

PSEUDOTYPED VIRUSES PERMIT RAPID DETECTION OF NEUTRALIZING ANTIBODIES IN HUMAN AND EQUINE SERUM AGAINST VENEZUELAN EQUINE ENCEPHALITIS VIRUS

ANDREY A. KOLOKOLTSOV,† ERYU WANG,† TONYA M. COLPITTS, SCOTT C. WEAVER, AND ROBERT A. DAVEY*
Departments of Microbiology and Immunology, Pathology and Center for Emerging Infectious Diseases, University of Texas Medical Branch, Galveston, Texas

Abstract. Virus envelope proteins are the primary targets of neutralizing antibody responses. The epitopes recognized differ sufficiently between virus subtypes and species to distinguish viruses and provide an important basis for disease diagnosis. Venezuelan equine encephalitis virus (VEEV) causes acute febrile illness in humans and has high mortality in equines. The most specific detection methods for serum antibodies use live virus in neutralization assays or in blocking enzyme linked immunosorbent assays. However, work with Venezuelan equine encephalitis virus requires biosafety level 3 containment and select agent security in the United States. We report two new assays for detection of Venezuelan equine encephalitis virus neutralizing antibody responses, based on virus pseudotypes. The first provides detection by marker gene expression after 20 hours and is particularly suited for high-throughput screening; the second uses a new, rapid virus entry assay to give readouts within 1 hour. Both assays are safe, sensitive, and in general recapitulate neutralizing antibody titers obtained by conventional plaque reduction assays. Each is suitable as a rapid primary screen for detection of neutralizing antibodies against Venezuelan equine encephalitis virus.

INTRODUCTION

Venezuelan equine encephalitis virus (VEEV), a member of the family *Togaviridae*, genus *Alphavirus*, is found in tropical and subtropical regions of South, Central, and North America where it cycles between mosquito vectors and rodent populations. The horse and other equines act as efficient amplifying hosts of some strains, from which humans can be infected via mosquitoes. In equines, mortality ranges from 38–80%, placing a heavy burden on the agricultural industry, especially in South America where heavy dependence on equines as beasts of burden is prevalent.¹ In humans, infection produces an acute febrile illness lasting up to 2 weeks, with nonspecific flu-like symptoms that are usually self-limited. Children are at greater risk, with 5–15% developing neurologic disease often resulting in neurologic sequelae, sometimes lasting for life.¹ Severe leucopenia can also result, making individuals susceptible to secondary infections.² Overall mortality in human cases averages about 0.5% and no effective drug treatment or approved vaccine is available for people.³

To design better vaccines against VEEV and to diagnose natural infection to control outbreaks, it is important to rapidly detect and evaluate sero-conversion, especially neutralizing antibodies. The most direct method for specific identification of neutralizing antibodies is by plaque reduction neutralization tests (PRNT) using live virus. However, because VEEV is highly infectious by aerosol and is regulated as a select agent, few laboratories are equipped to perform such assays with wild-type viruses. Instead, a vaccine strain of a 1AB serogroup VEEV, TC-83, is commonly used but differences exist in exposed epitopes as compared with the Trinidad donkey parent and other field isolates of 1AB strains.⁴ Neutralizing antibodies against VEEV complex subtypes that have low cross-reactivity with TC-83 such as Everglades, 1D, and 1E may also go undetected.

Of the 6 VEEV subtypes, subtype IA/B and IC viruses are typically associated with disease outbreaks. Both are antigenically related and can only be distinguished using monoclonal antibodies or by genome sequencing. While some neutralizing monoclonal antibodies target the E1 envelope glycoprotein, most bind directly to the E2 envelope glycoprotein and inhibit binding of virus particles to cells, as well as inhibit hemagglutination activity. Four major epitopes have been previously identified that overlap on E2.⁵

Recently, we produced retroviral pseudotypes of VEEV, based on murine leukemia viruses, which bear the envelope proteins (envs) of VEEV on their surfaces.⁶ Unlike live virus, these particles cannot replicate but can enter cells, after which a marker gene becomes expressed. Our initial characterization, using VEEV-specific monoclonal antibodies, indicated that the appropriate native antigenic epitopes are present on the pseudotype particle surface.⁶ Given that Murine leukemia virus (MLV) vectors have a long track record in the clinical setting and pose little risk for human infection, the VEEV env-pseudotyped vectors may make suitable substitutes for live VEE virus in a PRNT. This would improve laboratory safety and security, as well as provide a stable and readily available reagent for performing such assays. MLV also have strong reporter gene expression that may generate sufficient signal for high-density assays on 96-well or 384-well plates. To investigate the applicability of pseudotypes for VEEV neutralizing antibody detection, PRNT assays were performed using live VEEV and serum titers were compared with those obtained using pseudotypes. Two types of assays were used with the pseudotypes. The first was a reporter gene expression assay in 96-well format where firefly luciferase was the reporter. The second assay was a new virus entry assay system that measures release of virus encapsulated luciferase into cells during virus entry. This real-time entry detection system promises to provide the fastest possible method for neutralization assays, with signals being detected as early as 15 minutes after contact of virus with cells.⁷ Here, we compare the neutralization titers using the traditional PRNT method with fully infectious virus to the pseudotype-based assays. To our knowledge, this is the first time a direct comparison has been

* Address correspondence to Robert Davey, Department of Microbiology and Immunology, University of Texas Medical Branch, 301 University Blvd, Galveston, TX 77555. E-mail: radavey@utmb.edu
† These authors contributed equally to this work.

performed between these two systems using both human and animal serum samples.

