Application of an Engineered Streptavidin-Binding Single Chain Fragment Variable Antibody in Light Addressable Potentiometric Immunoassays for Identification of VEEV

Wei-Gang Hu[1], H. Gail Thompson[1], Azhar Z. Alvi[1#], Les P. Nagata[1], Mavanur R. Suresh[2], and R. Elaine Fulton[1]*

[1] Chemical and Biological Defence Section, Defence R&D Canada-Suffield, Box 4000, Station Main, Medicine Hat, Alberta, Canada T1A 8K6

[2] Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2N8

[#] Current address: SYNX Pharma Inc., 1 Marmac Drive Toronto, Ontario, Canada M9W 1E7

**Corresponding author*

Ms. R. Elaine Fulton

Chemical and Biological Defence Section

Defence R&D Canada-Suffield

Box 4000, Station Main, Medicine Hat, Alberta

Canada T1A 8K6

Tel: 403-544-4630, Fax: 403-544-3388

Email: Elaine.Fulton@drdc-rddc.gc.ca

Abbreviation

B-mAb, biotinylated mAb
BSA, bovine serum albumin
BW/BT, biological warfare / bioterrorist
DMEM, dulbecco minimum essential medium
ELISA, enzyme-linked immunosorbent assay
F-pAb, fluoresceinated pAb
HRP, horseradish peroxidase
IFA, immunofiltration enzyme assay
LAPS, light addressable potentiometric sensor
LOD, limit of detection
mAb, monoclonal antibody
MCR, molar coupling ratio
MIR, molar incorporation ratio
NaCl, sodium chloride
NaH_2PO_4, Sodium dihydrogen phosphate
pAb, polyclonal antibody
PBS, phosphate buffered saline
PBST, PBS containing 0.1% tween-20
scFv, single chain fragment variable antibody
S/N, signal to noise
VEE, Venezuelan equine encephalitis virus
WEE, western equine encephalitis virus

Abstract

A genetically biotinylated single chain fragment variable antibody (scFv) against Venezuelan equine encephalitis virus (VEE) was applied in a system consisting of an immunofiltration enzyme assay (IFA) with a light addressable potentiometric sensor (LAPS) for the rapid identification of VEE. The IFA involved formation of an immunocomplex sandwich consisting of VEE, biotinylated antibody, fluoresceinated antibody and streptavidin, capture of the sandwich by filtration on biotinylated membrane, and labeling of the sandwich by anti-fluorescein urease conjugate. The concentration ratio of biotinylated to fluoresceinated antibodies was investigated and optimized. By the IFA/LAPS assay, the limit of detection of VEE was approximately 30 ng/ml, similar to that achieved when chemically biotinylated monoclonal antibody was applied. Total assay variance of the IFA/LAPS assay for both intra- and inter-assay precision was less than 20%. Assay accuracy was measured by comparing VEE concentrations estimated by IFA/LAPS standard curve to those obtained by conventional protein assay. VEE concentrations were found to differ by no more thatn 10%. The IFA/LAPS assay sensitivity was approximately equal to that of a conventional enzyme-linked immunosorbant assay utilizing polystyrene plates and a chromogenic substrate, however, less time and effort were required for performance of the IFA/LAPS assay. More importantly, use of genetically biotinylated scFv in the IFA/LAPS assay obviates the need for chemical biotinylation of antibody with resultant possible impairment of the antigen-binding site. Furthermore, the potential for batch-to-batch variability resulting from inequality in the number of biotin molecules labeled per antibody molecule, is eliminated.

Keywords: Identification; Venezuelan equine encephalitis virus; Genetically biotinylated recombinant antibody; Immunofiltration assay; Light addressable potentiometric sensor

1. Introduction

Venezuelan equine encephalitis virus (VEE), belonging to alphavirus genus of the family *Togaviridae*, is associated with encephalitis (Johnson et al., 1968; Franck and Johnson, 1970). Although natural infection with this virus results from mosquito bites, the virus is also highly infectious by aerosol (Johnston and Peters, 1996). Thus, VEE is a potential biological warfare / bioterrorist (BW/BT) agent of concern. The use of biological agents as BW/BT weapons is not a new concept, and alleged uses of biological agents as military or terrorist weapons can be found throughout history (Christopher, et al., 1997). The most recent case of use of the biological agent, anthrax, as a BT agent occurred in USA last year (Blendon, et al., 2002). Identification of biological agents involves either finding the agent in the environment or medical diagnosis of the agent and/or its effect(s) on human or animal victims. Early and rapid detection and identification of a biological agent is crucial for immediate and specific treatment of affected individuals and in order to limit the epidemic spread of associated disease.

