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Light Addressable Potentiometric Immunoassays for Identification of Biological Agents: NATO SIBCA Exercise I

H.G. Thompson, R.E. Fulton, G.R. Fisher and L.L. Stadnyk
Defence Research Establishment Suffield

Defence R&D Canada

Technical Report

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Abstract

In March 1999, the NATO Subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) conducted the first international training exercise on identification of biological agents. Eleven NATO national laboratories participated: Canada, France, Germany (two laboratories), Hungary, Italy, Netherlands, Norway, Poland, United Kingdom, and United States. Participating laboratories were sent five sample unknowns, four of which contained biological agents and one blank containing buffer only. Participants were advised that biological agents could consist of any one of the following 10 cobalt-irradiated organisms: *Bacillus anthracis*, *Yersinia pestis*, *Vibrio cholerae*, Venezuelan equine encephalitis virus (VEE), *Francisella tularensis*, *Brucella melitensis*, *Burkholderia mallei*, yellow fever virus, vaccinia virus, or *Coxiella burnetii*. The participating laboratory for Canada was the Defence Research Establishment Suffield (DRES). For the training exercise, DRES screened sample unknowns by two different antibody-based identification technologies, one of which was the Threshold™ assay. Threshold™ assays for seven of the 10 possible biological agents were developed: *Bacillus anthracis, Brucella melitensis, Francisella tularensis, Burkholderia mallei, Yersinia pestis,* VEE, and vaccinia virus. Two agents, *Bacillus anthracis* and *Yersinia pestis* were identified while the SIBCA sample containing VEE yielded a false negative. This latter result is consistent with results of the other NATO countries participating in the training exercise.

Résumé

En mars 1999, le sous-groupe de l'OTAN Échantillonnage et identification des agents biologiques et chimiques (SIBCA) a conduit son premier exercice international de formation d'identification d'agents biologiques. Onze laboratoires représentant dix nations: le Canada, la France, l'Allemagne (2 laboratoires), la Hongrie, l'Italie, les Pays-Bas, la Norvège, la Pologne, la Grande-Bretagne et les États-Unis ont participé à cet exercice international de formation. Chaque laboratoire a reçu cinq échantillons «inconnus », quatre d'entre eux contenaient des agents biologiques et un blanc contenant seulement un tampon. Les participants ont été informés que les échantillons pouvaient contenir l'un des dix agents désactivés ^{60}Co: *Bacillus anthracis*, *Yersinia pestis*, *Vibrio cholerae*, le virus de l'encéphalite équine vénézuélienne (VEE), *Francisella tularensis*, *Brucella melitensis*, *Burkholderia mallei*, le virus de la fièvre jaune, le virus de la vaccine ou *Coxiella burnetii.* Les deux agents du *Bacillus anthracis* et de la *Yersinia pestis* ont été correctement identifiés alors que les échantillons SIBCA contenant le VEE ont produit un faux négatif. Ce dernier résultat correspond aux résultats provenant des autres pays de l'OTAN qui participaient à cet exercice de formation.

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Executive summary

Introduction

NATO Forces may be required to carry out military or peace keeping operations in areas of the world where there is a threat of attack with biological agents or where the occurrence of biological attack is suspected or confirmed. Under such circumstances, NATO Forces would be expected to take samples of materials suspected of containing biological agents and to forward these samples to respective national laboratories, where procedures would be carried out to identify the agent unknowns. In order to assess national capabilities for identification of biological agents in samples, the NATO Subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) organized an international training exercise in which participating nations were requested to identify, within a given time period, agents in sample unknowns.

The first SIBCA training exercise for biological agents i.e., SIBCA exercise I, was held in March 1999. Eleven national laboratories participated in the exercise: Canada, France, Germany (two laboratories), Hungary, Italy, Netherlands, Norway, Poland, United Kingdom, and United States. Participant nations were advised that biological agents could consist of any one of the following 10 cobalt-irradiated organisms: *Bacillus anthracis*, *Yersinia pestis*, *Vibrio cholerae*, Venezuelan equine encephalitis virus (VEE), *Francisella tularensis*, *Brucella melitensis*, *Burkholderia mallei*, yellow fever virus, vaccinia virus, or *Coxiella burnetii*. The participating laboratory for Canada was the Defence Research Establishment Suffield (DRES). DRES screened sample unknowns by two different antibody-based identification technologies, one of which was the Threshold™ assay. This report describes the results obtained on screening of the SIBCA trial I samples by Threshold™ assay.

Results

Threshold™ assays for *Bacillus anthracis, Brucella melitensis, Francisella tularensis, Burkholderia mallei, Yersinia pestis,* VEE, and vaccinia virus were assessed for optimal performance prior to the training exercise. Limits of detection were determined for *Brucella melitensis, Francisella tularensis* and *Burkholderia mallei* assays.

Comparison of results obtained by Threshold™ assay with the known content of the agents in exercise samples indicated that two agents, *Bacillus anthracis* and *Yersinia pestis* were identified while the SIBCA sample containing VEE yielded a false negative. This latter result is consistent with results of the other NATO countries participating in the training exercise.

Significance of results

Results demonstrated that Threshold™ technology is a sensitive assay tool for identification of biological agents of concern to the military community. DRES participation in NATO SIBCA exercises provides a measure for evaluation of in-house capabilities for identification of biological agents in sample unknowns.

Future goals

DRES will continue to develop assays for the Threshold™ to complete the identification capability of the Threshold™ for agents of concern to Canadian Forces (CF). In addition, samples in matrices other than liquid will be studied including those in various types of soil and on vegetative material. The effect of battlefield interferents will also be investigated.

Thompson, H.G., Fulton, R.E., Fisher, G.R. and Stadnyk, L.L. 2001. Light Addressable Potentiometric Immunoassays for Identification of Biological Agents: NATO SIBCA Exercise I. DRES TR 2001-035. Defence Research Establishment Suffield.

Sommaire

Introduction

Les Forces canadiennes (FC) peuvent être appelées à exécuter des opérations militaires ou de maintien de la paix dans les régions du monde où il existe une menace sérieuse d'utilisation d'agents biologiques ou quand l'existence d'une attaque biologique a été confirmée ou est suspectée. Dans de telles circonstances, on s'attend à ce que les Forces de l'OTAN prennent des échantillons suspectés de contenir des agents biologiques et de référer ces échantillons aux laboratoires nationaux respectifs où ils seront soumis aux procédures d'identification des agents inconnus. Pour évaluer la capacité des laboratoires de l'OTAN à identifier les agents biologiques à partir d'échantillons, le sous-groupe du panel VII de l'OTAN Échantillonnage et identification des agents biologiques et chimiques (SIBCA) a parrainé un certain nombre d'exercices de formation durant lesquels chaque laboratoire participant a dû identifier des agents à partir d'échantillons inconnus, durant une période de temps limitée.

Les premiers exercices de formation SIBCA pour les agents biologique c à d. les exercices SIBCA I, se sont tenus en mars 1999. Onze laboratoires représentant dix nations: le Canada, la France, l'Allemagne (2 laboratoires), la Hongrie, l'Italie, les Pays-Bas, la Norvège, la Pologne, la Grande-Bretagne et les États-Unis ont participé à cet exercice international de formation. Chaque laboratoire a reçu cinq échantillons «inconnus », quatre d'entre eux contenaient des agents biologiques et un blanc contenant seulement un tampon. Les participants ont été informés que les échantillons pouvaient contenir l'un des dix agents désactivés ^{60}Co: *Bacillus anthracis*, *Yersinia pestis*, *Vibrio cholerae*, le virus de l'encéphalite équine vénézuélienne (VEE), *Francisella tularensis*, *Brucella melitensis*, *Burkholderia mallei*, le virus de la fièvre jaune, le virus de la vaccine ou *Coxiella burnetii.* Le laboratoire désigné pour représenter le Canada était le Centre de recherches pour la défense, Suffield (CRDS). Le CRDS a criblé des échantillons inconnus en utilisant deux technologies d'identification à base d'anticorps dont le bio-test Threshold™. Les résultats obtenus par les bio-tests Threshold™ de criblage des échantillons SIBCA Essai I sont décrits dans ce rapport.