MATERIALS AND METHODS

Viruses and cells. All assays were performed with either human 293HEK fibroblast cells or baby hamster kidney (BHK) cells. Cells were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 8% fetal bovine serum (FBS, Gemini Bioproducts, Woodland, CA). 293FT cells (Invitrogen, Carlsbad, CA) were used to generate env pseudotyped MLV and were grown in DMEM with FBS and 0.5 mg/ml of Geneticin (Invitrogen).

Viruses used were representative strains from the 1AB subtype, Trinidad Donkey (TRD),⁸ and the epizootic 1C field isolate, strain 3908.⁹ Each was cultivated on BHK cells and titered by plaque assays before PRNT.

Serum samples. Sera were obtained from convalescent patients with documented VEEV etiologies, as determined by viral isolation and characterization. The specificity of each was assumed from known distributions of antigenic subtypes of VEEV and previous results using an epitope blocking ELISA.¹⁰ Sera were also obtained from horses experimentally infected with VEEV strains ID 66637 or ZPC738, IE 68U201, and IC strains 243937 or SH3. Human samples were acquired after informed consent was obtained from donors with approval from UTMB and Naval Medical Research Detachment institutional review boards. All sera were complement-inactivated prior to use by heating to 56°C for 30 minutes; half was used for PRNT with live virus and the remainder with pseudotypes. Rabbit antisera specific to Pixuna and Mucambo viruses in the VEE complex, as well as Eastern equine encephalitis virus (EEEV) were provided by Robert Tesh from the World Reference Center for Emerging Viruses and Arboviruses, UTMB.

Production of Venezuelan equine encephalitis virus pseudotypes. Plasmids are described in detail in our previous work.⁶ Pseudotyped MLV were produced by transfection of 293FT cells with plasmids encoding the E3-E1 envs of VEEV (pVEEV-env), the MLV gag-pol genes (pGAG-POL), and a marker plasmid encoding firefly luciferase under control of the MLV LTR promoter and packaging (Ψ) sequence (p Ψ -luciferase). Similarly, a pseudotyped virus bearing the env of VSV was produced by substituting the VEEV env expression plasmid with that for VSV-G (pVSV-G, Clontech, Mountain View, CA). Transfection was by the calcium phosphate method¹¹ and supernatants containing virus were collected 2 days post-transfection. After removing cell debris with a 0.45- μ m filter, the supernatant was either frozen or used immediately to infect target cells, typically 293 cells. The method is depicted in Figure 1. Target cell infection was measured 20 hours after infection using a Turner Veritas (Turner Biosystems, Inc, Sunnyvale, CA) plate reader. Cells had been lysed by freeze-thaw and addition of Steady-glo luciferase assay buffer (Promega, Madison, WI). Stability of virus was tested by comparing luciferase activities of virus that had been kept at -80°C for 3 years to that previously recorded.

Rapid entry assay. Plasmids are described in detail in previous work.¹² In principle, the rapid entry assay measures release of encapsulated luciferase from pseudotyped virus particles upon fusion of virus and cell membranes. This gives a rapid (< 1 h) and quantitative readout of virus entry into

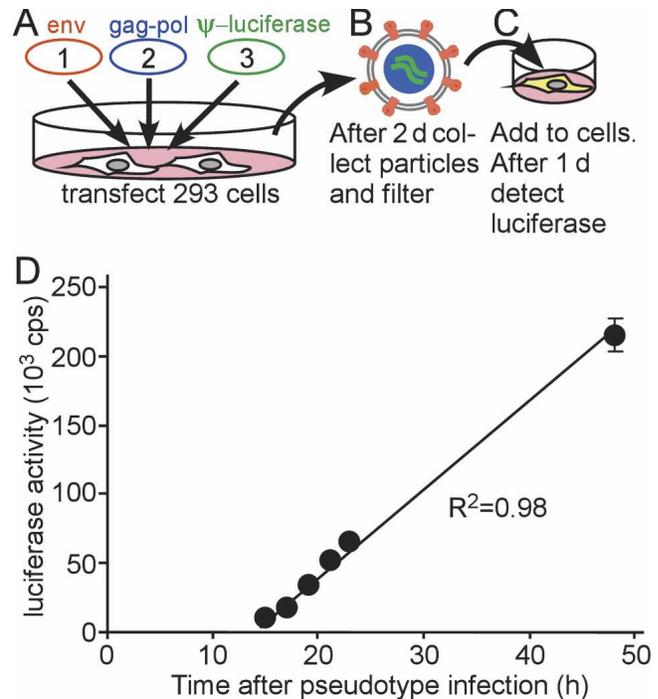


FIGURE 1. Generation of pseudotyped virus and determination of luciferase reporter gene expression kinetics. (Top) 293 cells were used to generate the pseudotyped viruses as they are readily and reproducibly transfected using standard calcium phosphate-based methods. A, Three plasmids encoding 1) the VSV or VEEV envelope protein (env), 2) MLV core/polymerase (gag-pol), and 3) a luciferase reporter gene under control of a viral promoter and packaging signal (Ψ -luciferase) are transiently transfected into cells. B, After 2 days, the culture supernatant containing the virus is filtered. C, The collected virus can be used directly in assays or frozen for later use. D, Cells in 96-well plates were incubated with MLV particles pseudotyped with VEEV TRD envs. Luciferase reporter activity was then assayed at the indicated times to produce a time course of luciferase expression. Values are the average of duplicates \pm SD. (Most error bars are not visible due to high reproducibility of the signal.) Curves were fitted to the data by non-linear regression analysis and the best fit was a straight line ($R^2 = 0.98$). This figure appears in color at www.ajtmh.org.

cells. Measurement is made in real-time and entry can be detected as early as 15 minutes after virus contact with cells. Virus containing luciferase was made by transfecting cells as described for pseudotype production, with the addition of a plasmid encoding a VEEV env-luciferase fusion protein (pVEEV env-luc). This plasmid was identical to the pVEEV env plasmid except that firefly luciferase was fused to the c-terminus of the E1 protein via an influenza hemagglutinin (HA) epitope peptide linker of approximately 30 amino acids, as described in previous work.¹² VSV-G pseudotyped particles containing luciferase were made similarly, as in previous work.⁷ Particles were concentrated from filtered culture supernatants by centrifugation at 100,000 \times g for 1 h through 20% sucrose and resuspension in DMEM to 1/50 the original supernatant volume. This step purified intact virus particles used in the assay away from the cell debris and free luciferase that add to the signal noise. We have also found that centrifugation of particles in a standard microcentrifuge at 16,000 \times g for 1 hour will pellet > 50% of the virus particles and provide sufficient material to perform assays.