Antibodies are critical reagents used in several biodetection platforms for the identification of biological agents (Libby and Wada, 1989; Lee, et al., 1993; Gehring, et al., 1998; Lee, et al., 2000; Carlson, et al., 2002; Emanuel, et al., 2000). Monoclonal antibody (mAb) 1A1A4 is specific for the envelope glycoprotein of VEE. This mAb has been shown to be highly reactive to VEE as well being able to neutralize the virus (Roehrig and Mathews, 1985). An anti-VEE mA116 single chain variable fragment antibody (scFv) was cloned from mAb 1A4A1 (Alvi et al., 2003). This scFv showed an affinity to VEE comparable to that of the parental mAb 1A4A1 and therefore is a good candidate as immunodiagnostic reagent.

The identification system employed in this study utilizes a flow-through immunofiltration-enzyme assay (IFA) in conjunction with a light addressable potentiometric sensor (LAPS) and has been previously described for identification of biological agents (Libby and Wada, 1989; Lee, et al., 1993; Gehring, et al., 1998; Lee, et

al., 2000). The IFA/LAPS system described to date utilized biotin-streptavidin-mediated capture filtration of immunocomplexes in which one of the analyte-specific antibodies required chemical biotinylation. The process of chemical biotyinylation is time-consuming and commonly associated with impairment of the antibody antigen-binding site on the antibody (Miralles, et al., 1991). To eliminate the need for chemical biotinylation, we genetically fused a gene encoding a streptavidin-binding peptide to an anti-VEE scFv gene (Hu, et al., 2002). The resulting recombinant fusion antibody not only retained VEE antigen-binding specificity similar to that of the parental mAb, but also possessed streptavidin-binding activity (Hu, et al., 2002). In the present study, we have demonstrated the feasibility of applying the genetically biotinylated scFv in the IFA/LAPS system for rapid identification of VEE. We have compared the sensitivity of the IFA/LAPS assay for identification of VEE, when genetically biotinylated scFv and the parental mAb, biotinylated chemically, were used. Finally, we have investigated the precision, accuracy, and specificity of the IFA/LAPS assay incorporating genetically biotinylated scFv for rapid identification of VEE. The assay has been proven to be highly sensitive, specific, and reproducible.

2. Materials and Methods

2.1. Reagents and solutions

Sodium dihydrogen phosphate (NaH_2PO_4) and Triton X-100 were obtained from BDH Chemicals (Toronto, ON). Bovine serum albumin (BSA), sodium chloride (NaCl), sodium hydroxide, Tween-20 and urea were obtained form Sigma-Aldrich Canada Ltd (Oakville, ON). Streptavidin was obtained from Scripps Laboratories (San Diego, CA). Biotinylated BSA-block nitrocellulose membrane sticks and antibody labeling reagents (*N*-hydroxysuccinimide esters of carboxyfluorescein and dinitrophenyl biotin) were purchased from Molecular Devices Corp (Menlo Park, CA). Anhydrous dimethylformamide (DMF) was purchased from Biolynx Inc (Brockville, ON). Sephadex G-25 columns were obtained from Amersham Pharmacia (Baie d' Urfe', QC). Washing buffer consisted of 10 mM NaH_2PO_4 (pH 6.5), 150 mM NaCl, and 0.05% Tween-20. Assay buffer consisted of 10 mM NaH_2PO_4 (pH 7.0), 150 mM NaCl, 0.025% Triton X-100, and 0.1% BSA. The substrate solution was 100 mM urea in the wash buffer (pH 6.5).