Résultats

L'efficacité optimale des bio-tests Threshold™ a été évaluée antérieurement aux exercices de formation pour les *Bacillus anthracis, Brucella melitensis, Francisella tularensis, Burkholderia mallei, Yersinia pestis,* VEE, et le virus de la vaccine. Des limites de détection ont été déterminées pour les bio-tests de *Brucella melitensis, Francisella tularensis* et *Burkholderia mallei..*

La comparaison des résultats, obtenus par les bio-tests Threshold™, des agents provenant des échantillons indique que les deux agents *Bacillus anthracis* et *Yersinia pestis* ont été correctement identifiés mais que l'échantillon SIBCA contenant le VEE a produit un faux négatif. Ce dernier résultat correspond aux résultats des autres pays de l'OTAN qui ont participé à cet exercice de formation.

La portée des résultats

Ces résultats démontrent que la technologie Threshold™ est un outil sensible de bio-tests pour l'identification des agents dont se préoccupe la communauté militaire. La participation du CRDS aux exercices SIBCA de l'OTAN fournit un moyen d'évaluation de ses capacités internes en matière d'identification des agents à partir d'échantillons inconnus.

Les buts futurs

Le CRDS continuera à mettre au point les bio-tests Threshold™ qui complèteront la gamme des capacités d'identification de la technologie Threshold™ pour les agents dont se préoccupe les Forces canadiennes (FC). De plus, les échantillons dans les matrices autres que les liquides seront étudiés dont les types variés de sol et de matériaux végétatifs. Les effets des substances interférentes sur les champs de bataille seront aussi étudiés.

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Introduction

NATO troops have a mandate to be able to operate under the threat of chemical or biological attack. In order to provide effective protection and therapy against Biological Warfare (BW) agents, a reliable means of detection and identification of these threat agents is required.

In 1999, the NATO subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) conducted an international training exercise on the identification of biological agents. The United States Dugway Proving Ground (US DPG), Utah, agreed to host the training exercise. Eleven national laboratories from Canada, France, Germany (two laboratories), Hungary, Italy, the Netherlands, Norway, Poland, the United Kingdom, and the United States participated in the international training exercise. The objective of the exercise was to evaluate the capability of the NATO laboratories to detect and identify cobalt (^{60}Co)-inactivated BW materials.

Each participating laboratory was sent five "unknown" samples, four of which contained biological material and one blank containing phosphate buffered saline (PBS) only. Participants were informed that the biological material could consist of any of the following ten ^{60}Co-inactivated agents: *Bacillus anthracis, Yersinia pestis, Vibrio cholerae,* Venezuelan equine encephalitis virus (VEE), *Francisella tularensis, Brucella melitensis, Burkholderia mallei,* yellow fever virus, vaccinia virus, or *Coxiella burnetii.* Participant laboratories were requested to complete their analyses within 10 working days.

In Canada, the participating laboratory was the Defence Research Establishment Suffield (DRES). One of the techniques used by DRES in the analyses was an antibody-antigen based method employing the Threshold™ unit, a Light Addressable Potentiometric Sensor (LAPS) which monitors pH change. By this method, reagent antibodies and antigens are incubated together in a single step. The resulting "sandwich" immuno-complexes are filtered through biotin-embedded nitrocellulose membrane (Threshold™ stick) and are immobilized on the membrane by means of biotin-streptavidin interactions. A urease-conjugated antibody is subsequently captured by the "immuno-sandwich" and the membrane is wetted with urea. The membrane is placed in contact with the LAPS and the rate of change of pH with respect to time at the surface of the silicon sensor is monitored by the rate of change with respect to time of the surface potential as uV/sec [1]. A diagrammatic representation of the reaction scheme as performed by Threshold™ assay is provided in Figure 1.

The operation of the Threshold™ was first described by Hafeman et al [1]. The instrument has been used for a variety of microorganisms including salmonella [2] and *Escherichia coli* [3] and such toxins as ricin and saxitoxin [4][5]. It has also been investigated as a tool for rapid identification of biological warfare agents [6]. Threshold™ assays have been developed in-house for traditional chemical warfare agents as well as numerous microorganisms, including *Francisella tularensis* [7], *Brucella melitensis* [8], and Newcastle disease virus [9][10]. In a study that investigated the configuration of antibodies in Threshold™ assays, Threshold™ assays for a variety of protein toxins, chemical warfare agents, and microorganisms were reported [11]. In other studies, nonspecific interactions of streptavidin and urease-conjugated antibodies and the antigenicity of *Bacillus globigii* spores were examined [12][13].