To perform the rapid entry assay, luciferase-containing par-

ticles were incubated with 5×10^5 cells. One hour after addition of virus, luciferase activity was measured after freeze-thaw lysis of cells (does not affect virus) and addition of Steady-glo luciferase assay buffer (Promega, Madison, WI). Unless indicated otherwise, virus concentration was adjusted to give approximately 2000 counts per second (cps) per assay point. The background count of the luminometer was 10–20 cps.

Neutralization assays. For assays using both live virus and pseudotype-based virus, viruses were incubated at 37°C with serially diluted serum (as indicated) for 30 minutes prior to addition to cells. After 1 hour incubation, the supernatant was replaced with fresh medium (DMEM with fetal bovine serum) and cells were incubated for the indicated time before assay readout.

i) PRNT assay. For assays with live VEEV, PRNT assays were performed as described previously¹³ with titers expressed as the reciprocal of the antiserum dilution required to reduce virus plaque numbers by 80%. After infection with virus, cells were incubated in 6-well plates for 2–3 days with 0.4% agarose overlays and were then stained with 0.05% crystal violet dye in 50% methanol and plaques were counted.

ii) Pseudotype neutralization. For assays with pseudotyped virus, a gene reporter or a rapid entry assay was used. The reporter gene assay used detection of luciferase activity after its expression in pseudotype transduced cells. This reporter gene assay was performed in a 96-well plate with cells incubated for 20 hours after infection with serum-treated virus, before readout. In contrast, for the rapid entry assay, cells (10^5) were incubated with serum-treated virus in tubes for 1 hour, which was sufficient to obtain a reproducible signal. For each assay the drop in luciferase signal, compared with untreated or cells treated with control serum, was used to determine the neutralizing activity of each serum. The concentration of serum that decreased luciferase activity by $\geq 80\%$ was used as the end point with the reciprocal of this serum dilution-factor given as the neutralizing titer. At least 2 independent experiments were performed for each of the assays described and the range of titers observed is given where applicable.

Curve fitting and statistical analysis. Curves were fitted to kinetic data using non-linear regression analysis and kinetic and goodness of fit parameters calculated using Graphpad software (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). For each dataset, exponential, first (straight line) order, and second order polynomial curves were tested. The curve with the highest R^2 value was cited. Graphpad software was also used for statistics analysis. Data were compared by one way ANOVA and included the Tukey-Kramer post test.

RESULTS

Current PRNT methods detect alphavirus neutralizing antibodies effectively, but take 2–3 days to obtain a readout and require the use of live virus. They also consume large volumes of serum and are difficult to perform in a high-throughput format, primarily due to the need to see a distinctive plaque. VEEV is one example of a number of North American encephalitic viruses where such assays are further complicated

because live virus is highly contagious by aerosol and is a select agent, making access to reference stocks difficult.

We recently developed MLV and lentivirus pseudotypes that bear the envs of VEEV subtypes 1AB and 1C on their surface. Preliminary work indicated that the envs are generally indistinguishable in function and monoclonal antibody neutralization of virus was similar to that reported for wild-type virus.⁶ The only detectable difference was that pseudotyped particles have fewer envs on their surface as compared with live virus. However, this should not influence the ability of pseudotypes to function in a neutralization assay, as most likely they would be more sensitive to antibody neutralization. MLV pseudotypes cannot propagate as most of the retroviral genome is replaced with a reporter gene, such as luciferase. They are assembled by supplying individual plasmids encoding matrix/capsid genes plus polymerase, envs, and a reporter gene with packaging signals on separate plasmids. To obtain an infectious virus, multiple independent recombination events are needed. Even at this stage MLV are only infectious toward mice; no evidence of infection of healthy humans has been reported. Indeed, current NIH guidelines recommend that these vectors can routinely be used at biosafety level 2 or level 1 when replication competent virus is not detectable.¹⁴ Here, we tested if the pseudotypes could be used as an alternative to wild-type virus in neutralization assays using human and horse serum.

Retrovirus pseudotypes were made bearing the envs of VEEV or VSV (see Fig. 1). The VSV-G pseudotyped virus served as a negative control in our assays to detect nonspecific effects of serum on MLV infection. MLV pseudotypes can enter cells, integrate a reporter gene into the host chromosome, and then permanently drive expression of the reporter gene through action of cellular transcription factors on the viral LTR promoter. For the VEEV pseudotype, expression of luciferase (see Fig. 1, lower) was detectable as early as 15 hours and increased at later time points. Curve fitting indicated that the increase in signal was linear over time ($R^2 = 0.98$), with a theoretical starting point of 14 hours and increasing by 5500 ± 200 cps/h of incubation of cells. The VSV vector had similar kinetics (not shown). Out of convenience subsequent assays were performed at 20 hours post inoculation of pseudotyped virus but could be done sooner as signal for each virus was sufficient ($> 10^4$ cps) by 17 hours. The stability of each pseudotype was tested by comparing the luciferase activity of each after a single freeze-thaw cycle for virus stored for 3 years at -80°C to that recorded when fresh. While initial freezing reduced the titer of the VSV and VEEV pseudotypes by 3-fold and 5-fold respectively, activities for the 3-year-old stocks were similar to that of the initial frozen stock (within 2-fold, data not shown). This indicated that beyond the initial impact of freezing, the pseudotypes remained stable for prolonged periods of time.

