Microbead electrochemiluminescence immunoassay for detection and identification of Venezuelan equine encephalitis virus

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ABSTRACT

An electrochemiluminescence (ECL) immunoassay, incorporating chemically biotinylated and ruthenylated antibodies down-selected from a panel of monoclonal and polyclonal reagents, was developed to detect and identify Venezuelan equine encephalitis virus (VEEV). The limit of detection (LOD) of the optimized ECL assay was 10^3 pfu/ml VEEV TC-83 virus and 1 ng/ml recombinant (r) VEEV E2 protein. The LOD of the ECL assay was approximately one log unit lower than that of a sandwich enzyme-linked immunosorbent assay (ELISA) incorporating the same immunoreagents. Repetition of ECL assays over time and by different operators demonstrated that the assay was reproducible (coefficient of variation 4.7–18.5% month-to-month; 3.3–8.8% person-to-person). The VEEV ECL assay exhibited no cross-reactivity with two closely related alphaviruses or with 21 heterologous biological agents. A genetically biotinylated recombinant VEEV antibody, MA116SBP, was evaluated for utility for detection of rE2; although functional in the ECL assay, the LOD was two log units higher (100 ng/ml vs 1 ng/ml) using MA116SBP than when chemically biotinylated antibody was used. The ECL assay detected VEEV at the lowest LOD (highest sensitivity) hitherto reported in the published literature and ECL assay results were generated in ~60 min compared to a 6-8 hr period required for ELISA. Results have demonstrated a sensitive, rapid, and fully automated ECL immunoassay for detection and identification of VEEV.

Keywords: Venezuelan equine encephalitis virus; Envelope E2 protein; Electrochemiluminescence immunoassay; Enzyme-linked immunosorbent assay

1. Introduction

Venezuelan equine encephalitis virus (VEEV), a positive-sense single-stranded RNA alphavirus, is an important mosquito-borne pathogen in humans and equines (Weaver et al., 2004). Epizootic VEEV infections cause debilitating disease with a high fatality rate in equines. In humans, VEEV infection is associated with a potentially life-threatening severe febrile illness and neurological disease appears in ~5% of cases (Griffin, 2001). The incidence of human infection during equine epizootics can reach 30% and mortality associated with encephalitis in children is as high as 35%. The outbreaks in Venezuela and Colombia in 1995 affected around 75,000 people and more than 300 fatal encephalitis cases occurred (Rivas et al., 1997). VEEV is environmentally stable and highly infectious by aerosol inhalation, making it a potential biological weapon threat. As such, it is crucial that rapid and sensitive immunoassays be developed for detection of VEEV in environmental and clinical samples. A number of immunoassay techniques utilizing polyclonal or monoclonal antibodies (Wang et al., 2005) or recombinant antibodies (Duggan et al., 2001; Hu et al., 2002, 2004; Kirsch et al., 2008) has been described for the detection of VEEV, including enzyme-linked immunosorbent assay (ELISA), radioimmunosorbent assay (RIA), light addressable potentiometric (LAP) assay, and dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA) (Roehrig et al., 1982; Smith et al., 2001; Hu et al., 2002, 2004). Typically, these assays require multiple operational steps and long incubation periods; acquisition and analysis of assay results can take many hours.

Electrochemiluminescence (ECL) immunoassay is a magnetic bead-based

technology for conducting immunoassays with improved assay performance (Deaver, 1995; Yang et al., 1994). There are three components in an ECL immunoassay: (i) a biotinylated capture antibody (Cab), pre-bound to streptavidin-coated magnetic beads, (ii) a detector antibody (Dab), labelled with ruthenium-trisbipyridal, for the emission of light when electrochemically stimulated, and (iii) an analyte, which reacts with the capture and detector antibodies resulting in an antigen-antibody sandwich. In ECL reactions, a precursor molecule (tripropylamine) is activated on an electrode surface resulting in an electron transfer reaction. This transfer initiates the excitation of ruthenium-trisbipyridal which ultimately results in the emission of a photon at 620 nm.

Assay techniques utilizing ECL technology have a number of advantages over conventional assay methods e.g., ELISA and RIA: Limits of detection (LOD) in ECL assays are lower (higher sensitivity), due to high luminescent signal to noise ratios (Gatto-Menking et al., 1995; Yu et al., 2000; Garber and O'Brien, 2008; Yoshimura et al., 2008; Rossi et al., 2008; Kuhle et al., 2010). The dynamic range for analyte detection extends over a wide range (five orders of magnitude) (Yang et al., 1994; Kijek et al., 2000; Yu et al., 2000). The time and labour required to complete ECL assays are reduced compared with conventional immunoassays. The ECL assay is a non-separation technique, thus does not require plate coating, washing, or aspiration steps. Sample reading is rapid; the ECL instrumentation used in the present study requires approximately one minute per sample to read. Labelled ECL reagents are exceptionally stable and ECL assays are robust and tolerant of analyte detection in the presence of a variety of sample matrices. These features make the ECL detection system an attractive alternative to conventional immunoassay techniques. In addition, the instrumentation

used in this study is a military hardened version, making it especially useful for military field use.

This study describes the development of an ECL assay for VEEV. A panel of VEEV antibodies was screened for optimal performance using VEEV strain TC-83 whole virus and VEEV recombinant (r) envelope E2 protein as target antigens. ECL assays were optimized for antibody concentration and assay LOD were determined in comparison to ELISA incorporating the same antibody reagents. ECL assays were assessed for reproducibility and precision over time and when conducted by different operators. Assay specificity for VEEV was evaluated by screening against closely related alphaviruses and 21 heterologous bacteria, virus, or toxin agents. A genetically biotinylated recombinant antibody to VEEV was compared to a chemically biotinylated antibody for utility for detection of VEEV rE2.

2. Materials and methods

2.1. Instrumentation

ECL measurements were performed using a M-SERIES® M1MR analyzer (BioVeris Corp., Gaithersburg, MD).

2.2. Antigens

2.2.1. Homologous antigen

Live VEE TC-83 virus, purified as previously described (Hu et al., 2004), was kindly provided by Dr. J. Wu, DRDC Suffield. Recombinant E2 protein was expressed in *E. coli* and purified at DRDC Suffield (W-G. Hu et al., unpublished data).

2.2.2. Heterologous antigens

Gamma-irradiated Western equine encephalitis virus (WEEV) and Eastern equine encephalitis virus (EEEV) were purchased from the US Critical Reagents Program (CRP) (Aberdeen Proving Ground, MD). Yellow fever virus, attenuated vaccine (strain 17D), was purchased from Aventis Pasteur Inc. (Toronto, ON). Dengue and Klebsiella pneumoniae were purchased from American Type Culture Collection (Manassas, VA). Inactivated (^{60}Co irradiated) Brucella melitensis (suis), Bacillus anthracis (vollum),

Bacillus globigii (spores), Francisella tularensis, Yersinia pseudotuberculosis, Yersinia pests (JAVA 9), Escherichia coli, Bacillus cereus, Vaccinia virus (Lister), Bacillus thuringiensis, Aspergillus niger, Erwinia herbicola, Pseudomonas aeruginosa, Coxiella burnetii, and MS2 were all acquired from US Army Dugway Proving Ground (DPG) (Dugway, UT). SEB toxin was purchased from Toxin Technology (Sarasota, FL), botulinum toxoid A was purchased from WAKO Chemicals Inc. (Richmond, VA), and ricin A chain was purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON).