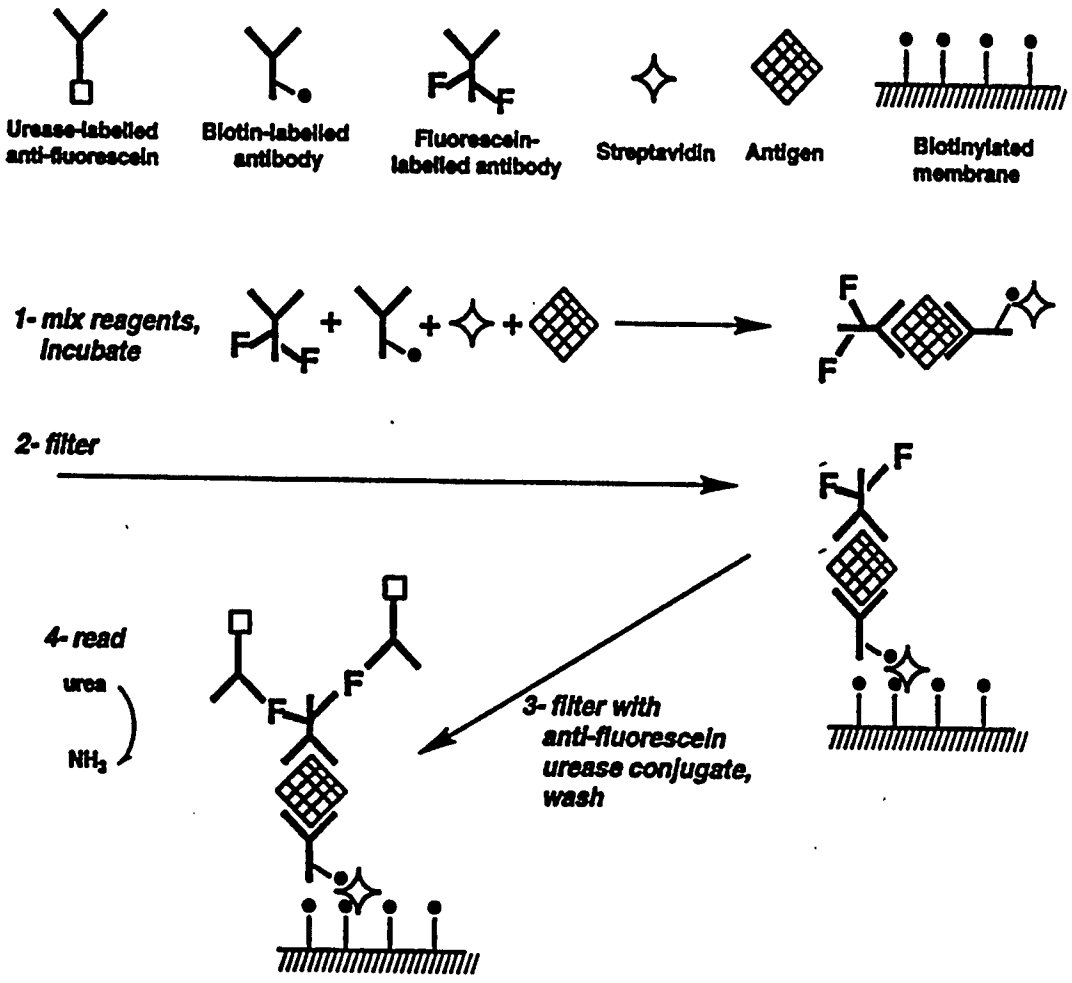

Figure 1. Reaction assay scheme for Threshold™ assays

This report describes the results obtained on screening of the SIBCA trial I samples by Threshold™ assay. Assays for only *Bacillus anthracis, Burkholderia mallei, Brucella melitensis, Francisella tularensis, Yersinia pestis*, vaccinia virus, and VEE were performed on the Threshold™. Agents identified by Threshold™ on SIBCA I samples were compared with the known agent content of samples as revealed by DPG following the exercise. Results indicate that all biological agents present in sample unknowns, for which Threshold™ assays were available, were identified correctly with the exception of VEE.

Materials and methods

SIBCA test samples

Samples of ^{60}Co-irradiated biological materials were prepared at US DPG and dispatched to participant SIBCA laboratories during March 1999. Each SIBCA participant laboratory was sent five "unknown" samples, four of which contained biological material and one blank containing PBS only. The DRES sample numbers were 110, 137, 143, 157 and 160. Samples were in 10 mL volumes in PBS and contained bacteria at a concentration of 10^6 - 10^7 cfu/mL or virus/rickettsia at 10^7 - 10^8 pfu/mL (Bruce Harper, personal communication). Immediately upon receipt at DRES, samples were aseptically aliquoted into 2 mL volumes. Aliquots for immediate use were stored at 4°C while the remaining aliquots were transferred to a -70°C freezer for longer term storage. Participating laboratories were requested to complete their analysis within 10 working days.

Threshold assays

Materials

Sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$) and Triton X-100 were obtained from BDH Chemicals (Toronto, ON). Bovine serum albumin (BSA), sodium chloride (NaCl), sodium hydroxide (NaOH), Tween-20, and urea were obtained from Sigma Chemical Co., (St. Louis, MO). Streptavidin was obtained from Scripps Laboratories (San Diego, CA) and reconstituted in distilled water to 10 mg/mL. Biotinylated Threshold ™ sticks, N-hydroxysuccinmide ester of dinitrophenyl biotin, and N-hydroxysuccinimide ester of carboxyfluorescein were from Molecular Devices Corp. (Menlo Park, CA).

Antigens and antibodies

Positive control antigens (Table 1) and anti-analyte antibodies (Table 2) were from the DRES collection. Antibodies produced by SciLab Consulting Inc. were purified on Bio-Gel^R protein G Fast Flow gel columns (Bio-Rad Laboratories, Mississauga, ON) by High Performance Liquid Chromatography (HPLC) [14]. Antibodies produced by Canadian Bioconcepts Inc. were purified on Bio-Rad Protein A™ columns by Low Pressure Liquid Chromatography [15]. Biotin-labelled and fluorescein-labelled antibodies (Table 3) were prepared according to the procedure outlined in the Threshold™ Operator's Manual. Lyophilized anti-fluorescein urease-conjugated antibody was purchased from Molecular Devices Corp. Prior to use, urease-conjugated antibody was reconstituted in 30 mL of assay buffer to a stock concentration of 7.5 ug/mL.

Table 1. *Positive control antigens used in Threshold™ assays of SIBCA sample unknowns*

Organism	Description	Working conc.	Amount per test site
B. anthracis[a]	Thraxol[R] vaccine (Sterne strain) from ADRI[1] (Lethbridge, AB); formalin inactivated by DRES	-	-
B. mallei[b]	Mallein complement fixation (CF) antigen (ophthalmic); serial no. 91-94; expiry date 95.12.31 from ADRI (Nepean, ON)	1 ng/μL	100 ng
B. melitensis	Strain 16M from ADRI (Nepean, ON); formalin-inactivated by DRES	2 ng/μL	200 ng
F. tularensis	Live vaccine strain (LVS) from USAMRIID2 (Frederick, MD); formalin-inactivated by DRES	2 ng/μL	200 ng
Vaccinia virus	Vaccine, lot 1556-12, expiry date Jan 96 from Connaught Labs (Willowdale, ON)	$2x10^{-3}$ doses/μL[c]	0.2 doses
VEE[a]	Vaccine, inactivated (TC84), Salk Institute (Swiftwater, PA) from USAMRIID	-	-
Y. pestis	Avirulent strain NCTC 5923 (*Pasteurella pestis*, ATCC #19428); formalin-inactivated by DRES	5 ng/μL	500 ng

1. Animal Disease Research Institute

2. United States Army Medical Research Institute of Infectious Diseases

[a] Not used in final assays - see "Results" section for explanation

[b] Prepared daily

[c] 1 dose ~6.25×10^5 pfu [19] (as determined at a later date)

Table 2. *Anti-analyte antibodies used in Threshold™ assays*

Antibody	Species	Source	Immunizing Antigen
B. anthracis: Lot # SC97Ant002 Serial #[a] CABAC71P0090797	rabbit	SciLab[b]	Mixture spore strains (Sterne, Ames, Vollum), US DPG, ^{60}Co-irradiated
B. mallei: Lot # SC97M1001 Serial # 1CaBur21P0090797	goat	SciLab	Mallein CF antigen (ophthalmic), serial # 91-94, expiry date 95.12.31, ADRI (Nepean, ON)
B. melitensis: Lot # SC95BM001 Serial # CaBru62P0280897	goat	SciLab	16M, ADRI (Nepean, ON) formalin-inactivated
F. tularensis: Serial # CaFra60P014079	rabbit	Bioconcepts[c]	*Pasteurella tularensis* live vaccine, National Drug Co., USA
Vaccinia virus[d]: Lot # SC97V001 Serial # CAVac61P0090797	goat	SciLab	vaccinia vaccine, live attenuated, Connaught Labs (Willowdale, ON)
VEE virus: Lot # SCVEE001 Serial # CAVen6P0000529	rabbit	SciLab	VEE vaccine TC83, attenuated, (Salk Institute), ^{60}Co-irradiated, from USAMRIID
Y. pestis Lot # SC97YP001 Serial # CAYER3810/08/99	goat	SciLab	ATCC strain 5923 (*Pasteurella pestis*), formalin-inactivated

a. DRES MOU database serial no.

b. SciLab Consulting Inc., Redcliff, AB [8]

c. Canadian Bioconcepts Inc., Saanichton, BC [9]

d. Concentration of 2.5 x 10^7 pfu/mL if reconstituted as directed [19] (as determined at a later date)