2.2. Viruses

VEE strain TC-83 and Western equine encephalitis virus (WEE) strain B11 were prepared as follows. Viruses were cultured in Vero monkey kidney cells or in baby hamster kidney cells (America Type Culture Collection, Rockville, MD). Cells were maintained in complete Dulbecco Minimum Essential Medium (DMEM) (Invitrogen Canada Inc., Burlington, ON) containing 5% fetal calf serum. When cells were confluent, the medium was decanted and replaced by a volume of virus inoculum, in DMEM, sufficient to just cover the monolayer. The virus inoculum was spread over the monolayer every 15 min and was allowed to adsorb to the cells for a total of 1 hr. Fresh complete medium was added and the flasks were incubated for several days to one week. When 90% cytopathic effect was reached, cells and medium were harvested and centrifuged at 10,000g for 30 min. To the pooled supernatants, polyethylene glycol

(~6,000 MW) (7 % final concentration) and NaCl (2.3% final concentration) were added and the suspensions were allowed to incubate with stirring for 15-20 hr at 4 °C. The mixtures were then centrifuged at 10,000g for 30 min and the resulting supernatants discarded. The virus pellet was resuspended in a small volume of phosphate buffered saline (PBS) and stored at -70 °C to await purification. The virus particles were purified by sucrose density gradient centrifugation by standard techniques. Samples were centrifuged at 100,000g at 4 °C for 3.5 h. Fractions containing virus were pooled and the pooled virus was dialyzed against PBS. Protein concentration of the purified virus was determined by protein assay and virus purity was assessed by polyacrylamide gel electrophoresis. WEE was cultured and purified in Defence R&D-Suffield BL-3 laboratory following Health Canada and Canadian Food Inspection Agency guideline. It was inactivated with β propiolactone and safety tested prior to removal to BL-2 laboratory.

2.3. Antibodies

Genetically biotinylated scFv to VEE was constructed from mAb 1A4A1 to VEE, expressed in bacteria and purified previously (Hu, et al., 2002). The hybridoma cell line producing mAb 1A4A1 was kindly provided by Dr. J.T. Roehrig (Division of Vector-borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Colins, CO). MAb 1A4A1 was purified from vectraCell™ bioreactor (BioVectra™dcl**,** Charlottetown, Prince Edward Island) cultures using protein G affinity chromatography (Biolynx Inc.). Polyclonal antibody (pAb) to VEE was purified from rabbit serum using protein G affinity chromatography. Anti-fluorescein urease conjugate was purchased from Molecular Devices Corp.

2.4. Instrumentation

The LAPS used in this study was the Threshold™ Unit, marketed by Molecular Devices Corp. The instrument was controlled by a computer and Threshold™ version 2 software. Reactions took place on membrane sticks (pore size 0.45 micron) (Molecular

Devices Corp.) and the Threshold™ Unit could process a maximum of four membrane sticks simultaneously (eight reaction test spots per stick).

2.5. Preparation of biotinylated mAb and fluoresceinated pAb

Biotinylated mAb (B-mAb) and fluoresceinated pAb (F-pAb) were prepared according to procedures outlined in the Threshold™ manual. Briefly, antibodies were adjusted to 1 mg/ml in PBS. MAb 1A4A1 was biotinylated chemically via *N*-hydroxysuccinimide ester of dinitrophenyl biotin (reconstituted in DMF) for 2 hr at room temperature; the molar coupling ratio (MCR) of biotin hapten to antibody was 10:1. The pAb was fluoresceinated via *N*-hydroxysuccinimide ester of carboxyfluorescein (reconstituted in DMF) for 2 hr at room temperature; the MCR of fluorescein hapten to antibody was 20:1. Unreacted haptens were removed using PD-10 columns (Amersham Pharmacia Biotech) equilibrated in PBS. The number of moles of hapten covalently bound per mole of antibody was determined for each conjugate using the molar incorporation ratio (MIR) described in the Threshold™ manual. The MIRs of B-mAb and F-pAb were 3.8 and 4.0, respectively. The labeled antibodies were stored at 4 °C until used.

2.6. Assay procedure

A volume of 100 µl of VEE (0-20 µg/ml) was incubated with 100 µl of genetically biotinylated scFv (B-scFv) or B-mAb (1-5 µg/ml) and 100 µl of F-pAb (1-5 µg/ml) at room temperature for 30 min. At the end of the incubation period, 1,000 µl (0.6 µg/ml in assay buffer) streptavidin was added to the reaction mixture and mixed thoroughly. The resulting mixtures were filtrated through the membrane sticks (mounted in filter bases) on the Threshold™ Unit under low vacuum (complete filtration in ~10 min). The sticks were washed with 2 ml wash buffer while filtering under high vacuum (complete filtration in ~8 min). One ml of anti-fluorescein urease conjugate was then added to the membrane sticks and filtrated under low vacuum (complete filtration in ~10 min), followed by the addition of 2 ml of wash buffer and filtration under high vacuum (complete filtration in ~8 min). The membrane sticks were removed from the filtration compartment, and inserted into the reader chamber containing the LAPS and the substrate