2.3. Monoclonal and polyclonal antibodies

Hybridomas 1A4A1 and 1A3A9 were kindly provided by Dr. J.T. Roehrig (Mathews and Roehrig, 1982; Roehrig et al., 1982; Roehrig and Mathews, 1985). The hybridomas were grown in BD Cell^{TM} MAb Basal Medium (BD-Biosciences, Mississauga, ON) supplemented with 10% FETALCLONE$^{®}$ fetal calf serum (HyClone Laboratories, Logan UT), 2mM L-glutamine (Invitrogen, Burlington, ON), and 1% HAT Media Supplement Hybri-Max$^{®}$ (Sigma-Aldrich Canada). The hybridomas were weaned from FBS medium and transferred to a CELLine^{TM} 1000 flask (BD-Biosciences) and incubated at 37 ^{o}C under 5% CO_2 for two weeks. Supernatant was harvested from the CELLine^{TM} 1000 flask and the monoclonal antibody (mAb) was then purified using a Melon^{TM} Gel Monoclonal IgG Purification Kit (Pierce, Ottawa, ON) according to the manufacturer's instructions.

Rabbit and goat anti-VEEV polyclonal antibodies (pAb) were previously developed and purified for IgG under a DRDC Suffield contract by SciLab Consulting Inc.

(Redcliff, AB). Both the rabbit and the goat pAb had been purified on a Bio-Gel® Protein G column (Bio-Rad Laboratories, Mississauga, ON) by High-Performance Liquid Chromatography (Spectral Physics, San Jose, CA).

2.4. Genetically biotinylated recombinant antibody

A genetically biotinylated recombinant VEEV single-chain variable fragment antibody, MA116SBP, was previously generated at DRDC Suffield (Alvi et al., 1999, 2002, 2003; Hu et al., 2002).

2.5. ECL assay reagents

The following ECL assay reagents and buffers were purchased from BioVeris Corp.: Biotin-LC-Sulfo-NHS ester, Ruthenium (II) tris-bipyridine-NHS ester, Streptavidin-coated Dynabeads® M-280, M-SERIES® Positive Calibrator, M-SERIES® Negative Calibrator, StabilCoat® Immunoassay Stabilizer, BV-CLEAN™ Plus solution, BV-GLO™ Plus solution, BV-DILUENT™ solution, BV-STORE™ solution, and BV-SANITIZE™ solution.

2.6. Matrix powders and soils

Flour (white, enriched, all purpose) (Safeway brand), cornstarch (Safeway brand), baking powder (Safeway brand), baking soda (Safeway brand), laundry detergent (Tide

Original, Procter and Gamble), coffee creamer (Coffee-Mate®, Carnation, Nestlé), skim milk, and powdered sugar were all purchased from Safeway Canada Ltd. (Medicine Hat, AB). Talcum powder, powdered cleanser, and spackling powder were purchased through Foreign Military Sales from the US Critical Reagents Program. The four soil samples including sand, sand loam, loamy sand, and clay loam were from the DRDC Suffield Experimental Proving Ground and had been previously characterized by the Alberta Environmental Centre (Vegreville, AB).

2.7. Preparation of labeled antibodies

Prior to labeling, all antibodies were desalted using NAP-5 columns (Amersham Biosciences, Baie d'Urfé, QC) according to the manufacturer's instructions. In separate labeling reactions, the four antibodies (1A4A1 mAb, 1A3A9 mAb, rabbit pAb, and goat pAb) were each biotinylated with biotin-LC-Sulfo-NHS ester at a molar ratio of 10:1 (Biotin:Ab) and ruthenylated with ruthenium (II) tris-bipyridine-NHS ester at 7.5:1 (Ru:Ab) by labeling procedures recommended by BioVeris. In brief, the antibodies and respective labeling solutions were incubated separately and simultaneously using an end-over-end shaker with gentle rotation for 60 min at RT. The labeling reactions were quenched by the addition of 20 µl of 2 M glycine and then incubated at RT for an additional 10 min. Uncoupled biotin and ruthenium esters were removed by dialysis in four changes of sterile phosphate buffered saline (PBS) (pH 7.4) at 4 ^{o}C over two days. The protein concentrations of labeled antibodies were determined using a Micro BCA™ assay (Pierce) or a ND-1000 Spectrophotometer (LabX, Midland, ON) with bovine serum

albumin (BSA) (Pierce) as the standard. Aliquots of each labeled antibody were stored at 4 ^{o}C until used.

2.8 Preparation of capture antibodies

For the preparation of Cab (antibody-functionalized beads), biotinylated antibodies were pre-bound to streptavidin-coated Dynabeads® (100 µg antibody per ml of Dynabeads^{R}). Biotinylated antibodies and beads were incubated together with an end-over-end rotation for 60 min at RT. Unbound antibody was removed by washing the beads three times with sterile PBS-0.1% Tween 20 (PBST) using a MPC^{TM}-S magnetic particle concentrator (Dynal Biotech ASA, Oslo), followed by three washes with sterile PBS.

2.9. ECL immunoassay

Cab (biotinylated antibody pre-bound to streptavidin-coated Dynabeads®) was diluted in StabilCoat® Immunoassay Stabilizer and the detector antibody (Dab) (ruthenylated antibody) (Ab-Ru) was diluted in PBS-2% BSA-0.1% Tween 20. Antigen was diluted in PBS-0.3% Tween 20. ECL reaction solutions were prepared in 0.75 ml round-bottom tubes (Matrix Technologies) (VWR International, Mississauga, ON) by adding 25 µl of Cab, 25 µl of Dab, and 50 µl of antigen solution (or 50 µl of PBS-0.3% Tween 20 for the antigen negative control). The tubes were placed in a specialized 96-well assay plate (BioVeris Corp.), which was then loaded into the M1MR analyzer, where it was incubated with shaking for 15 min at RT and then analyzed.

2.10. ELISA

Initial ELISA experiments were performed in sandwich format to optimize the concentrations of the Cab, Dab, and indicator antibody (Iab) (data not shown). For the sandwich ELISA, the format utilized the rabbit-Cab/1A4A1-Dab antibody pair identical to that used in the ECL assay. Nunc Maxisorp^{TM} flat-bottomed 96-well plates (Life Technologies, Burlington, ON) were coated overnight at 4^{o}C with 3 μg/ml of Cab (rabbit pAb-biotin) in carbonate bicarbonate buffer, pH 9.6. The plates were washed five times with PBST in an ELx50 plate washer (Biotek Instruments, Winooski, VT) and then blocked with 2% BSA for 1 h at 37 ^{o}C. After five washes with PBST, plates were incubated with TC-83 virus at 10^{1}–10^{6} pfu/ml or VEEV rE2 protein at 0.01–10,000 ng/ml for 1 h at 37 ^{o}C. Following five washes with PBST, the plates were incubated with 2 μg/ml of Dab (1A4A1-Ru) for 1 h at 37 ^{o}C. The plates were again washed five times with PBST and then incubated with a 1:2000 dilution of horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Caltag Laboratories, Burlingame, CA). Finally, the plates were washed five times with PBST and developed for 30 min at RT with 3,3',5,5'-tetramethylbenzidine (TMB) microwell perioxidase substrate system (KPL, Gaithersburg, USA). The reactions were read at an absorbance of 635 nm by a microplate autoreader (Molecular Devices, Sunnyvale, CA, USA).