The pseudotyped viruses were then used to establish that they could be effectively neutralized by immune serum and that neutralization was specific to the VEEV env and not due to nonspecific interaction with the common MLV core. Seven sera were selected from humans and horses. One was from a vaccinated horse and another from a vaccinated laboratory worker and were previously shown to have neutralizing antibodies by PRNT. Four other sera were from unvaccinated laboratory workers and another was from an unvaccinated horse that had not been previously exposed to VEEV. Each

was used to define the neutralization properties of pseudotypes bearing envs from the Trinidad donkey (TRD) strain of VEEV or VSV (Figure 2; the human non-immune serum behaved similarly and so only 2 are shown). Mouse ascites fluid from mice inoculated with VSV (Indiana strain) was also tested on the VSV-G pseudotyped virus only and served as a positive control for this virus. For the VSV env pseudotype, the VEEV immune and non-immune serum behaved similarly. Each human sera had neutralizing activity that peaked at 1:20 (lowest dilution tested), reducing the luciferase signal by $64 \pm 19\%$. At 1:40 this was reduced to $41 \pm 16\%$. The signal from the VEEV env pseudotyped virus was also inhibited by the 1:20 dilution of non-immune human serum with surprisingly little variation between serum samples, reducing the luciferase signal by $79 \pm 2\%$. At 1:40 this was reduced to 66%

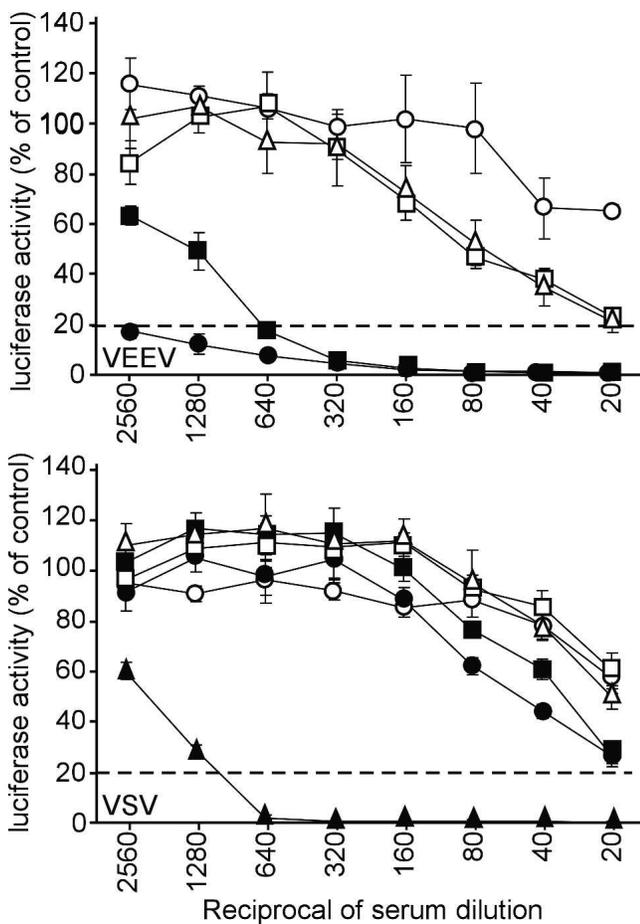


FIGURE 2. Titration of immune and non-immune serum and determination of neutralizing activity against VEEV (upper) and VSV (lower) env pseudotyped viruses. To determine if pseudotyped particles could be used in a neutralization assay, virus was incubated with the indicated dilutions of immune or non-immune serum in a 96-well plate. The mixture was then added to cells and incubated for 20 hours and luciferase activity was measured. Four replicates were performed for each and the average \pm SD is shown. Serum were from VEEV vaccinated human and horse (solid squares and circles respectively), unvaccinated humans (open squares and triangles), and unvaccinated horse (open circles). Anti-VSV (Indiana strain) mouse ascites fluid (ATCC #VR-1238AF) was used as a positive control with the VSV pseudotypes (solid triangles). The dashed horizontal line indicates the 80% reduction in signal threshold (compared with the average of untreated controls) used as the end point to determine neutralization titer.

± 2 . In contrast, the two VEEV immune serum significantly reduced VEEV pseudotype infection beyond the inhibition seen with non-immune serum ($P < 0.001$ at the 1:20 dilution), with dilutions of 1:640 and 1:2560 yielding $> 80\%$ signal reduction. Similarly, the anti-VSV ascites fluid reduced VSV luciferase activity by $> 80\%$ at a dilution of 1:640. These findings indicated that the VEEV pseudotype was potentially a useful tool for determining neutralization titers (NTs) but due to the effects of non-immune serum on the VEEV pseudotype, only NTs of > 40 were significant and indicated the presence of neutralizing antibody.

Assay specificity was tested by using sera from mice immunized with well-characterized species of the VEEV complex, or from a naturally infected human patient. The human sera had been previously characterized by PRNT and virus identified by isolation. For these assays, serum titers from PRNT with live virus in 6-well plates were compared with titers from pseudotype assays performed in a 96-well format (Table 1). We used 2 strains of VEEV that differed by subtype (IAB and IC) and are antigenically representative of common epizootic VEEV subtypes. Corresponding pseudotypes bearing the envs of each were also constructed. In this set of experiments the VSV control was neutralized to $57 \pm 16\%$ of the untreated control signal at a serum concentration of 1:40, which was similar to previous experiments. Non-immune serum behaved similarly. In contrast, VEEV TRD reactive serum effectively reduced assay signals or plaque numbers to give titers of 320 and > 1280 for the PRNT and pseudotype assays respectively (80% reduction in plaque number or luciferase signal was used as end point). The difference seen in the titers between the PRNT and the pseudotype assay indicated that the pseudotypes were more readily neutralized.