solution, urea. The pH change with respect to time at the surface of the sensor was monitored as the rate of change of the surface potential with respect to time in μV/sec (completion in ~2 min). The rate of pH change depends on the number of urease-containing antibody-antigen sandwiches immobilized on the membrane stick during the filtration capture process. The time for whole assay was less than 90 min. The IFA/LAPS scheme for the Threshold™ unit is depicted in Fig. 1.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The antigen-binding activity of B-scFv to VEE antigen was determined by an ELISA. Nunc maxisorp™ flat-bottomed 96-well plates (Life Technologies, Burlington, ON) were coated overnight at 4 °C with whole VEE (strain TC-83) at various concentrations of 0.2-60 μg/ml, in carbonate bicarbonate buffer, pH 9.6, containing 0.02% sodium azide. The plates were washed five times with PBS containing 0.1% tween-20 (PBST) and then blocked twice in 2% BSA for 1 hr at 37 °C. After five washes with PBST, plates were incubated for 1 hr at 37°C with a fixed concentration of 10 μg/ml of B-scFv, diluted in PBST. Following five washes with PBST, plates were incubated for 1 hr at 37°C with 1:1,000 dilution of horseradish peroxidase (HRP)-conjugated streptavidin in PBST. Finally, the plates were washed five times with PBST and developed for 30 min at room temperature with a substrate consisting of 2,2'-azino-di-(3-ethyl-benzthiazoline-sulfonic acid) diammonium salt and hydrogen peroxidate (Kirkegaard and Perry Laboratories, Gathersburg, MD). The reactions were read at an absorbance of 405 nm by a microplate autoreader (Molecular Devices).

2.8 Protein assay

Virus protein concentrations were estimated spectrophotometrically with a micro BCA protein assay kit purchased from Biolynx Inc.

3. Results

3.1. Optimal ratio between B-scFv or B-mAb and F-Ab for detection of VEE

A preliminary assay was first performed using a range of VEE concentrations (10 to 2,000 ng/spot) and antibody concentrations (ng/spot) in the following B-scFv:F-pAb ratios: 200:200, 200:100, 100:200, and 100:100. From the preliminary result, a VEE concentration of 500 ng/spot, providing a mid-range LAPS signal was chosen for further experiments. A series of IFA/LAPS experiments was performed to investigate the effect of the ratios of B-scFv and F-pAb on the output response of the assay. The aspect of the response of interest was the signal to noise ratio (S/N). S represented the output of LAPS from VEE. N was the background of the assay, that was, the output of the LAPS for the reagents without VEE. The S/Ns obtained using varying B-scFv/F-pAb concentratiosns (ng/spot) with a constant concentration of VEE antigen (500 ng/spot) were determined (Table 1). The B-scFv/F-pAb ratio giving the highest S/N was found to be 250:500 (ng/spot). Similar experiments were performed to determine the optimal concentration ratios of B-mAb and F-pAb. The B-mAb to F-pAb concentration ratio giving the highest S/N with fixed concentration of VEE was, similarly, found to be 250:500 (ng/spot) (Table 1).

3.2. Standard curve and sensitivity

To prepare ILA/LAPS standard antigen concentration curves, two-fold serial dilutions of VEE ranging from 125 to 2,000 ng/spot (1.25 to 20μg/ml) in assay buffer were titrated using optimal ratios of B-scFv or B-mAb and F-pAb (250:500 ng/spot) (Fig.2).

Assay sensitivity, or limit of detection (LOD), was defined as the lowest concentration of VEE producing an output signal greater than the background plus three standard deviations of the background, i.e., the lowest concentration that could be measured accurately and precisely. The LOD of the IFA/LAPS incorporating B-scfv for detection of VEE was 0.29 μg/ml, while that incorporating B-mAb was 0.34 μg/ml.

An ELISA incorporating B-scFv and HRP-conjugated streptavidin was conducted in order to compare its sensitivity of detection of VEE with ILA/LAPS system incorporating B-scFv. The LOD of the ELISA was about 0.25 μg/ml (Fig. 3).