2.11. ECL and ELISA data analysis

LOD was determined by calculating a cutoff value that was equal to 20% above the

mean ECL or ELISA reading for the negative control. Therefore, a signal was considered positive if the ECL or ELISA ratio of the sample signal reading to background reading (S/B) was 1.2 or greater.

3. Results

3.1. Screening and optimization of antibody pairs for VEEV ECL assay

Four Abs (1A4A1 mAb, 1A3A9 mAb, rabbit pAb, and goat pAb) were each labeled with biotin-LC-sulfo-NHS ester and ruthenium (II) tris-bipyridine-NHS ester. A total of 16 combinations of antibody pairs was tested for utility in the ECL assays at a Cab dilution of 1:25, Dab concentration of 4 μg/ml, TC-83 virus concentration of 10^5 pfu/ml, and VEEV rE2 concentration of 100 ng/ml. Amongst the 16 antibody pairs, three pairs (1A4A1-Cab/1A4A1-Dab, 1A4A1-Cab/1A3A9-Dab, and 1A3A9-Cab/1A4A1-Dab) exhibited high reactivity with TC-83 virus, five pairs exhibited moderate reactivity, and eight other pairs produced only background ECL signals (data not shown). Using VEEV rE2 as the antigen, four antibody pairs (rabbit-Cab/1A4A1-Dab, goat-Cab/1A4A1-Dab, 1A4A1-Cab/1A4A1-DAb, and 1A4A1-Cab/rabbit-Dab) exhibited high reactivity, five pairs exhibited moderate reactivity, and the remaining seven pairs produced only background ECL signals (data not shown). In total, approximately 50% of the antibody pairs tested had little or no reactivity in the ECL assay with either TC-83 virus or rE2. Based on these results, three antibody pairs (1A3A9-Cab/1A4A1-Dab with high reactivity to TC-83 virus, rabbit-Cab/1A4A1-Dab with high reactivity to recombinant E2, and 1A4A1-Cab/1A4A1-Dab with high reactivity to both TC-83 virus and rE2) were chosen for further optimization in the ECL assay.

Experiments were performed to optimize the concentrations of Cab and Dab for each of the three selected antibody pairs. Concentrations of Cab and Dab that yielded the

highest S/B ratio and the lowest LOD were determined to be optimal. If two or more concentrations produced comparable results, the lowest concentration of antibody was selected for use. Cabs were first optimized by testing dilutions of 1:16, 1:25, and 1:50 with 4 μg/ml of Dab against three different concentrations of antigen (VEEV TC-83 virus at 10^3, 10^4 and 10^5 pfu/ml, and VEEV rE2 protein at 1, 10, and 100 ng/ml). Then, Dabs were optimized by testing concentrations of 4, 10, and 20 μg/ml with the optimal dilution of Cab against the same three concentrations of antigen. As a result of these experiments, the three antibody pairs were optimized for both VEEV TV83 virus and VEEV E2 protein as follows: antibody pair 1A4A1-Cab/1A4A1-Dab: Cab 1:35 and Dab 10 μg/ml; 1A3A9-Cab/1A4A1-Dab: Cab 1:25 and Dab 10 μg/ml; and rabbit-Cab/1A4A1-Dab: Cab 1:16 and Dab 4 μg/ml (data not shown). These concentrations of Cab and Dab were used with the three noted antibody pairs in all subsequent experiments.

3.2. ECL LOD for TC-83 virus and VEEV recombinant E2

Experiments were performed to determine the assay LOD and dynamic range for each optimized antibody pair. Results showed that antibody pair 1A4A1-Cab/1A4A1-Dab produced the lowest assay LOD for both TC-83 virus and rE2 protein; with this antibody pair, the LOD was 10^2–10^3 pfu/ml for TC-83 virus and 1 ng/ml for rE2 (Fig. 1A and B and Table 1). The LOD for antibody pair 1A3A9-Cab/1A4A1-Dab was approximately10^3 pfu/ml for TC 83 and 10 ng/ml for rE2 protein, while antibody pair rabbit-Cab/1A4A1-Dab produced a LOD of 10^4–10^5 pfu/ml for TC83 and 1 ng/ml for rE2 protein (Fig. 1A and B and Table 1). Antibody pair 1A4A1-Cab/1A4A1-Dab exhibited

an assay dynamic range of 4–5 orders of magnitude, from approximately 10^2–10^6 pfu/ml for TC-83 whole virus and 0.1–10,000 ng/ml for rE2 protein (Fig. 1A and B).

3.3. *Comparison of ECL and ELISA*

To compare assay LOD of ECL and ELISA, a sandwich ELISA, which used a rabbit-Cab/1A4A1-Dab antibody pair format identical to that used in the ECL assay, was performed for detection of TC-83 whole virus and VEEV rE2. The ELISA LOD obtained with this antibody pair was 10^6 pfu/ml TC-83 whole virus and 10 ng/ml rE2 (Table 2). These values were one to two log units higher than that observed in the ECL assay when the same antibody pair was used (i.e., ECL LOD 10^4–10^5 pfu/ml for TC83 virus and 1 ng/ml for rE2 protein). Thus, the LOD of the VEEV ECL assay was approximately 10 times lower (higher sensitivity) than that of a comparable sandwich ELISA for detection of VEEV whole virus and VEEV rE2 protein.

3.4. *ECL LOD for VEEV recombinant E2 using recombinant antibody MA116SBP*

To eliminate the need for chemical biotinylation of antibody, a genetically biotinylated recombinant antibody, MA116SBP, was tested in the ECL assay for utility for detection of rE2 protein. MA116SBP was pre-bound to streptavidin-coated Dynabeads® following the same procedures as described above for mAbs and pAbs. MA116SBP Cab at a dilution of 1:25 was paired with each of four Dabs, 1A4A1-Dab, 1A3A9-Dab, rabbit-Dab, and goat-Dab, each at a concentration of 4 μg/ml, and evaluated

in the ECL assay with VEEV rE2 at a concentration of 100 ng/ml. Among the four antibody pairs, MA116SBP-Cab/1A4A1-Dab pair produced the highest S/B values for rE2 antigen (data not shown), thus this antibody pair was chosen for optimization of Cab and Dab. Cab was optimized by testing dilutions of 1:8, 1:16, 1:25, 1:50 with 4 μg/ml Dab and three concentrations of VEEV rE2 (1, 10, and 100 ng/ml), followed by optimization of Dab by testing concentrations of 4, 10, and 20 μg/ml against the optimal dilution of Cab (1:8) and VEEV rE2 at the same antigen concentrations. The assay LOD obtained using genetically biotinylated MA116SBP as Cab was compared with that obtained using chemically biotinylated Cab. Under optimal conditions, with the Cab dilution of 1:8 and the Dab concentration of 10 μg/ml, the LOD for rE2 obtained using the pair MA116SBP-Cab/1A4A1-Dab was 10–100 ng/ml (Fig. 2). This value was two to three logs higher than that observed when antibody pairs 1A4A1-Cab/1A4A1-Dab and rabbit-Cab/1A4A1-Dab were used to detect rE2 (Fig. 1B and Table 1).