The assay was then applied to determine its effectiveness at detecting cross-reactive neutralizing antibodies to other alphaviruses in the VEE complex and further compared with standard PRNT assays with live virus. Representative sera reactive to Everglades, Mucambo and Pixuna viruses were tested (see Table 1). For Everglades-reactive serum, neutralization was evident by PRNT (titer of 80) and with the VEEV pseudotypes (> 1280). Mucambo-reactive serum did not neutralize live virus but gave titers of 160 and 320 with the VEEV pseudotypes. This is consistent with E2 protein epitopes shared between TRD and these VEE complex subtypes.¹⁵ Mucambo and Everglades subtypes are reported to share 2 and 4 epitopes (as identified using monoclonal antibodies) respectively with VEEV strain TRD.¹⁶ The corresponding titers using PRNT and the pseudotype assay appear to reflect this relationship. The Pixuna reactive sera gave an unexpectedly potent neutralization (> 1280) of the VEEV pseudotypes but did not affect live VEEV (< 20) in the PRNT assay. This may reflect the exposure of a conserved, but buried cross-reactive epitope that is specifically bound by antibodies in the Pixuna reactive serum.

Other sera reactive to more distantly related alphaviruses were also tested. Eastern equine encephalitis (EEEV), Mayaro virus, and Barmah Forest virus-reactive sera were used. EEEV is the sister antigenic complex with VEE in the alphavirus genus, and Mayaro is a more distantly related New World alphavirus, while Barmah Forest is a distantly related Old World alphavirus. In each case no cross-reactivity was evident by PRNT, but low-level neutralization was observed using the pseudotypes with EEEV and Mayaro reactive sera.

TABLE 1

Neutralization titers of serum raised against virus species of the VEEV complex as determined by PRNT assays with live virus and VEEV env pseudotypes*

Serum reactivity	Antigenic classification	PRNT. VEEV strain TRD (1AB)	PRNT. VEEV 3908 strain (1C)	Pseudotype. TRD env	Pseudotype. 3908 env
Non-immune	—	<20, <20	<20, <20	<40, <40	<40, <40
VEEV TRD	1AB	320, 320	320, 320	>1280, >1280	>1280, >1280
Everglades†	II	80, 160	80, 80	>1280, >1280	>1280, >1280
Mucambo	III	<20, <20	<20, <20	160, 160	320, 640
Pixuna	IV	<20, <20	<20, <20	>1280, >1280	>1280, >1280
Mayaro‡	—	<20, <20	<20, <20	40, 80	40, 80
EEEV	—	<20, <20	<20, <20	<40, 40	40, 40
Barmah Forest	—	<20, <20	<20, <20	<40, <40	<40, <40

* Neutralizing antibody titers were determined using PRNT with VEEV strains TRD or 3908 or with the pseudotype luciferase reporter gene assay with virus bearing the envs of each. Serum was from mice immunized with the indicated virus unless stated otherwise. Serum was used at serial 2-fold dilutions. Titers are given as the reciprocal of the serum dilution that reduced plaques or luciferase reporter activity by $\geq 80\%$ with live virus or pseudotyped virus respectively. Results from 2 independent assays are shown.

† Everglades reactive serum was from infected cotton rats.

‡ Mayaro reactive serum was from an infected human patient.

No reactivity was evident with Barmah Forest reactive serum for either live VEEV or the pseudotypes. Apart from the exception of Pixuna reactive serum, neutralization patterns seen with the pseudotyped viruses correlated to those from PRNT assays using live VEEV. Importantly, since the pseudotype-based assay can be performed in a 96-well format (and potentially higher density formats, like 384-well plates) it is particularly suited for high-throughput primary screening of serum to detect neutralizing antibodies.

We then applied the pseudotype assay to screen for neutralizing antibodies in human and horse serum. Again, a 96-well format was used that could be adapted to a high-throughput patient or veterinary diagnostic screening scenario. Sera were reacted with pseudotyped virus and then cells were added and cultured. After 20 hours, cells were lysed and luciferase gene expression was read. Typical signals ob-

tained for untreated virus were 2×10^4 cps, which corresponds to approximately 200 colony forming units of virus (data not shown), and was readily detected in our 96-well plate reading luminometer. We tested 5 human sera from individuals living in VEEV-endemic areas of Mexico, 4 laboratory workers (in addition to that from Figure 2) previously vaccinated with the TC-83 vaccine strain (a derivative of strain TRD), and 7 horses experimentally infected with different VEEV strains (indicated in Table 2). One horse was also bled at day 0, 8, and 14 after inoculation with virus to observe seroconversion. In general, where end points were obtained, the pseudotypes were neutralized more effectively than wild-type virus, and gave titers that were at least 2-fold higher than for the PRNT using live virus (see Table 2). In each case the NTs reflected the results obtained from the PRNT assay. Of the 14 serum that were positive by PRNT, all were detected as being posi-

TABLE 2

Neutralization titers of human and horse serum determined by PRNT using live VEEV strains Trinidad (TRD) or 3908 or corresponding envelope pseudotypes*