Table 3. *Anti-analyte conjugated antibodies used in the Threshold™ assays*

Conjugate	Stock Concentration (mg/mL)	Molar Incorporation Ratio
Anti-*B. anthracis*-F	0.433	6.0
Anti-*B. anthracis*-B	0.210	3.0
Anti-*B. mallei*-F	0.391	6.9
Anti-*B. mallei*-B	0.173	4.0
Anti-*B. melitensis*-F	0.477	6.3
Anti-*B. melitensis*-B	0.248	3.7
Anti-*F. tularensis*-F	1.194	3.5
Anti-*F. tularensis*-B	0.907	2.3
Anti-vaccinia-F	0.502	6.5
Anti-vaccinia-B	0.308	3.8
Anti-VEE-F	0.328	7.5
Anti-VEE-B	0.159	0.9
Anti-*Y. pestis*-F	0.782	5.5
Anti-*Y. pestis*-F	0.289	2.5

F=fluorescein

B=biotin

Reagents

Wash buffer consisted of 10 mM sodium phosphate (pH 6.5), 150 mM NaCl, and 0.05% Tween-20. Assay buffer consisted of 10 mM sodium phosphate (pH 7.0), 150 mM NaCl, 0.025% Triton X-100, and 0.1% BSA. Wash and assay buffers were stored at 4°C. The substrate solution was 100 mM urea in wash buffer (pH 6.5), prepared daily. The following reagents were added together in the following order to make up the cocktail mixture (total volume of 1 mL): assay buffer, streptavidin, biotin-labelled antibody, and fluorescein-labelled antibody (Table 4).

Table 4. *Amounts of analyte-specific fluoresceinated-antibody, analyte-specific biotinylated-antibody, and streptavidin A per test site in Threshold™ assays*

	F-Ab (ng)	**Biotin-Ab (ng)**	**SA (ng)**
B. anthracis	50	50	500
B. mallei	50	100	500
B. melitensis	50	100	500
F. tularensis	200	50	500
Vaccinia virus	100	50	500
VEE	100	100	500
Y. pestis	200	100	500

F-Ab: fluoresceinated antibody

B-Ab: biotinylated antibody

SA: streptavidin

Equipment

The assay apparatus was a commercially available LAP sensor marketed under the name Threshold™ Unit (Molecular Devices Corp.). The instrument is capable of simultaneously processing four membrane sticks consisting of eight reaction test sites per stick. The instrument is controlled by an IBM PS/2 model 30 microcomputer and custom software supplied by Molecular Devices Corp.

Assay methods

Assay optimizations and limits of detection

Prior to the SIBCA exercise, assays for *Brucella melitensis, Francisella tularensis, Burkholderia mallei,* vaccinia virus, and *Yersinia pestis* were optimized by the following method. A preliminary assay was first performed using a range of antigen concentrations and antibody conjugate concentrations (ng/spot) in the following ratios: 100:100, 100:50, 50:100, and 50:50 (concentration of fluoresceinated antibody:biotinylated antibody). From the results of the preliminary assay, an antigen concentration providing a mid range Threshold ™ signal was chosen for further experiments (Table 5). Refinement of optimized antibody conjugate concentration was then achieved by varying the respective antibody conjugate concentrations while holding the antigen concentrations constant. The optimal concentrations of conjugated antibodies were those that produced the greatest positive signal to background ratio while maintaining a relatively low signal for the zero antigen control. Test and zero antigen control samples were tested in replicates of four. Four Threshold™ sticks were then assayed simultaneously with six different antigen concentrations on each stick. Reactions were incubated at room temperature for 10 minutes. The signals for each concentration were averaged and a plot of concentration versus mean signal was produced. The limit of detection (LOD) was determined as the numerical value corresponding to the intersection of the antigen concentration curve with the curve describing zero antigen concentration plus two standard deviations.

Table 5. *Concentrations of antigens used in optimization of Threshold™ assays*

Antigen	**Concentration (ug/mL, unless otherwise indicated)**
B. anthracis	optimization not done
B. mallei	5
B. melitensis	5
F. tularensis	0.5
Vaccinia[a]	2×10^{-1} dose/mL
VEE	optimization not done
Y. pestis	10

[a] 1 dose ≈ 6.25×10^5 pfu [19] (as determined at a later date)

SIBCA unknown analyses

A volume of 125 µL of each test analyte (unknown sample), antigen (positive control), and assay buffer (negative control) were pipetted into separate 0.5 mL microfuge tubes. Unless otherwise specified, sample unknowns were assayed undiluted. A volume of 125 µL of cocktail reaction mixture was added to each of the tubes, mixed by pipette, and allowed to incubate at room temperature for 10 minutes. During the incubation step, biotinylated membrane sticks were placed into the Threshold™ filtration unit and were pre-wetted by filtering, under high vacuum, 500 µL of wash buffer per test site. At the completion of the 10 minute incubation step, 200 µL of each of the reaction mixtures were pipetted into appropriate locations on the membrane filtration unit and filtered under low vacuum. The vacuum was set to "special" and 100 µL (750 ng) of anti-fluorescein antibody was added to each test site. The reagent was removed by filtration and then each test site was washed once under high vacuum with 500 µL of wash buffer. The membrane sticks were removed from the filtration compartment, then inserted into the reader compartment containing the LAP sensor and the substrate solution. The rate of 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. A typical configuration for a sample assay is depicted in Figure 2. The layout of the assays was designed to maximize use of the eight assay locations on each stick while, at the same time, ensuring that each unknown that was reacted with a specific antibody was accompanied by its corresponding positive and negative controls on the same stick. By this means, inequalities in the nitrocellulose membrane that could potentially render invalid inter-stick comparisons are minimized.

Anti-Anthrax & Sample # 137	empty	Anti-Brucella mel. & Sample # 137	Anti-Francisella tul. & Sample # 137	empty	Anti-mallei & Sample # 137	Anti-Vaccinia & Sample # 137	empty	Anti-VEE & Sample # 137	Anti-Y. Pestis & Sample # 137	empty	empty
empty	not usueable	Anti-Brucella mel & positive control	Anti-Francisella tul. & positive control	not usueable	Anti-mallei & positive control	Anti-Vaccinia & positive control	not usueable	empty	Anti-Y. pestis & positive control	not usueable	empty
Anti-Anthrax & negative control	empty	Anti-Brucella mel & negative control	Anti-Francisella tul. & negative control	empty	Anti-mallei & negative control	Anti-Vaccinia & negative control	empty	Anti-VEE & negative control	Anti-Y. pestis & negative control	empty	empty
Stick A			Stick B			Stick C			Stick D		

Figure 2. *Typical sample assay configuration depicting reaction test site locations on four Threshold™ sticks assayed simultaneously*

Results

Limits of detection

Threshold™ assays for *Coxiella burnetii, Vibrio cholerae* and yellow fever virus could not be performed due to lack of reagents. For the remaining agents (vaccinia virus, *Yersinia pestis, Francisella tularensis, Brucella melitensis, Bacillus anthracis, Burkholderia mallei,* and VEE), antibody conjugates used in the Threshold™ assays were assessed for optimal performance prior to the training exercise and were used in optimal concentration proportions with known positive control antigens to determine the LODs of the respective assays. LODs of assays for *Brucella melitensis*, *Francisella tularensis* and *Burkholderia mallei* were 70 ng/mL, 155 ng/mL, and 54 ng/mL, respectively. A graphical example of the quadratic function used to determine the LOD is presented in Figure 3.