Antibody pair MA116SBP-Cab/1A4A1-DAb exhibited a dynamic range of approximately three orders of magnitude, from approximately 100–100,000 ng/ml for detection of rE2 protein (Fig. 2). By comparison, antibody pairs 1A4A1-Cab/1A4A1-Dab and rabbit-Cab/1A4A1-DAb each exhibited a dynamic range of approximately four orders of magnitude (1–10,000 ng/ml) for detection of rE2 (Fig. 1B).

The findings suggest that, although the genetically biotinylated recombinant antibody MA116SBP was functional as Cab in ECL, the assay LOD obtained by its use for detection of VEEV rE2 protein was higher (lower sensitivity) than that obtained using chemically biotinylated Cab.

3.5. Assay reproducibility and precision

The assay reproducibility and precision were evaluated by titrating samples of VEEV whole virus from month-to-month and from person-to-person (Fig. 3 and Tables 3 and 4). For month-to-month reproducibility, LOD assays were performed on three separate occasions over a period of five months (May 2007 to September 2007) with the same antibody pair (1A4A1-Cab/1A4A1-Dab), stored at 4 ^{o}C over the five-month period, and the same 10-fold dilutions of VEEV TC83, freshly prepared for each experiment (Fig. 3). For person-to-person reproducibility, two different persons conducted standard LOD assays using the same antibody pair (1A4A1-Cab/1A4A1-Dab) and same 10-fold dilutions of VEEV TC83, prepared freshly by each person on the day of the experiment. The coefficient of variation for the month-to-month assay precision determinations ranged from 4.7%–18.5% (Table 3) and for the person-to-person assay precision determinations from 3.3%–8.8% (Table 4).

3.6. Assay specificity

The specificity of the ECL assay was evaluated by testing two closely related members of the alphavirus genus, WEEV and EEEV, and 21 unrelated bacterial, viral, and toxin agents. Using the antibody pair 1A4A1-Cab/1A4A1-Dab, no cross-reactivity was detected with either WEEV or EEEV whole viruses (Fig. 4A and B). While VEEV produced a positive S/B ECL signal at a concentration of 10^4 pfu/ml, WEEV and EEEV exhibited only a background level signal even at a concentration of 10^7 pfu/ml (Fig. 4A

and B). Similarly, there was no cross-reactivity observed with 21 unrelated agents, including unrelated viruses: dengue, MS2, vaccinia Lister, and yellow fever (Fig. 5). These results demonstrated that, with respect to the related and unrelated microorganisms tested, this ECL assay was specific for VEEV.

3.7. Interference assay with powder and soil matrices

To evaluate matrix effects in the ECL assay, 15 environmental matrices, including 11 different powders and four types of soils, were examined in either non-spiked or 10^4 pfu/ml VEEV-spiked ECL assays. All powder and soil suspensions were used in the assay at a final concentration of 1 mg of powder or soil per ml of PBS-0.3% Tween 20. The positive control was prepared in PBS-0.3% Tween 20 containing 10^4 pfu/ml TC-83 whole virus and the negative control was PBS-0.3% Tween 20 only. A signal was considered positive with interference effect if the S/B ratio was 20% above or below the mean negative control (for non-spiked) or mean positive control (for VEEV-spiked). As shown in Fig. 6, talcum powder, powdered cleanser, sand, sand loam, loamy sand, and clay loam tested positive by increasing the background in the non-spiked ECL assay. In the VEEV-spiked assay, sand and loamy sand exhibited significant interference by quenching the ECL signal by 31%, relative to the positive control (Fig. 6). There was no significant interference detected with 13 other powders and soils in the VEEV-spiked assay. These results indicated that, at the level of significance examined, two powders and four soils interfered with the ECL assays by increasing background or quenching the ECL signals.

4. Discussion

A fully automated ECL immunoassay has been developed to detect VEEV TC-83 whole virus and VEEV rE2 protein.. The sensitivity of this ECL assay was the highest (lowest LOD) hitherto reported in the published literature. Use of the 1A4A1-Cab/1A4A1-Dab antibody combination yielded a LOD for TC-83 whole virus of 10^2–10^3 pfu/ml and for VEEV rE2 protein of 1 ng/ml. In a comparison of the ECL assay and ELISA in which the same antibody pairs and assay format were used, rabbit-Cab/1A4A1-Dab yielded a LOD of 10^4–10^5 pfu/ml whole virus by ECL assay and 1 ng/ml rE2 protein; in comparison, the ELISA LOD was 10^6 pfu/ml for TC-83 whole virus and 10 ng/ml for rE2 protein. In a previously published study, the LOD for the Trinidad donkey strain of VEEV was reported to be 1.25×10^7 pfu/ml by ELISA and 3.13×10^6 pfu/ml by DELFIA (Smith et al., 2001). In the current study, ECL assay results were generated rapidly (30–60 min, including 15 min incubation) compared with the time required to perform a comparable ELISA (6–8 hrs). Results also indicated that the biotin- and ruthenium-labeled VEEV mAb and pAb Cab and Dab could be stored at 4 ^{o}C for at least 10 months without deterioration of ECL signal. This finding is consistent with a previous report stating that biotin- and ruthenium-labeled antibodies were stable at 4 ^{o}C for up to one year (Kijek et al., 2000).

There are two envelope glycoproteins on VEEV, E1 and E2. Eight epitopes on the VEEV E2 glycoprotein ($E2^{a–h}$) have been identified (Mathews and Roehrig, 1982; Roehrig et al., 1982; Roehrig and Mathews, 1985). MAbs 1A4A1 and 1A3A9 have been

previously well characterized and found to be specific for $E2^c$ and $E2^g$ epitopes, respectively (Roehrig et al., 1982; Roehrig and Mathews, 1985). Studies on the spatial arrangement of these epitopes have indicated that the $E2^c$ and $E2^g$ epitopes are closely linked on VEEV TC-83 and that an anti-$E2^c$ antibody (e.g., 1A4A1) and an anti-$E2^g$ antibody (e.g., 1A3A9) will compete with each other due to steric hindrance (Roehrig and Mathews, 1985). In the present study, antibody pairs 1A3A9-Cab/1A4A1-Dab and 1A4A1-Cab/1A4A1-Dab exhibited high reactivity with TC-83 whole virus, suggesting that 1A4A1 and 1A3A9 did not compete with each other and that the competition of 1A4A1 with itself did not affect binding. This observation also suggests that the amount of Cab and Dab relative to the amount of available antigenic epitope was not limiting. This is not unexpected as there are multiple copies of E2 on the surface of the VEEV whole virus. By comparison, antibody pair 1A3A9-Cab/1A4A1-Dab pair exhibited poor reactivity when rE2 was used as antigen. This observation suggests that one or more of the antibody pair may have been directed against a conformational epitope on the whole virus or that rE2 may have lacked important glycosylation and/or may have been improperly folded for optimal activity with 1A3A9-Cab. The antibody pair rabbit-Cab/1A4A1-Dab exhibited good reactivity with rE2 but, when compared to antibody pair 1A4A1-Cab/1A4A1-Dab, relatively poor reactivity with TC-83 whole virus. Overall, our data showed that, in the context of developing an ECL assay, some antibody pairs had good reactivity with both whole virus and recombinant antigen, while other antibody pairs had good reactivity with only whole virus or recombinant antigen, but not both. This finding suggests that, while recombinant component antigen can be a useful substitute for whole pathogen antigen in development of immunoassays with some

antibody combinations, this may not always be the case, thus illustrating the importance of utilizing the whole pathogen as antigen when developing assays for clinical or environmental use.