Serum Identity	Virus subtype	PRNT using strain TRD (1AB)	PRNT using 3908 strain (1C)	Pseudotype titer. TRD env	Pseudotype titer. 3908 env
Human†					
168	1E	80, 160	40, 40	640, 1280	>640, >640
503	1E	80, 80	80, 80	>640, >640	>640, >640
545	1E	40, 160	40, 40	160, 640	80, 160
505	NEG	<20, <20	<20, <20	40, 80	40, 40
486	NEG	<20, <20	<20, <20	80, 80	20, 40
VAC1	1AB	<20, <20	<20, <20	40, 40	40, 80
VAC2	1AB	160, 160	40, 40	160, 160	160, 320
VAC3	1AB	40, 40	40, 40	320, 320	160, 160
VAC4	1D	80, 160	80, 80	>1280, >1280	1280, 1280
Horses‡					
Non-immune	—	<20, <20	<20, <20	<40, <40	<40, <40
876	1C 243937	>640, >640	160, 160	>1280, >1280	>640, >640
968	1D 66637	>640, >640	320, 320	>1280, >1280	>640, >640
DP1	1E 68U201	>640, >640	320, 320	>1280, >1280	>640, >640
DP2	1E 68U201	>640, >640	160, 160	>1280, >1280	>640, >640
SW7	1C SH3	>640, >640	320, 320	>1280, 1280	>640, >640
SW4	1D ZPC738	80, 80	80, 80	80, 80	20, 40
Timed horse bleeds					
Horse 01 Day 0	1D 66637	<20, <20	<20, <20	<20, 40	<20, 40
Horse 01 Day 8	1D	>640, >640	160, 160	>1280, >1280	320, 640
Horse 01 Day 14	1D	>640, >640	320, 320	>1280, >1280	320, 1280

* Neutralizing antibody titers were determined using PRNT with VEEV strains TRD or 3908 or with the pseudotype luciferase reporter gene assay with virus bearing the envs of each. Serum was used at serial 2-fold dilutions. Titers are given as the reciprocal of the serum dilution that reduced plaques or luciferase reporter activity by $\geq 80\%$ with live virus or pseudotyped virus respectively. Results from 2 independent assays are shown.

† Human sera were from people living in regions with endemic VEEV or from vaccinated laboratory workers (VAC1-3) or infected from a laboratory accident with a subtype 1D strain (VAC4).

‡ Horses were experimentally infected with the indicated subtype strains of VEEV.

tive by the pseudotype assay. Indeed, 2 samples taken from individuals living in a VEEV endemic area (serum 486 and 505), previously indicated to be negative by PRNT had low-level reactivity using the pseudotype assay (titer of 40-80 using the TRD pseudotype). A vaccinated individual (VAC1) also gave a low NT (40-80) with the pseudotypes but was negative by PRNT. These were likely true positives as the titers obtained with normal human serum did not exceed 1:20. Also, the VSV-G control pseudotype gave a neutralization activity on average of 64% and 41% at 1:20 and 1:40 respectively with each of the sera tested in this set of experiments (data not shown).

Recently, an advance was made in being able to measure the entry of alphaviruses in real time using a luciferase-based virus entry assay.¹² The assay took advantage of encapsulation of luciferase enzyme directly into pseudotyped virus particles. Only when the pseudotype virus membrane fused to the cell membrane (upon entry) is luciferase enzyme released and detected by perfusing cells with luciferase assay buffer (Figure 3, upper panel). This approach offers the potential of detecting neutralizing antibodies in the fastest time possible for a virus-based method. In our previous work entry was detected as quickly as 15 minutes after virus contact with cells.

A time course was first done to establish the earliest time that could be used in a neutralization assay. Continuous incubation of luciferase-containing pseudotypes with cells gave a signal that increased over time. Curves were fitted to the data by non-linear regression and indicated that an exponential growth curve with a signal doubling time of 32 ± 4 minutes was the best fit ($R^2 = 0.98$, Figure 3, middle panel). From this a time of 1 hour with 10^5 cells was sufficient to obtain a signal that was highly reproducible and had a signal to noise ratio of > 100 (2200 cps with a typical instrument background of 20 cps). While assays were performed in tubes, a 96-well plate assay could be achieved with extended incubation times (2 h) to obtain sufficient signal from smaller cell numbers (typically 10^4 cell per well).

Neutralization assays were then performed using selected serum from our prior screen of the pseudotypes. These included human serum (503) and horse serum (day 14 post-infection). NTs were 80 and 160 respectively (Figure 3, lower panel). In each case the VSV control was unaffected by the serum, showing that the signal inhibition was specific for the VEEV particles. NTs were calculated based on an 80% reduction in signal from the untreated cells. We found that the NTs obtained were within 2-fold of those from the PRNT assay. These observations indicate that the entry assay provides a rapid method to titer neutralizing VEEV antibody responses.

DISCUSSION

Neutralizing antibodies are important for effective humoral immune responses against virus pathogens and effectively block initial infection and then cell-cell spread. Many vaccines rely on neutralizing antibody responses for protection. Neutralizing responses are also commonly used to differentiate virus infections, as surface antigens tend to be virus-specific and even subtype-specific. To date, most assays to detect neutralizing antibodies rely on use of fully infectious virus, which has 2 significant disadvantages. Representative

reference stocks of virus must be maintained, which may be difficult given that antigenic drift could occur during passage in cell culture.^{5,17} Secondly, as is the case with VEEV, working with live virus is dangerous. Other restrictions imposed by regulatory agencies also prohibit easy access to reference strains. Use of the TC-83 vaccine strain can overcome some of the health risk; however, this virus may not be useful for detection of VEEV subtypes of low antigenic relatedness. Another drawback of a standard PRNT assay is the need to visualize distinct plaques in cell monolayers. Since this is mostly done with the naked eye, with plaques being 1–2 mm in diameter the assays require at least a 12-well plate format, making high-density screens impractical. A strong need then exists for alternatives to assays with live virus that can be used for detection of neutralizing antibodies in high-throughput formats.

