viruses in rabbit sera using a recombinant antigen and virus-specific monoclonal antibodies. *J Vet Med B Infect Dis Vet Public Health* 50: 265–269.
 20. Berge TO, Banks IS, Tigertt WD, 1961. Attenuation of Venezuelan equine encephalomyelitis virus by *in vitro* cultivation in guinea pig heart cells. *Am J Hyg* 73: 209–218.
 21. Walder R, Suarez OM, Calisher CH, 1984. Arbovirus studies in the Guajira region of Venezuela: activities of eastern equine encephalitis and Venezuelan equine encephalitis viruses during an interepizootic period. *Am J Trop Med Hyg* 33: 699–707.
 22. Rico-Hesse R, Weaver SC, de Siger J, Medina G, Salas RA, 1995. Emergence of a new epidemic/epizootic Venezuelan equine encephalitis virus in South America. *Proc Natl Acad Sci USA* 92: 5278–5281.
 23. Powers AM, Brault AC, Kinney RM, Weaver SC, 2000. The use of chimeric Venezuelan equine encephalitis viruses as an approach for the molecular identification of natural virulence determinants. *J Virol* 74: 4258–4263.
 24. Wang E, Brault AC, Powers AM, Kang W, Weaver SC, 2003. Glycosaminoglycan binding properties of natural Venezuelan equine encephalitis virus isolates. *J Virol* 77: 1204–1210.
 25. Roehrig JT, Bolin RA, 1997. Monoclonal antibodies capable of distinguishing epizootic from enzootic varieties of subtype I Venezuelan equine encephalitis viruses in a rapid indirect immunofluorescence assay. *J Clin Microbiol* 35: 1887–1890.
 26. Wang E, Bowen RA, Medina G, Powers AM, Kang W, Chandler LM, Shope RE, Weaver SC, 2001. Virulence and viremia characteristics of 1992 epizootic subtype IC Venezuelan equine encephalitis viruses and closely related enzootic subtype ID strains. *Am J Trop Med Hyg* 65: 64–69.
 27. Gonzalez-Salazar D, Estrada-Franco JG, Carrara AS, Aronson JF, Weaver SC, 2003. Equine amplification and virulence of subtype IE Venezuelan equine encephalitis viruses isolated during the 1993 and 1996 Mexican epizootics. *Emerg Infect Dis* 9: 161–168.
 28. Beaty BJ, Calisher CH, Shope RE, 1989. Arboviruses. Schmidt NJ, Emmons RW, eds. *Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections*. Sixth edition. Washington, DC: American Public Health Association, 797–855.
 29. Aguilar PV, Greene IP, Coffey LL, Medina G, Moncayo AC, Anishchenko M, Ludwig GV, Turell MJ, O'Guinn ML, Lee J, Tesh RB, Watts DM, Russell KL, Hice C, Yanoviak S, Morrison AC, Klein TA, Dohm DJ, Guzman H, Travassos da Rosa AP, Guevara C, Kochel T, Olson J, Cabezas C, Weaver SC, 2004. Endemic Venezuelan equine encephalitis in northern Peru. *Emerg Infect Dis* 10: 880–888.
 30. Watts DM, Callahan J, Rossi C, Oberste MS, Roehrig JT, Wooster MT, Smith JF, Cropp CB, Gentrau EM, Karabatsos N, Gubler D, Hayes CG, 1998. Venezuelan equine encephalitis febrile cases among humans in the Peruvian Amazon River region. *Am J Trop Med Hyg* 58: 35–40.
 31. Estrada-Franco JG, Navarro-Lopez R, Freier JE, Cordova D, Clements T, Moncayo AC, Kang W, Gomez-Hernandez C, Rodriguez-Dominguez G, Ludwig GV, Weaver SC, 2004. Enzootic/endemic Venezuelan equine encephalitis virus, southern Mexico. *Emerg Infect Dis* 10: 2113–2121.
 32. Dickerman RW, Baker GJ, Ordóñez JV, Scherer WF, 1973. Venezuelan equine encephalomyelitis viremia and antibody responses of pigs and cattle. *Am J Vet Res* 34: 357–361.
 33. Walton TE, Johnson KM, 1972. Experimental Venezuelan equine encephalomyelitis virus infection of the bovine. *Infect Immun* 5: 155–159.
 34. Scherer WF, Dickerman RW, Campillo-Sainz C, Zarate ML, Gonzales E, 1971. Ecologic studies of Venezuelan encephalitis virus in southeastern Mexico. V. Infection of domestic animals other than equines. *Am J Trop Med Hyg* 20: 989–993.
 35. Paessler S, Fayzulin RZ, Anishchenko M, Greene IP, Weaver SC, Frolov I, 2003. Recombinant Sindbis/Venezuelan equine encephalitis virus is highly attenuated and immunogenic. *J Virol* 77: 9278–9286.
 36. Kolokoltsov AA, Weaver SC, Davey RA, 2005. Efficient functional pseudotyping of oncoretroviral and lentiviral vectors by Venezuelan equine encephalitis virus envelope proteins. *J Virol* 79: 756–763.