During the labeling of antibody with biotin-LC-Sulfo-NHS ester or ruthenium (II) tris-bipyridine-NHS ester, the N-hydroxysulfo-succinimide ester of biotin or ruthenium combines with the ε–amide group of lysine to form a stable amide bond (Miralles et al., 1991; Deaver, 1995). These reactions have been shown to lead sometimes to loss of antigen-binding activity of the antibody. In the present study, ELISA results showed no observable loss of binding activity of biotinylated or ruthenylated antibodies, in comparison with unlabeled antibodies, with either TC83 whole virus or rE2 antigen (data not shown). This observation agrees with previously published findings (Kijek et al., 2000). However, eight of the 16 antibody pairs tested (50%) had little or no reactivity with either TC-83 virus or rE2 in the ECL assay. This could have been a reflection of the sandwich format in which two antibodies may have competed for the same epitope on the antigen or may have been sterically hindered by each other. For example, antibody pairs 1A3A9-Cab/1A3A9-Dab, rabbit-Cab/rabbit-Dab, and goat-Cab/goat-Dab produced poor ECL signals. However, antibody pair 1A4A1-Cab/1A4A1-Dab pair was an exception, exhibiting excellent ECL signals for both TC-83 whole virus and recombinant E2 protein; the reason for this apparent inconsistency remains unclear.

The ECL microbead immunoassay described in this paper requires the biotinylation of antibody. Chemical biotinylation of antibody followed by removal of unbound biotin is time-consuming, typically requiring 3–4 days to complete. Furthermore, the degree of conjugation with biotin often varies, batch-to-batch (Miralles et al., 1991). To eliminate

these problems and the need for chemical biotinylation, a genetically biotinylated recombinant antibody, MA116SBP, was tested for utility in the ECL assay. MA116SBP was found to be functional as Cab in the ECL assay, but its use resulted in a higher assay LOD (lower sensitivity) than when a chemically biotinylated Cab was used (LOD = 100 ng/ml vs 1 ng/ml). This could have been due to (i) MA116SBP being improperly folded and hence lacking spatial conformation following expression in *E. coli*, (ii) the recombinant protein containing only 11 residues of the streptavidin-binding peptide rather than the full biotin molecule, or (iii) the antibody being a single-chain variable fragment rather than the full length antibody. Function of the genetically biotinylated recombinant antibody might be improved by constructing new expression vectors containing full length biotin and antibody or by expression in a eukaryotic expression system to gain proper post translational modification and spatial conformation. Further studies would be required to address these issues.

5. Conclusions

An ECL immunoassay reactive with VEEV TC83 whole virus and the E2 glycoprotein on the VEEV virion surface has been developed for detection and identification of VEEV. Antibody pairs, in all possible combinations, were down-selected from a panel of four monoclonal and polyclonal antibody reagents for utility in the assay. The LOD of the optimized ECL assay was 10^3 pfu/ml for TC-83 whole virus and 1 ng/ml for VEEV rE2 component. The LOD of the VEEV ECL assay for both TC83 whole virus and rE2 was approximately one log unit lower (higher sensitivity) than that of a sandwich ELISA incorporating the same immunoreagents. The ECL assay was reproducible over time and when conducted by different persons. The VEEV ECL assay exhibited no cross-reactivity with two closely related alphaviruses or with 21 unrelated heterologous agents. In experiments designed to evaluate the effect of sample matrices on assay performance, several powder and soil types were shown to interfere with the assay by increasing background or quenching of ECL signal. A genetically biotinylated VEEV recombinant antibody was evaluated in the assay for utility for detection of rE2, but was found to lack reactivity when compared to incorporation in the assay of a chemically biotinylated antibody reagent. This study has demonstrated a rapid, sensitive, and specific assay for detection and identification of VEEV in environmental or clinical samples.

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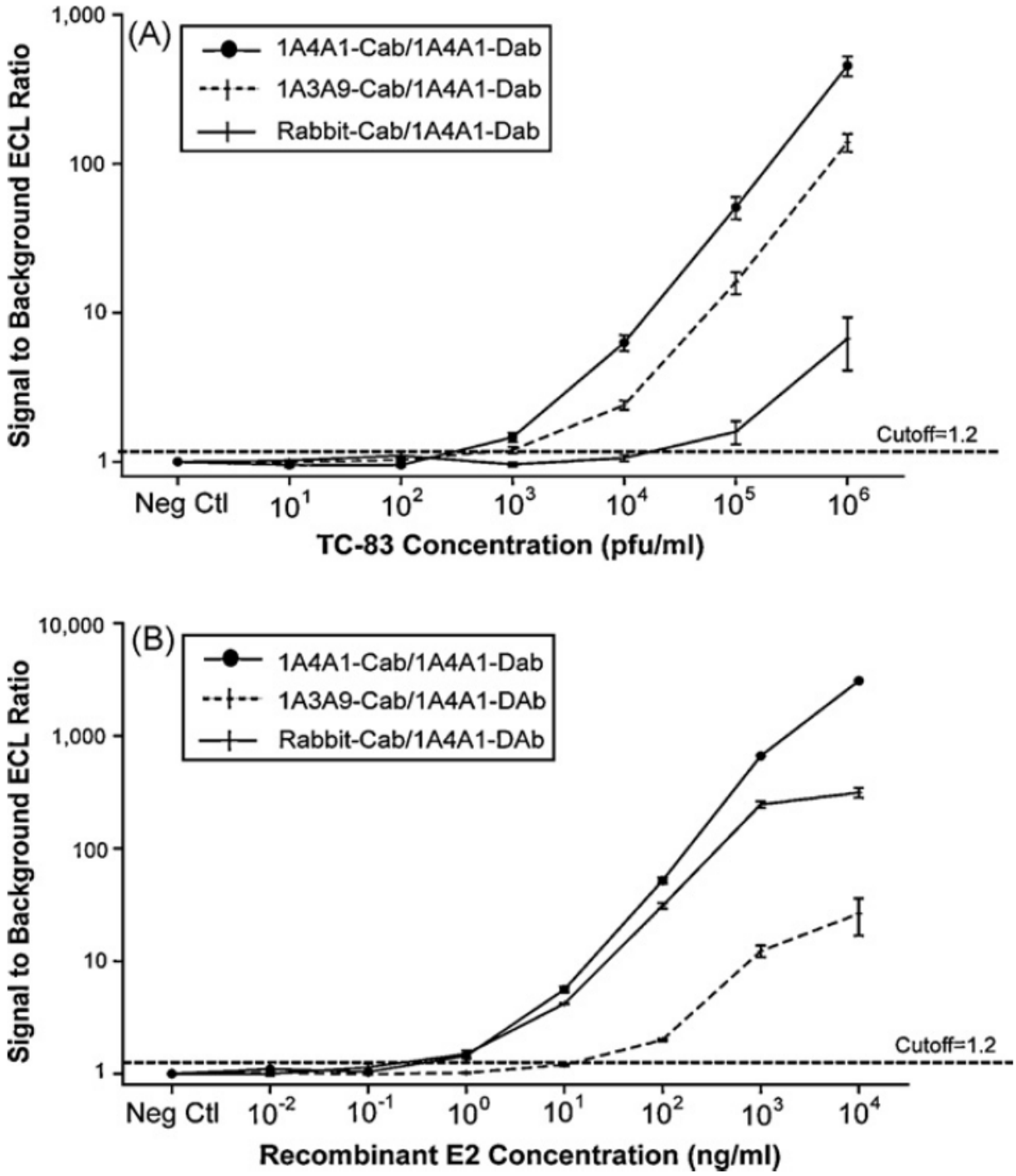