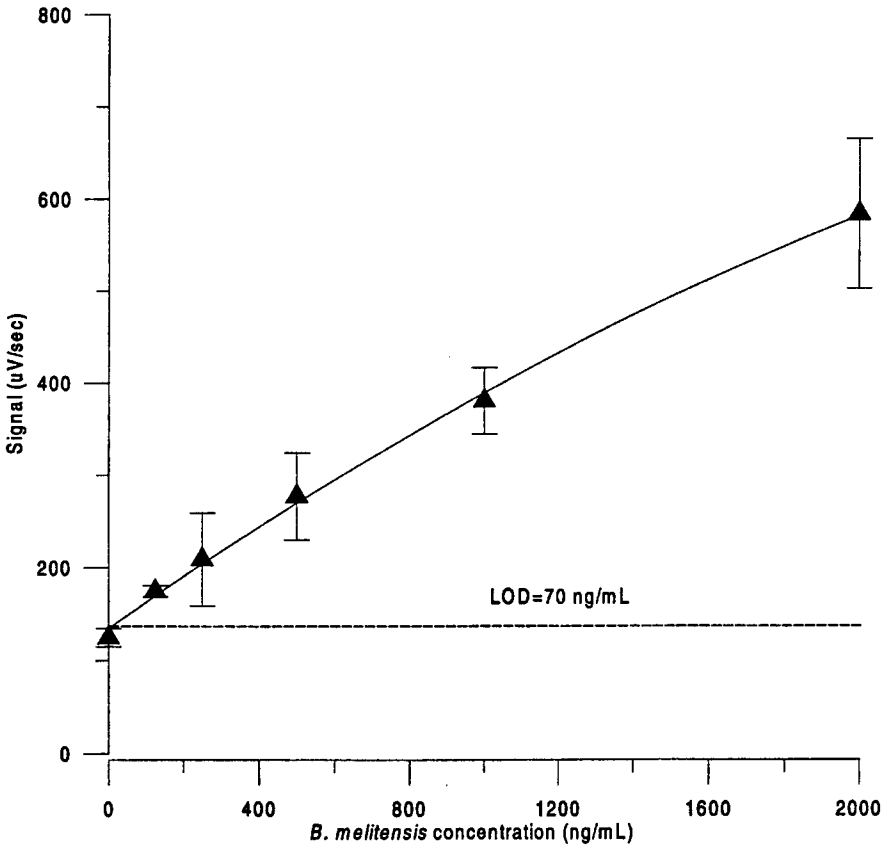

Figure 3. *LOD of B. melitensis by Threshold™ assay*

LODs for *Bacillus anthracis*, VEE, *Yersinia pestis*, and vaccinia virus could not be determined. In the preliminary optimization experiments, positive responses were not observed in either the *Bacillus anthracis* or VEE assays. For the *Bacillus anthracis* assay, it was unclear as to whether the absence of a signal was due to a problem with either or both of the antibody conjugates or with the *Bacillus anthracis* positive control antigen. In the case of VEE, the molar incorporation ratio determined for the anti-VEE biotin conjugate was observed to be very low (Table 3), suggesting that this antibody conjugate was suspect. Subsequent to the SIBCA exercise, a new biotin conjugate was made from the same VEE antibody and the VEE assay was evaluated using TC83 live virus vaccine as the positive control antigen. In these experiments, a positive response was obtained and the LOD for the VEE assay was determined to be 900 ng/mL . The LOD of vaccinia virus was not determined as, at the time of assay optimization, the concentration of the stock antigen was unknown except in terms of dose. Thus, generation of a standard curve from which to derive an LOD was left until further details as to antigen concentration could be ascertained.

The preliminary Threshold™ assays with *Yersinia pestis* showed this bacteria to be extremely "sticky." Blank sticks read after reading two *Yersinia pestis* sample sticks have resulted in readings as high as 755 uV/sec (unpublished data). Even at low concentrations, *Yersinia pestis* fouls the reader, necessitating a cleaning of the visible contamination of the reader between each stick read. Since time was a factor in preparing for the SIBCA I exercise, it was decided that the determination of a LOD for *Yersinia pestis* was not essential given the difficulty in working with this agent.

Analyses of SIBCA unknowns

A summary of the identification results obtained on the SIBCA sample unknowns by Threshold ™ assay and the agents known to be present in these samples is presented in Table 6. As well, the individual sample assay results are shown in Figures 4 to 8.

Positive control concentrations were chosen in an attempt to give a mid-range response on the Threshold ™. The positive controls for *Burkholderia mallei,* however, were considerably higher than desired. As the positive control signal was at the preferred level for the assay of SIBCA sample #143, the first unknown assayed, this concentration was maintained even though subsequent assays showed very high signals for the *Burkholderia mallei* positive control. *Burkholderia mallei* was prepared fresh daily, as preliminary work indicated that this agent was not stable over time when diluted. The average signal for a 1 ug/mL sample of *Burkholderia mallei* decreased from 1228+/- 61 uV/sec to a value of 178+/-8 uV/sec over the period of six weeks. The concentration of the positive control for *Yersinia pestis* was chosen so as to minimize the fouling of the reader. However, results indicated that this concentration was, in some assays, insufficient to produce a definitive positive response (Figures 4 and 5). There was no *Yersinia pestis* positive control for SIBCA sample # 110, the last SIBCA sample assayed, due to depletion of the antigen (Figure 8). Furthermore, no positive controls were used in the *Bacillus anthracis* and VEE assays, as the positive control antigens for these agents had produced no signal with respective antibody conjugates in preliminary assays conducted prior to the SIBCA exercise.

Samples unknowns #143 and #137 were identified by Threshold ™ assay as *Yersinia pestis* (Figure 4) and *Bacillus anthracis* (Figure 5), respectively. Sample #143 also reacted above background with antibody reagents for *Bacillus anthracis*, *Brucella melitensis*, *Francisella tularensis*, *Burkholderia mallei*, vaccinia virus, and VEE (Figure 4). These positive reactions were attributed to the "stickiness" of the *Yersinia pestis* antigen, as discussed above, and identification was made based on the magnitude of reactivity with *Yersinia pestis* antibodies. A strong signal obtained for SIBCA sample #137 with the *Bacillus anthracis* reagents implies that the lack of signal observed in preliminary *Bacillus anthracis* assays was due to antigen rather than antibody conjugate problems (Table 7). Samples #157, #160 and #110 did not react strongly with any of the antibody reagents. Readings that were minimally elevated above background were considered within the normal variance limits of the assay.

A comparison of agents identified by Threshold™ assay in SIBCA samples with those known present in sample unknowns (Table 6) indicated that, for agents for which Threshold™ assays were available, all were detected except VEE (false negative). There were no false positive identifications made from any of the five SIBCA samples.