Virus pseudotypes are virus particles that bear the envs of a donor virus over the core of another virus. The most commonly used pseudotypes are retrovirus and VSV core-based particles. These form when the virus core buds from cell membrane domains that are enriched for the virus envs. When the native env is deleted, other viral envs can substitute. Pseudotyping efficiency is related to the ability of the new env to localize to membrane domains where the donor core buds.^{18,19} Many env pseudotypes of Murine leukemia viruses have been described and are promising gene therapy vectors.²⁰ Importantly, in each case, the pseudotype adopts the host range, receptor utilization, and entry mechanism of the acquired env. Another advantage of pseudotypes is that they are rapidly generated using plasmid-based transfection of commercially available and well-characterized cell lines (usually derivatives of 293 HEK cells). From the current work, transient transfection of one 10-cm plate of 293 cells yields sufficient VEEV env or VSV env pseudotyped virus to perform 10 and 100 96-well plate assays, respectively (10^4 cps/well at 20 h; differences correspond to titer of the pseudotype). Virus can be rapidly assembled from plasmid stocks and virus stocks can be stored frozen at -80°C for at least 3 years without appreciable (2-fold) loss in activity. Producing virus stocks from plasmids also overcomes issues of antigenic drift that are associated with passage of replication competent virus in cell culture or animals.

Pseudotyped particles are also considered safe and non-contagious as the viral genome is almost completely replaced by a reporter gene. Indeed, current NIH guidelines indicate that MLV pseudotypes can be used at BSL-1 when no evidence of replication competent virus exists (as is typical).¹⁴ This is a significant advantage over other virus-based assays. In the present work we used the firefly luciferase reporter gene to replace the MLV genome. This enzyme produces light when its substrates, ATP, oxygen, and luciferin, are reacted together. Assay buffer is commercially available and many laboratories have equipment to sensitively measure the light output. To date, few groups have taken advantage of pseudotypes as potential diagnostic reagents. Exceptions are Hepatitis C virus²¹ and the SARS coronavirus.²² However, in both reports, no quantitative assessment was made to indicate how the NTs of the pseudotypes compared with those obtained through standard assays using live virus.

We show that VEEV envelope pseudotypes encoding firefly luciferase can be used to screen for neutralizing antibodies in serum of humans, horses, and rodents. In general, the neu-

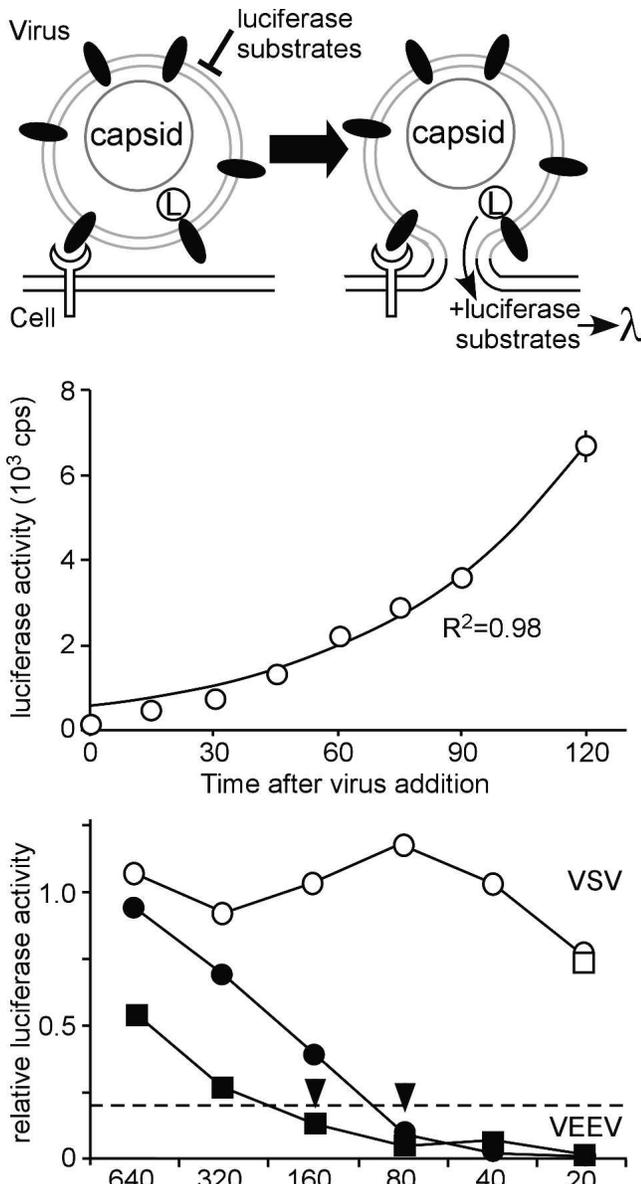


FIGURE 3. Rapid assay for detecting neutralizing antibodies. The principle of the assay is shown at top. Luciferase enzyme ("L" within circle) incorporated directly into virus particles is initially shielded from its substrates luciferin, ATP, and oxygen and gives no activity. This first step of entry is mediated by virus envs (black ovals), which bind to receptors and the virus becomes endocytosed. After endosomal acidification, the virus and cell membranes fuse. The luciferase can then access the cell cytoplasm and its substrates, including luciferin, which is transported into cells. Neutralizing antibody binding to the envs blocks receptor interaction and prevents entry. The assay was optimized by determining the time required to generate sufficient signal (**middle panel**). Cells were incubated in the presence of virus for the indicated times, then washed and freeze-thaw lysed. Curves were fitted by non-linear regression to the data with the best fit for an exponential growth curve ($R^2 = 0.98$) giving a signal doubling time of 32 ± 4 minutes. Neutralization assays (**lower panel**) were performed using a VEEV env pseudotyped particle (solid shapes) that contained luciferase and a VSV env pseudotype (open shapes) as control. Virus was incubated with the indicated dilutions of serum for 1 hour and then with 10^5 cells for 1 hour at 37°C . Cells were washed and lysed by freeze-thaw. Luciferase activity was then measured after addition of luciferase assay buffer. Arrowheads indicate dilution of serum that gave the NT. Serum were human 503 (circles), and experimentally infected horse at day 14 (squares). Only the highest concentration of horse serum was tested for nonspecific neutralization using the VSV pseudotype. The horizontal dashed line indicates the 80% decrease in signal that was used as the endpoint threshold.