Fig. 1. ECL LOD for TC-83 virus and rE2 protein using three antibody pairs. A) Titration of 10-fold dilutions of TC-83 whole virus (10^1–10^6 pfu/ml) against three antibody pairs, 1A4A1-Cab/1A4A1-Dab, 1A3A9-Cab/1A4A1-Dab, and rabbit-

Cab/1A4A1-Dab. B) Titration of 10-fold dilutions of rE2 (0.01−10,000 ng/ml) against three antibody pairs. The concentrations of Cab and Dab for each antibody pair were as follows: 1A4A1-Cab/1A4A1-Dab pair at a Cab dilution of 1:35 and Dab concentration of 10 μg/ml, 1A3A9-Cab/1A4A1-Dab pair at Cab dilution of 1:25 and Dab concentration of 10 μg/ml, and rabbit-Cab/1A4A1-Dab pair at Cab dilution of 1:16 and Dab concentration of 4 μg/ml. The data was from three separate experiments, each with three replicates of each concentration of antigen (n=9). Error bars represent one standard deviation of the mean. Neg Ctl: PBS-0.3% Tween 20 (n=18). Signal to Background ECL Ratio: average ECL reading divided by average ECL reading of negative control.

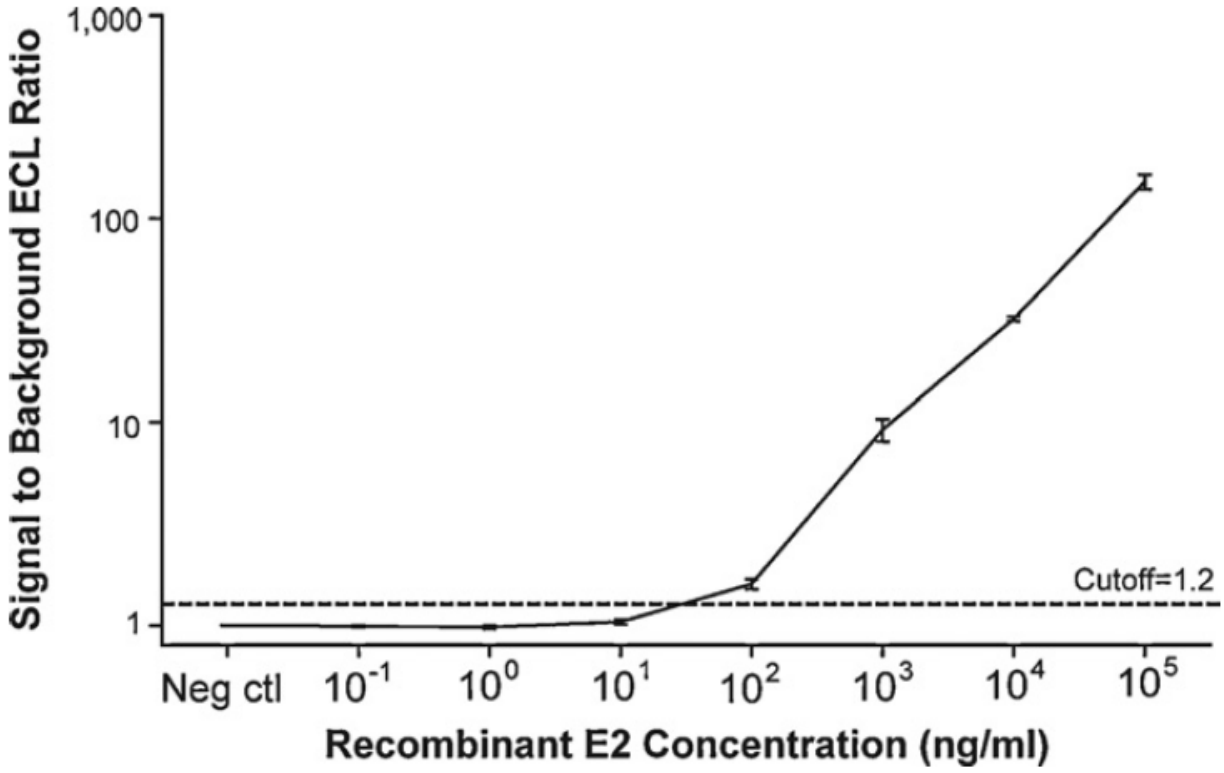

Fig. 2. ECL LOD for VEEV rE2 protein using recombinant Cab MA116SBP. Titration of 10-fold dilutions of rE2 (0.1−100,000 ng/ml) using the antibody pair MA116SBP-

Cab/1A4A1-Dab. MA116SBP-Cab dilution was at 1:8 and 1A4A1-Dab at 10 μg/ml. The data was from three separate experiments, each with three replicates of each concentration of antigen (n=9). Error bars represent one standard deviation of the mean. Neg Ctl: PBS-0.3% Tween 20 (n=18). Signal to Background ECL Ratio: average ECL reading divided by average ECL reading of negative control.