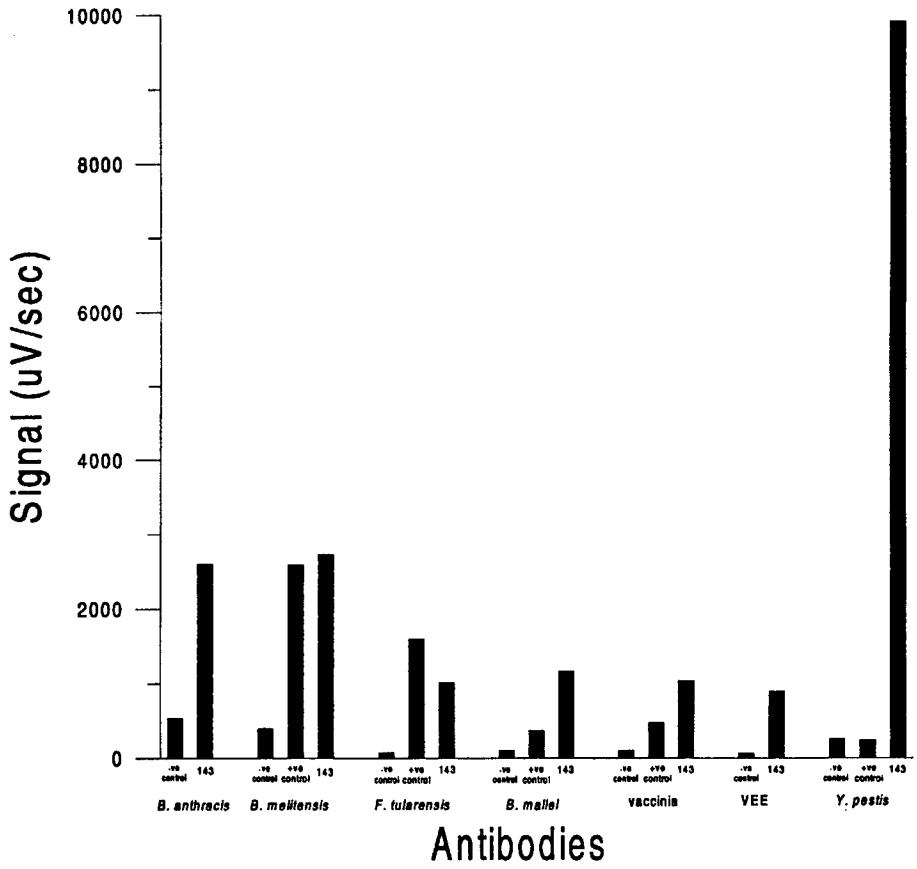

Figure 4. *Threshold™ analysis of SIBCA sample # 110*

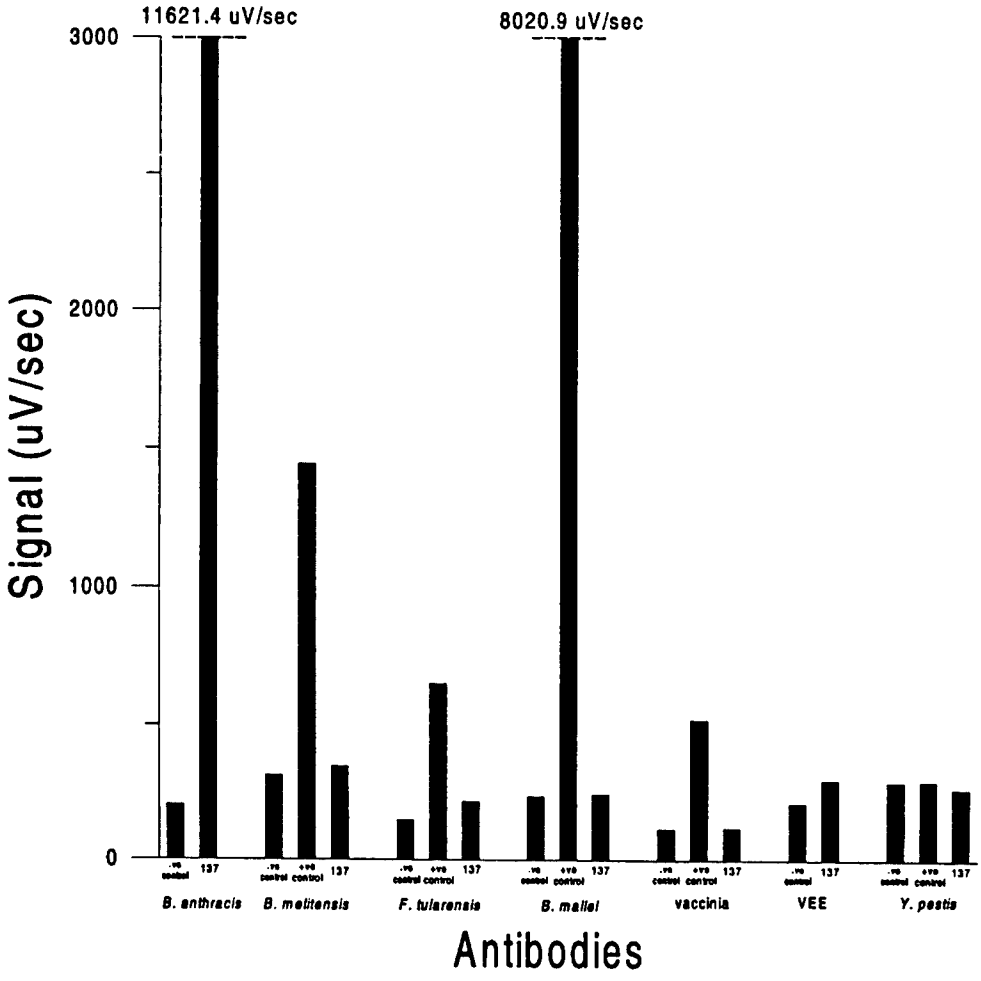

Figure 5. *Threshold™ analysis of SIBCA sample # 137*

Table 6. *Agents identified by Threshold™ assay compared to agents present in SIBCA I samples*

Sample no.	Agents present[1]	Concentration of agents present	Agents identified by Threshold™
143	*Y. pestis,* Lapaz strain	$3.7x10^7$ cfu/mL	*Y. pestis*
137	*B. anthracis,* vollum strain	$8.7x10^6$ cfu/mL; 6.1 ug/mL (protein)	*B. anthracis*
157	VEE virus, vaccine strain TC83	$7.5x10^8$ $TCID_{50}$/mL[a]; 0.5 ug/mL (protein)	none
160	*C. burnetii,* 9 mile strain, phase I	2.1x10E7 ID_{50}/mL; 2.3 ug/mL (optical density)	none
110	PBS		none

a. $7.5x10^7$ cfu/mL actually provided (Bruce Harper, DPG, personal communication)

Table 7. *Typical Threshold™ analysis of formalin-inactivated B. anthracis compared with results obtained on B. anthracis assay from SIBCA sample #137*

Sample	Concentration (ug/mL)	Signal (uV/sec)
Negative (no antigen) control	0	231.7
B. anthracis (formalin-inactivated)	0.1	303.2
	0.5	222.8
	1.0	222.5
	5.0	289.7
	10.0	218.1
	50.0	243.1
	100.0	240.6
SIBCA #137[2] (^{60}Co-inactivated)	Unknown	11,621.4