tralization patterns of the pseudotypes were similar to those seen with live virus by PRNT. In cases in which end points were obtained, the pseudotype assays gave titers at least 2-fold greater than by PRNT. Consistent with this result, we often observed neutralization of the pseudotyped particles when the PRNT assay did not detect activity, indicating the presence of low-level neutralizing antibodies. A drawback was seen in neutralization of both VSV and VEEV env-pseudotyped particles with "non-immune" serum, which with VEEV env virus reached the 80% signal reduction threshold at serum concentrations of 1:20. The VSV env bearing pseudotypes were neutralized more weakly. However, when serum containing known neutralizing antibodies was used, neutralization exceeded that of the non-immune serum. This neutralization may be the product of nonspecific anti-viral factors in the serum. Others have reported that pseudotypes produced in murine cells can be neutralized by human antibodies reactive to mouse-specific carbohydrate modifications of the envs.²³ Neutralization was also seen with non-immune serum in the PRNT assay using live virus, but it did not exceed the 80% reduction in plaque number that is the standard cut-off threshold.¹³ The "nonspecific" neutralization set the upper boundary of detection for the assay to serum concentrations $\leq 1:40$ where neutralization did not exceed 66% for any of the non-immune samples tested. We had expected that the VSV pseudotypes would be nonspecifically neutralized to a similar extent as seen with the VEEV env pseudotyped viruses and serve to measure this effect. However, 3 of 5 non-immune serum tested neutralized the VSV more weakly than the VEEV env pseudotyped virus. Another pseudotype bearing the envelope proteins of a different virus (e.g., another alphavirus) may perform this function better.

The assay was tested for the ability to detect cross-reactive neutralizing antibodies generated against members of the VEE complex and related alphaviruses. The VEE complex is made of 6 virus species that are antigenically related. Antisera generated against each virus cross-react to different extents.²⁴ Monoclonal antibodies are required to further distinguish subtypes and strains. Subtype I viruses share 1–4 env epitopes with subtypes II through V.^{16,25} Most differences are in epitopes present on E2 and correspond to amino acid sequence differences. The NT using pseudotypes was expected to reflect the antigenic relatedness of VEEV TRD or 3908 strains to the virus used to generate the serum. Everglades virus is closely related to VEEV and shares at least 4 monoclonal antibody-recognized epitopes.¹⁶ Consistent with this relatedness, antiserum against Everglades potentially neutralized VEEV TRD and 3908-based pseudotypes. Mucambo is a subtype III virus and shares only 2 epitopes, and Mucambo-reactive sera gave lower titers with both live and pseudotyped viruses. The exception was Pixuna reactive antiserum where the pseudotypes were efficiently neutralized but the live virus was unaffected. We speculate that the pseudotypes may expose a neutralizing epitope that is normally buried on live virus but was a dominant reactivity in the anti-Pixuna serum only. Epitopes on E1 are likely candidates as they are highly conserved between VEE complex members and even more distantly related alphaviruses.¹⁶ However, Mayaro, EEEV, and Barmah forest virus-reactive serum had little to no neutralizing activity for the pseudotypes indicating that for these antibodies against the neutralizing Pixuna epitope is not a major component. The findings indicate that the pseudotypes

may give false positive reactions for some sera containing neutralizing antibodies to related viruses, like Pixuna. However, more extensive work with human and horse serum validated the effectiveness of the assay to screen for anti-VEEV neutralizing antibodies. In general, the NT results using pseudotypes closely reflected those obtained with live virus in a PRNT, with all PRNT-positive serum being positive by the pseudotype assay as well. We conclude that the pseudotype assay may be best suited as a rapid and high-throughput primary screen to detect neutralizing antibodies to closely related members of the VEE complex but identification of strain or subtype-reactive antibodies will likely require further testing using conventional PRNT and other assays.

While the pseudotype assay can be performed in under 24 hours, we also showed that a luciferase-based rapid virus entry assay could also be used to determine an NT more quickly. Times as short as 1 hour were sufficient to obtain a signal and obtain a quantitative measure of entry inhibition by antibody. This assay gave NTs that were within 2-fold of the PRNT assay with live virus. This system is more difficult to adapt to a high-throughput format as it is a kinetic assay; thus small differences in addition of virus, cells, and serum can result in increased errors in the readout of the assay. However, the assay is most useful when a quick determination of neutralization titer is required and for primary screening of drugs or other substances that affect cell viability after long-term exposure.

Overall we expect our findings to be directly applicable to high-throughput screening of serum for VEEV-neutralizing antibodies. The assays that we discussed may be useful in the laboratory for detection of neutralizing antibodies in patients and for development of vaccines or identification of drugs that block VEEV entry.

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Authors' addresses: Andrey A. Kolokoltsov, Tonya M. Colpitts, and Robert A. Davey, 4.138, MRB, Department of Microbiology and Immunology, 301 University Blvd., Galveston, TX 77555. Eryu Wang and Scott C. Weaver, 4.128, Department of Pathology, Kieller Bldg., 301 University Blvd., Galveston, TX 77555.

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