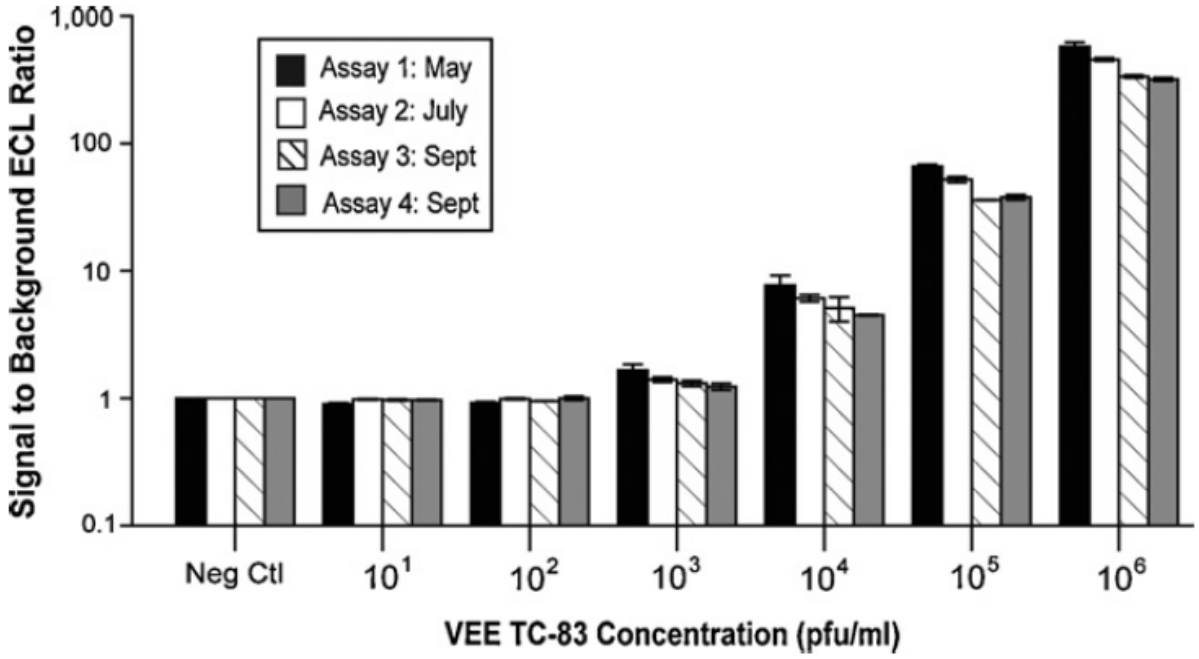

Fig. 3. Reproducibility of VEEV ECL assay from month-to-month and from person-to-person. The assays were performed with the antibody pair 1A4A1-Cab/1A4A1-Dab at a Cab dilution of 1:35 and a Dab concentration of 10 μg/ml. Assays 1, 2 and 3 represent three separate titrations of 10-fold dilutions of TC-83 whole virus (10^1–10^6 pfu/ml), performed by one person, over a period of five months, each with three replicates of each concentration of antigen (n=3 for each assay). Assay 4 represents a single titration of virus using the same antibody pair, performed in triplicate by a second person, in September 2007 (n=3). Error bars represent one standard deviation of the mean. Neg Ctl:

PBS-0.3% Tween 20 (n=3 for each assay). Signal to Background ECL Ratio: average ECL reading divided by average ECL reading of negative control.

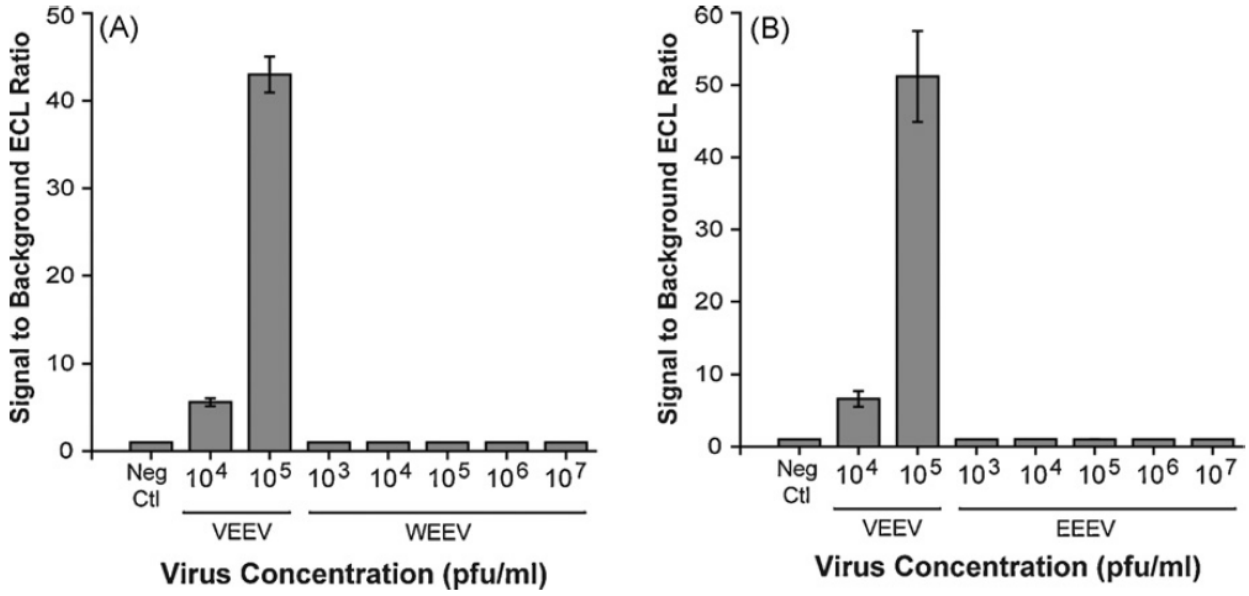

Fig. 4. Cross-reactivity assays with alphaviruses WEEV and EEEV. The assays were performed with the antibody pair 1A4A1-Cab/1A4A1-Dab at a Cab dilution of 1:35 and a Dab concentration of 10 μg/ml. The data was from two separate assays, each with three replicates of each concentration of agent (n=6). Error bars represent one standard deviation of the mean. Neg Ctl: PBS-0.3% Tween 20 (n=12). Signal to Background Ratio: average ECL reading divided by average ECL reading of negative control.