3.3. Assay precision

Intra- and inter-assay variabilities were evaluated by testing three different VEE concentration samples (20, 5, and 1.25 μg/ml) in six assays performed together on the same day and on 6 different days, respectively. Intra- and inter-assay coefficients of variation for the precision were determined by the ratios of standard deviations and means from six assays. They ranged from 15 to19% and 18%, respectively (Tables 2 and 3).

3.4. Assay accuracy

The ability of IFA/LAP system with B-scFv to quantitate VEE was performed. For five different test sample concentrations of VEE in the range of 1.25-20 μg/ml, the mean difference between the measured and the actual amounts was about 10%. A linear regression analysis yielded a high correlation (r^2=0.967) between the measured and actual amounts (Fig.4).

3.5. Assay specificity

The specificity of the IFA/LAPS assay incorporating B-scfv was evaluated by challenging the assay with one other member of alphavirus genus of the family *Togaviridae*, WEE. The results showed no cross-reaction in the assay between VEE and WEE (Fig.5).

4. Discussion

A genetically biotinylated scFv antibody against VEE was applied in a commercially available assay system employing IFA/LAPS for rapid detection of VEE. This study was performed to investigate the feasibility of replacing a murine mAb, biotinylated chemically, with a scFv antibody, biotinylated genetically, as immunodiagnostic reagent in the IFA/LAPS system to detect VEE.

The disadvantages of chemically biotinylating mAbs as immunodiagnostic reagents are numerous. The cost and time required for growth and maintenance of hybridoma cell lines and production and purification of mAbs, and the potential for the occurrence of genetic mutation during repeated cycles of cell growth, make routine production of mAbs from hybridoma cell lines difficult, expensive, and time-consuming. Chemical biotinylation of antibodies is also time-consuming. Most biotins bind to amino groups on proteins and the degree of labeling tends to differ batch-to-batch. The possibility also exists that the biological activity of the antibody may be affected by the labeling procedure (Miralles, et al., 1991). ScFv antibodies are comprised of variable regions of heavy and light chains of immunoglobulin, covalently connected by a peptide linker (Huston, et al., 1988). These small proteins generally retain the specificity and affinity for antigen similar to their parental mAbs and possibly bind to poorly accessible epitopes more efficiently due to their small size (Marin, et al., 1995; Bruyns, et al., 1996). The attractiveness of scFv antibodies is that they can be produced economically and stably in bacteria and can be manipulated via genetic engineering for different purposes, such as biotinylation (Luo, et al., 1998).

Before comparing the sensitivity of assays incorporating B-scFv and B-mAb in the IFA/LAPS system for detection of VEE, optimization of the concentration ratios of B-scFv or B-mAb and F-pAb was investigated. These ratios are influenced by the type and affinity of the antibodies and are important aspects of the assay. Optimizations of assay conditions such as buffer formulation, temperature, incubation time, etc., were not undertaken as these had been previously determined and were available in the

Threshold™ manual. It was found that the optimal concentration ratios of both B-scFv and B-mAb and F-pAb were 250:500 (ng/spot). By using these concentration ratios, standard curves relating IFA/LAPS signal and VEE concentration were constructed. Although signals in the IFA/LAPS assay incorporating B-mAb were two-fold higher than in assay incorporating B-scFv, the background noise in assay using B-mAb was twice as high as in assay using B-scFv, resulting in similar LODs whether B-scFv or B-mAb were used. The LOD with B-scFv was 0.29 μg/ml, while that with B-mAb was 0.34 μg/ml. These results indicated that genetically biotinylated scFv could replace chemically biotinylated mAb in the IFA/LAPS system. ELISA incorporating B-scFv and HRP-conjugated streptavidin showed LOD of the ELISA was about 0.25 μg/ml, comparable to that obtained in the ILA/LAPS system. Therefore, the sensitivity of ILA/LAPS with B-scFv is comparable to that of ELISA. However, the total time required and effort expended in this assay were significantly reduced.