[1] Information provided by DPG post SIBCA exercise

[2] Diluted 1/10

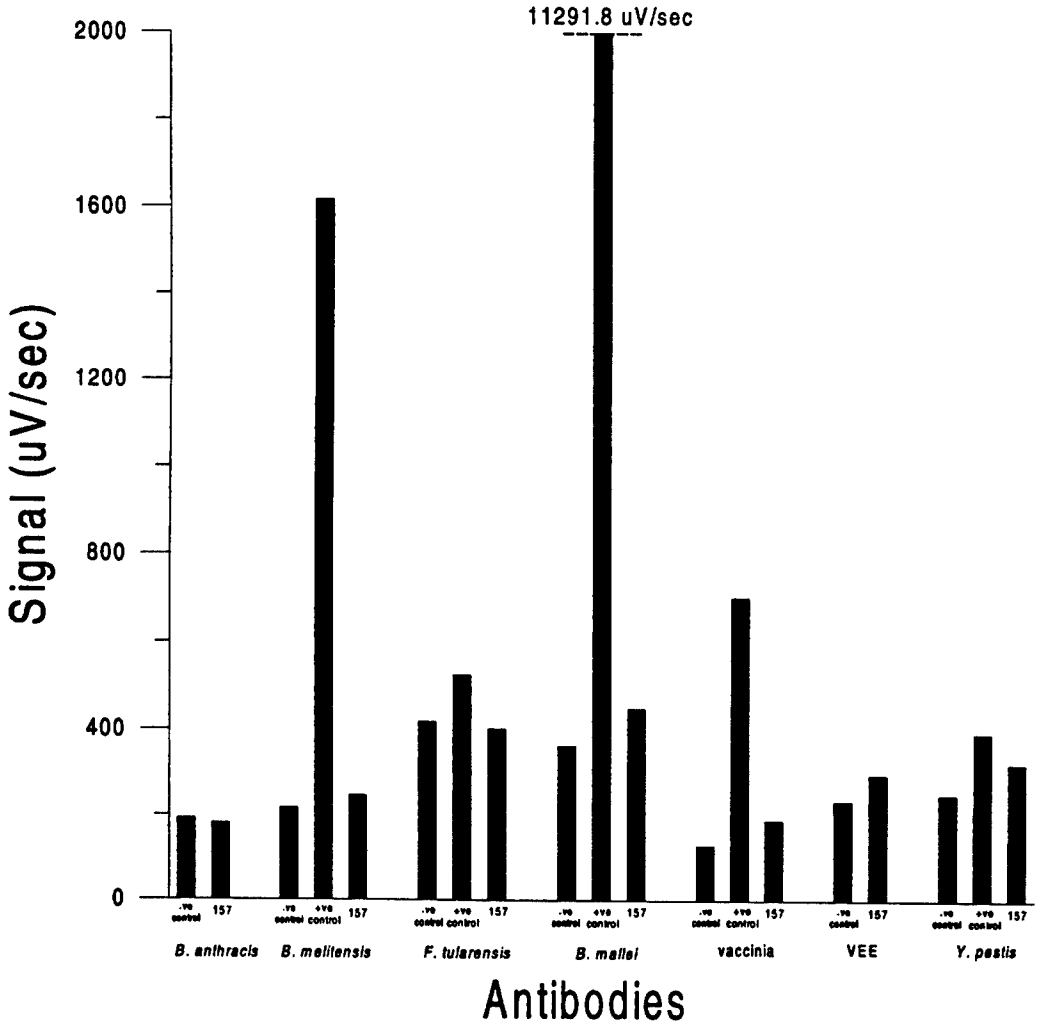

Figure 6. *Threshold™ analysis of SIBCA sample # 143*

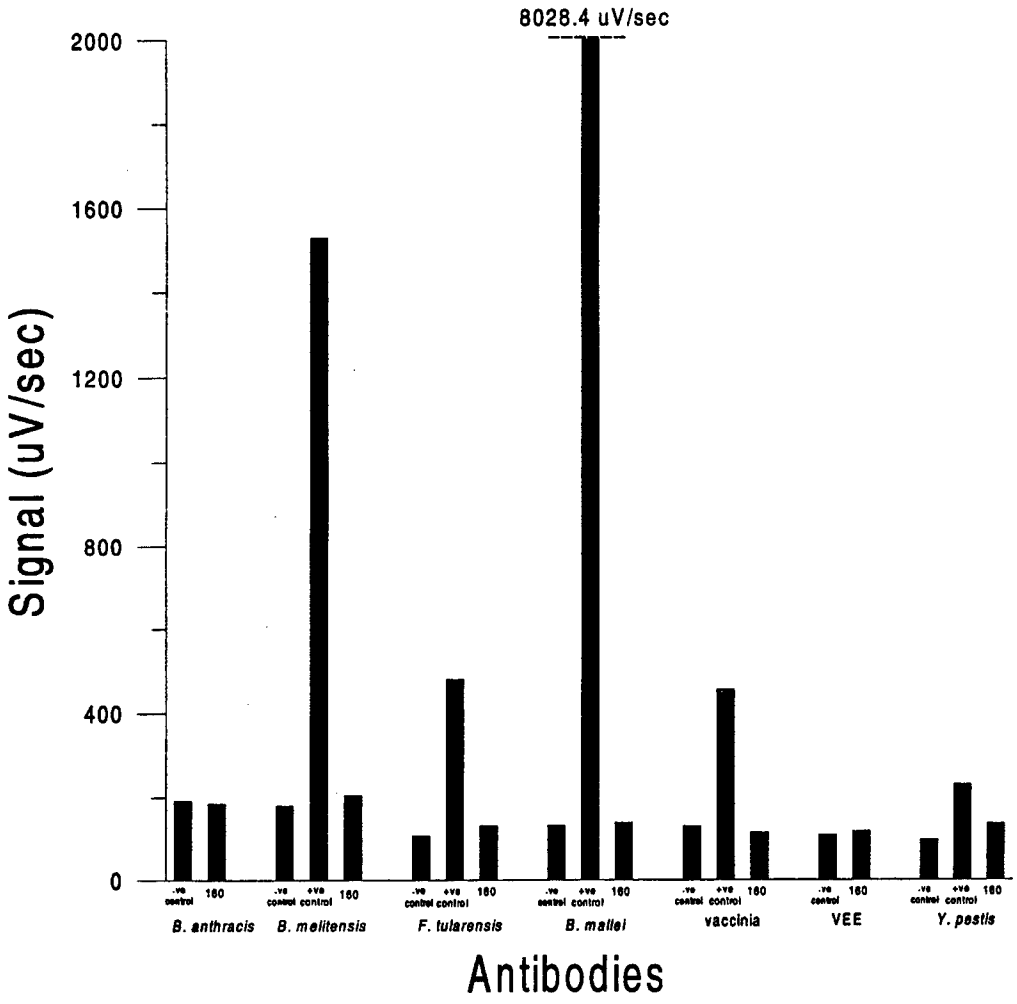

Figure 7. *Threshold™ analysis of SIBCA sample # 157*

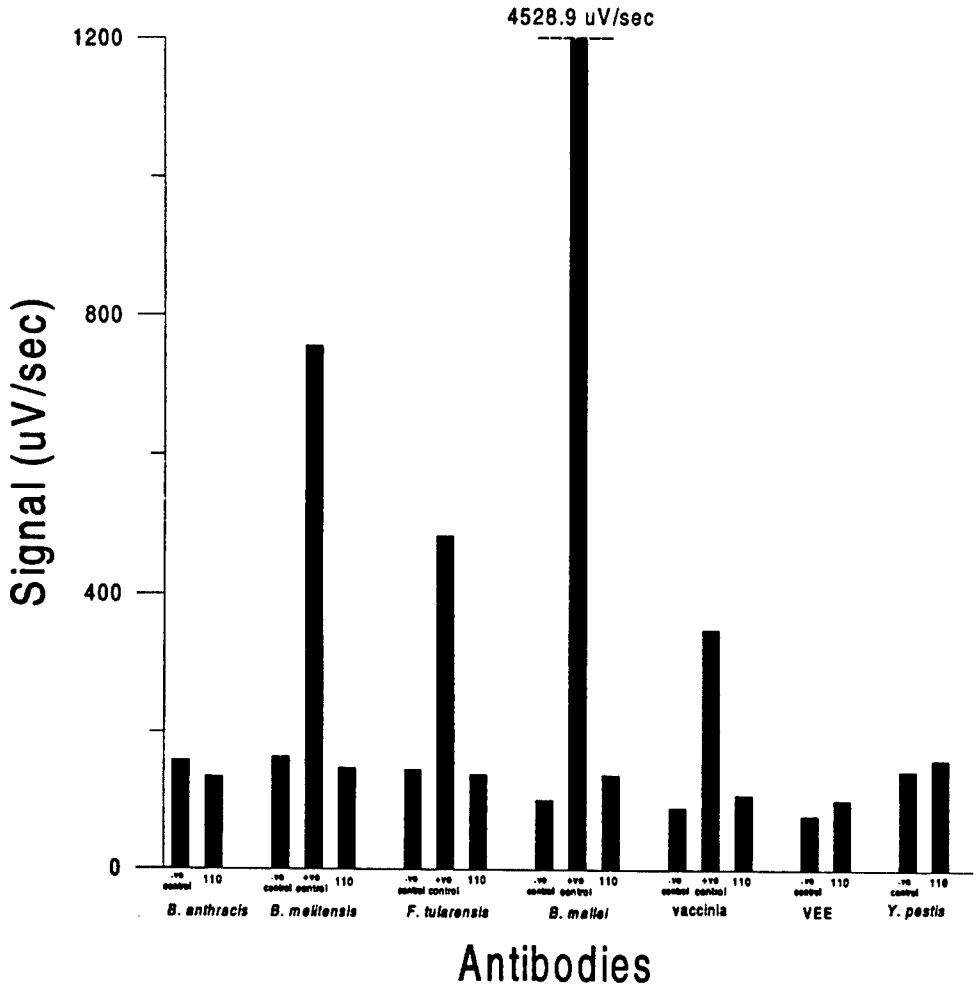

Figure 8. *Threshold™ analysis of SIBCA sample # 160*

Discussion

The approach to the Threshold ™ assay analyses of the SIBCA unknowns was one of a quick survey, rather than a more detailed study of the liquids provided, involving replicate assays. Since the approximate concentrations of agents in unknown samples were provided by DPG prior to the exercise, it was known in advance that expected signals would be well above background signals for all of the Threshold™ assays that had been optimized pre-trial. Thus, no uncertainty was anticipated in identifying any agent for which assays had been developed. In the event a true unknown sample were to arrive at DRES, all available information on the sample's origin would be taken into account in determining the approach to identify it. It is possible that a simple survey on the Threshold ™, as conducted for this exercise, might be performed first, followed by a more thorough investigation using replicate analyses and longer incubation times.

No signal for the *Bacillus anthracis* positive control, which had been inactivated by formalin treatment, was observed in the preliminary assays conducted prior to the exercise. This observation is consistent with an interpretation that the formalin-inactivated *Bacillus anthracis* may have undergone changes to the extent that altered epitopes on the agent surface were undetectable by antibodies raised against pristine (i.e., live or ^{60}Co-irradiated) *Bacillus anthracis* antigen. That inactivation of proteins with formalin can result in damage to surface epitopes is well documented and a more complete discussion of the subject is provided in the literature [16]. Previous studies at DRES on development of enzyme-linked immunosorbant assays for the Persian Gulf indicated that boiling or sonication of formalin-inactivated *Bacillus anthracis* was required in order to disperse or "unmask" the antigens [17]. However, this procedure, when applied to identification of *Bacillus anthracis* by Threshold™ assay, proved ineffective (unpublished data).

Similarly, no signal was observed for VEE in preliminary assays conducted prior to the SIBCA exercise. It was suspected that the biotin-conjugated antibody was at fault due to an observed low molar incorporation ratio of the biotin conjugate. It was also unknown whether the antigen, an inactivated TC84 vaccine, might have been inactive. In assays conducted following the SIBCA exercise, a new biotin-conjugated antibody reagent, prepared from the same antibody, was used to successfully detect live TC83 vaccine positive control antigen. In addition, sample #157, known to contain VEE, was tested for the presence of this virus with the new antibody conjugate. A negative response was obtained. This is consistent with the results obtained by the other NATO countries participating in the exercise, who could not observe the VEE by any immunoassay method. Only genetic methods enabled two countries to identify VEE in sample # 157 [18].