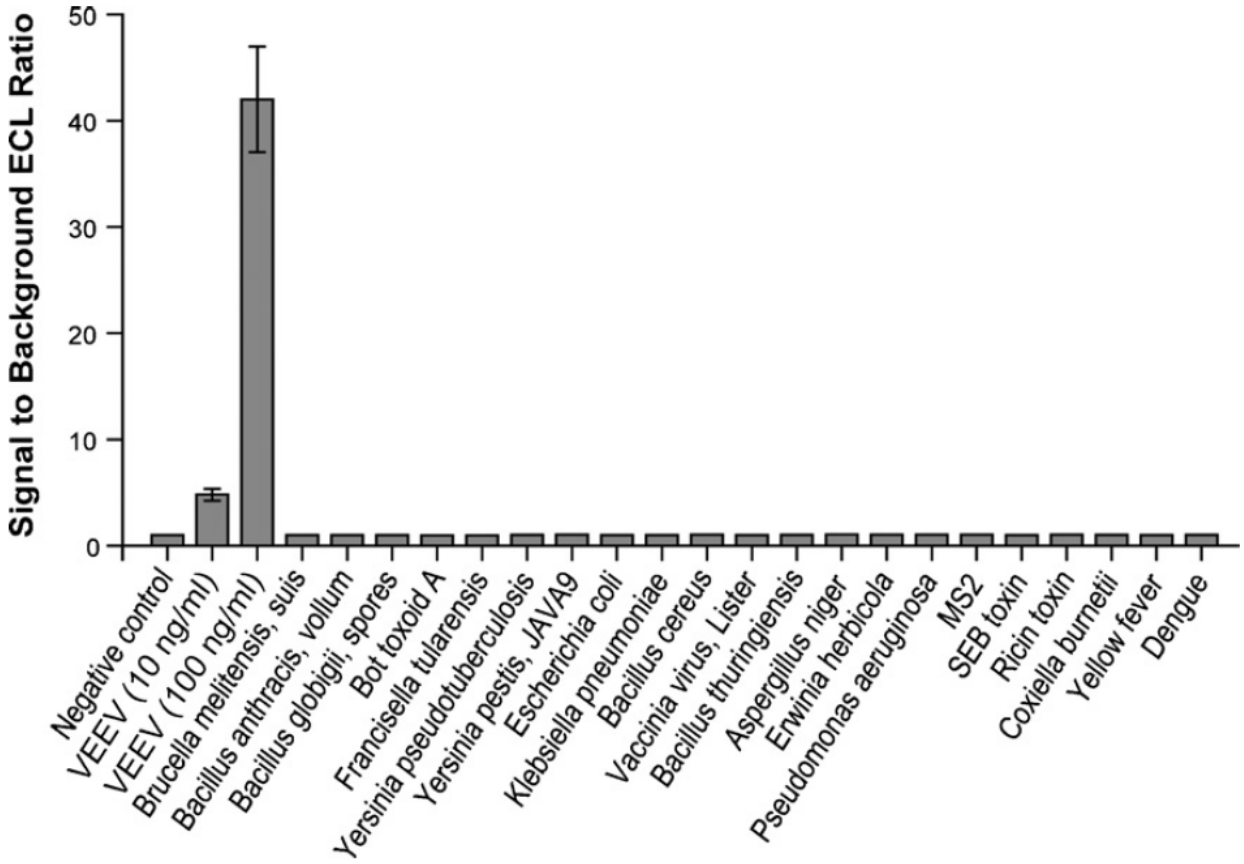

Fig. 5. Cross-reactivity assays with 21 unrelated agents. The assay was performed with the antibody pair 1A4A1-Cab/1A4A1-Dab at a Cab dilution of 1:35 and a Dab concentration of 10 μg/ml. A concentration of 1 μg/ml was used for most of the 21 agents with the exception of: Vaccinia, Lister: 10^6 pfu/ml; Aspergillus niger: 10^4 spores/ml; Staphylococcal enterotoxin B (SEB): 2 ng/ml; Ricin: 3 ng/ml; Yellow fever: 10^9 pfu/ml; Dengue: 10^4 pfu/ml. The data was from two separate assays, each with three replicates of each concentration of agent (n=6). Error bars represent one standard deviation of the mean. Negative control: PBS-0.3% Tween 20 (n=12). Signal to Background ECL Ratio: average ECL reading divided by average ECL reading of negative control.

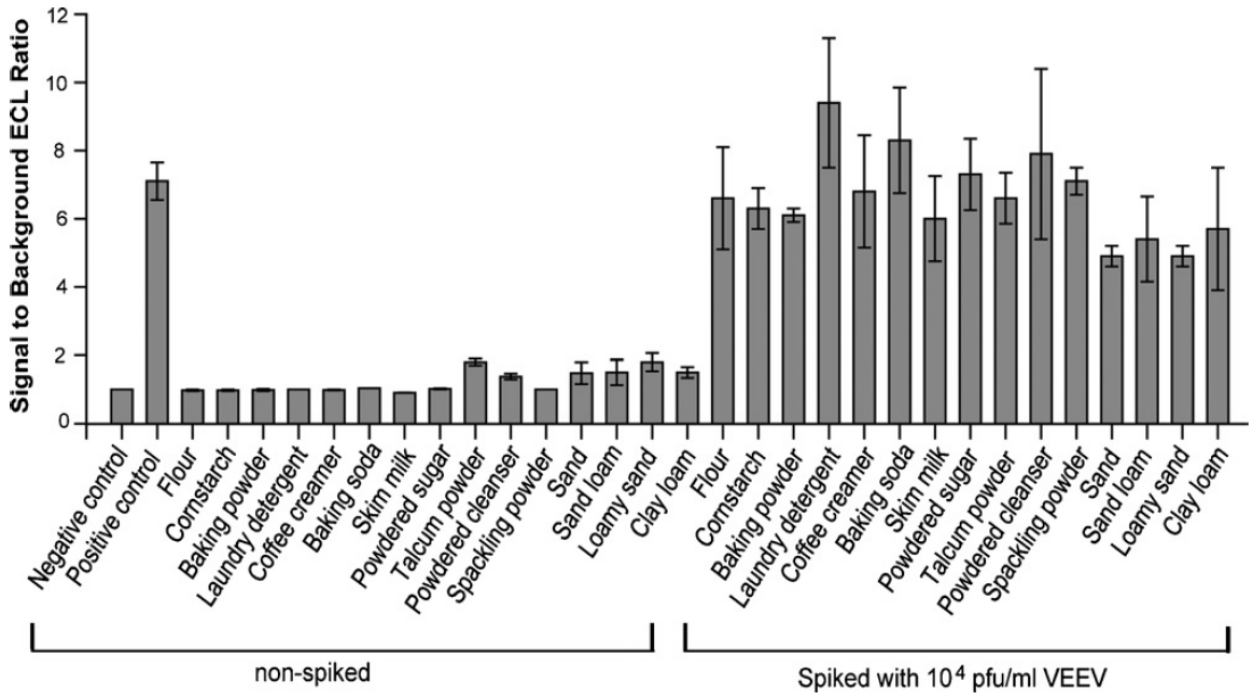

Fig. 6. Spiked and non-spiked interference assay. The assay was performed with the antibody pair 1A4A1-Cab/1A4A1-Dab at a Cab dilution of 1:35 and a Dab concentration of 10 μg/ml. The data was from two separate assays, each with two replicates of each concentration (n=4). Positive control: 10^4 pfu/ml TC-83 whole virus in PBS-0.3% Tween 20 (n=8). Error bars represent one standard deviation of the mean. Neg Ctl: PBS-0.3% Tween 20 (n=8). Signal to Background Ratio: average ECL reading divided by average ECL reading of negative control.

Table 1. LOD for VEEV TC83 and rE2 protein by ECL using three different antibody pairs[a]

1A4A1-Cab/1A4A1-Dab		1A3A9-Cab/1A4A1-Dab		Rbt-Cab/1A4A1-Dab	
ECL		ECL		ECL	
virus (pfu/ml)	rE2 (ng/ml)	virus (pfu/ml)	rE2 (ng/ml)	virus (pfu/ml)	rE2 (ng/ml)
10^2–10^3	1	10^3	10	10^4–10^5	1

[a]Data was from three separate experiments; each concentration of antigen was tested in triplicate (n=9).

Table 2. Comparison of LOD for VEEV TC83 and E2 protein by ECL and sandwich ELISA[a]

ECL		ELISA	
Virus (pfu/ml)	rE2 (ng/ml)	Virus (pfu/ml)	rE2 (ng/ml)
10^4–10^5	1	10^6	10

[a]Data was from three separate experiments; each concentration of antigen was tested in triplicate by ECL (n=9) and in duplicate by ELISA (n=6).

Table 3. Month-to-month reproducibility of VEEV ECL assay[a]

VEE (pfu/ml)	N	S/B	S.D.	CV (%)
10^1	9	0.97	0.058	6
10^2	9	1.01	1.101	9.9
10^3	9	1.87	0.247	13.2
10^4	9	9.0	1.410	15.7
10^5	9	79.3	14.64	18.5
10^6	9	608	28.45	4.7

[a]Data was from three separate experiments performed over a period of five months; each assay was performed with three replicates of each concentration of antigen (n=9). S/B: mean ECL reading of test sample divided by mean ECL reading of negative control.

Table 4. Person-to-person reproducibility of VEEV ECL assay[a]

VEE (pfu/ml)	N	S/B	S.D.	CV (%)
10^1	6	0.97	0	0
10^2	6	0.99	0.035	3.6
10^3	6	1.27	0.057	4.5
10^4	6	4.8	0.424	8.8
10^5	6	36.9	1.202	3.3
10^6	6	327	28.45	3.7

[a]Data was from two separate experiments performed by two different people; each assay was performed with three replicates of each concentration of antigen. S/B: mean ECL reading of test sample divided by mean ECL reading of negative control.