To further evaluate the IFA/LAPS system incorporating B-scFv, the precision, accuracy, and specificity of the assay were investigated. A standard curve relating protein concentration to IFA/LAPS signal was used to accurately access the concentration of VEE. Protein concentration, whether estimated by IFA/LAPS standard curve or by protein assay differed by no more than 10%. Intra-assay and inter-assay precision varied by less than 20%. In addition, the specificity of the assay was confirmed by showing no cross-reactivity with WEE, a virus with a genetic background and a disease spectrum very similar to that of VEE.

In conclusion, the IFA/LAPS incorporating B-scFv was shown to be reliable, reproducible, and sensitive for use in rapid identification of VEE. More importantly, use of this assay obviates the need for chemical biotinylation of antibody, with resultant potential impairment of the antibody antigen-binding site and differences in degree of labeling, from batch-to-batch.

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Table 1

Effects of concentration ratios of B-scFv or B-mAb and F-pAb on S/Ns of IFA/LAPS assays to detect VEE

B-scFv:F-pAb (ng/spot)	S/N (μV/s)	B-mAb:F-pAb (ng/spot)	S/N (μV/s)
125:500	5.63	125:500	6.37
250:500	8.78	250:500	7.60
500:500	4.80	500:500	3.18
125:250	5.82	125:250	6.11
250:250	6.50	250:250	5.22
500:250	4.51	500:250	4.86
125:125	3.43	125:125	3.89
250:125	5.14	250:125	5.02
500:125	4.70	500:125	3.38

Samples were tested in replicates of four.

Table 2

Intra-assay precision of ILA/LAPS assay incorporating B-scFv to detect VEE

VEE (μg/ml)	N	Mean (μV/sec)	Standard deviation (μV/sec)	Coefficient of Variation (%)
20	6	2156	309	15
5	6	844	160	19
1.25	6	243	38	16

The intra-assay precision was determined from the results of 6 replicates performed together on the same day for each VEE concentration.

Table 3

Inter-assay precision of ILA/LAPS assay incorporating B-scFv to detect VEE

VEE (μg/ml)	N	Mean (μV/sec)	Standard deviation (μV/sec)	Coefficient of Variation (%)
20	6	2076	370	18
5	6	918	175	18
1.25	6	240	43	18

The inter-assay precision was determined from the results of 6 assays performed on different days for each VEE concentration.

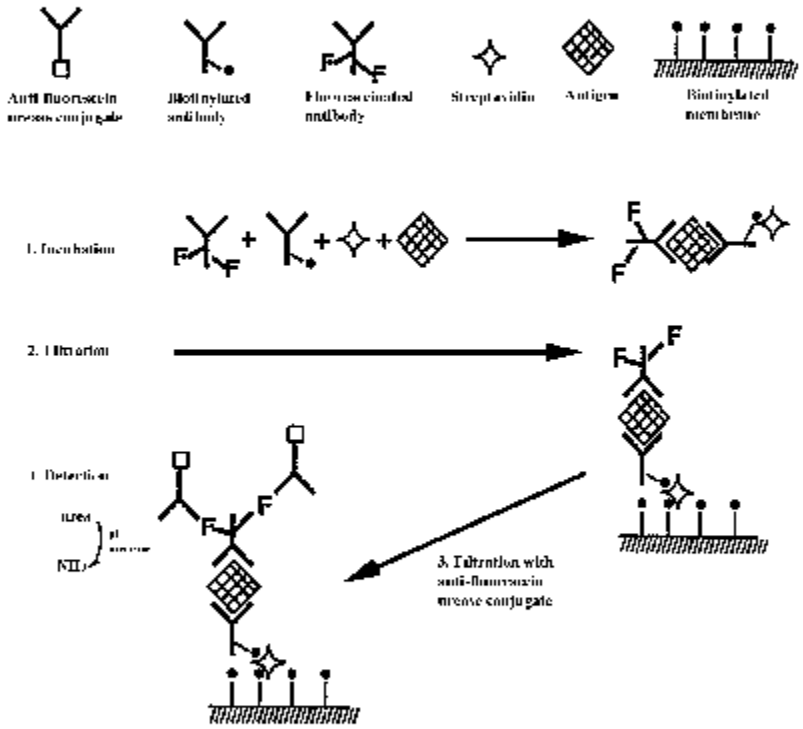

Fig.1

Fig.1. Schematic representation of IFA/LAPS system.