In the analyses of the SIBCA sample unknowns, there were instances where signals obtained with some anti-analyte antibodies were above background (Figures 4 to 8). However, without replicate analyses, no certainty of false positives could be ascertained. In light of the concentrations of agent that were expected to be present in SIBCA samples i.e.,10^6 - 10^7 cfu/mL bacteria or 10^7 - 10^8 pfu/mL virus/rickettsia, slight positive signals were not taken to be false positives. In the case of *Yersinia pestis*, unknown sample signals were well above background with all of the anti-analyte antibody conjugates, an observation that may be accounted for by the stickiness of this antigen, as discussed previously. A positive identification of *Yersinia pestis* was made on the basis of the substantially larger signal obtained with *Yersinia pestis* antibodies than with the other antibody conjugates (Figure 4).

Conclusions

Threshold ™ assays were developed for seven of the SIBCA trial agents: *Bacillus anthracis, Brucella melitensis, Francisella tularensis, Burkholderia mallei, Yersinia pestis,* VEE, and vaccinia virus. Comparison of results obtained by Threshold™ screening of the SIBCA sample unknowns with the known content of agent in SIBCA samples, indicated that for agents for which Threshold™ assays were available, only VEE virus was undetected. The inability to detect VEE is consistent with the results obtained by other NATO countries involved in the training exercise who employed immunological methods.

The Threshold™ instrument provides a reasonably rapid means of identifying unknown bacterial agents in liquid media and has shown its potential as an immunodiagnostic tool for identification of biological agents of military interest. Use of the Threshold™ in future training exercises should include replicate analyses in order to statistically validate the positive responses and rule out any potential problems of cross reactivity. LODs for assays not optimized will need to be determined. In addition, Threshold™ assays for additional agents of interest to the military will need to be developed. Due to problems encountered with formalin-inactivated agents, future assays will be developed, where possible, using ^{60}Co-inactivated agents. Furthermore, all Threshold™ assays will be assessed for specificity in the presence of related and unrelated BW agents, as well as potential battlefield interferents.

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In March 1999, the NATO Subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) conducted the first international training exercise on identification of biological agents. Eleven NATO national laboratories participated: Canada, France, Germany (two laboratories), Hungary, Italy, Netherlands, Norway, Poland, United Kingdom, and United States. Participating laboratories were sent five sample unknowns, four of which contained biological agents and one blank containing buffer only. Participants were advised that biological agents could consist of any one of the following 10 gamma-irradiated organisms: *Bacillus anthracis*, *Yersinia pestis*, *Vibrio cholerae*, Venezuelan Equine Encephalitis (VEE) virus, *Francisella tularensis*, *Brucella melitensis*, *Burkholderia mallei*, Yellow Fever virus, Vaccinia virus, or *Coxiella burnetii*. The participating laboratory for Canada was the Defence Research Establishment Suffield (DRES). For the training exercise, DRES screened sample unknowns by two different antibody-based identification technologies, one of which was the Threshold™ assay. Threshold™ assays for seven of the 10 possible biological agents were developed: *Bacillus anthracis, Brucella melitensis, Francisella tularensis, Burkholderia mallei, Yersinia pestis,* Venezuelan Equine Encephalitis virus, and Vaccinia virus and SIBCA samples were screened for these seven agents. Two agents, *Bacillus anthracis* and *Y. pestis* were identified while the SIBCA sample containing Venezuelan Equine Encephalitis virus yielded a false negative. This latter result is consistent with results of the other NATO countries participating in the training exercise.

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