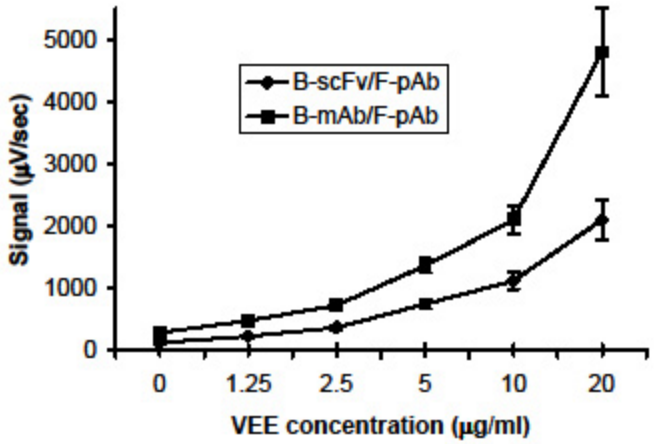

Fig.2

Fig.2. Standard curves for IFA/LAPS assay incorporating B-scFv and B-mAb in detection of VEE.

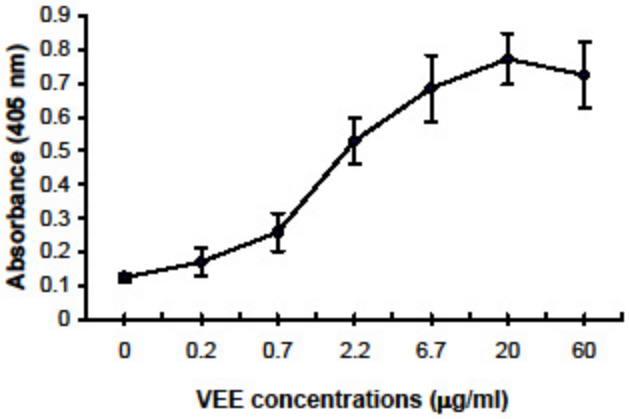

Fig. 3

Fig. 3. VEE antigen binding assay by ELISA incorporating B-scFv and HRP-conjugated streptavidin.

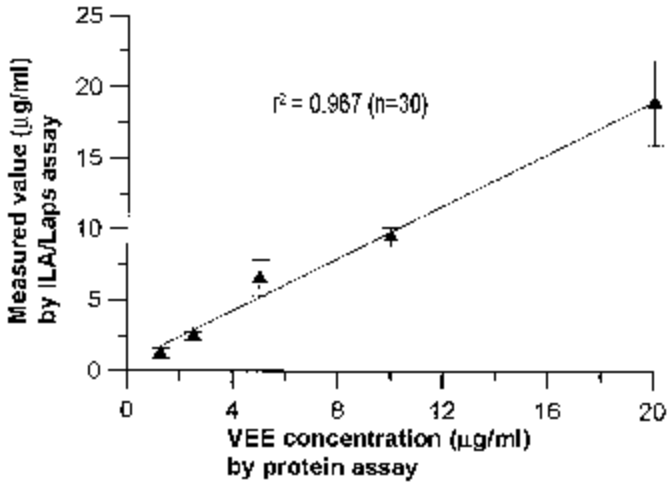

Fig.4

Fig. 4. Quantitation of VEE by IFA/LAPS with B-scFv. Each point represents the mean (n=6) of an independent determination of VEE concentration.

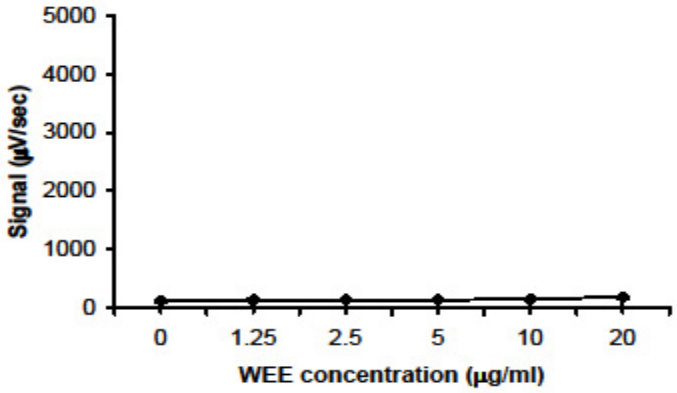

Fig.5

Fig.5. Evaluation of the specificity of IFA/LAPS assay incorporating B-scFv by challenging the assay with WEE.